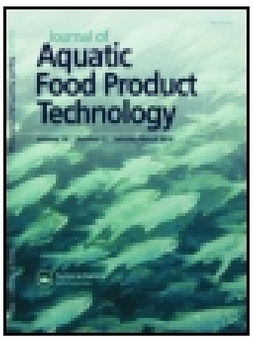

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Changes in the anti oxidant potential of Nori sheets during *in -vitro* digestion with pepsin.

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Short version of title: Anti oxidant activity of Nori sheets.

Keywords: Anti oxidant activity; DPPH; Nori; Porphyra; Pepsin

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Changes in the antioxidant potential of Nori sheets during *in -vitro* digestion with pepsin

Abstract:

Nori, the dried sheets of *Porphyra*, is a popular edible seaweed and a potential source of high quality protein, vitamins, and minerals. In the present study, the antioxidant potential of Nori has been investigated. For this purpose, an aqueous extract was obtained by soaking powdered Nori in KCl-HCl buffer and hydrolyzing it with pepsin. The antioxidant activity of

aqueous extract of crude as well as pepsin hydrolyzate was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, reducing power, total antioxidant capacity and lipid per oxidation by thiobarbituric acid reactive substances (TBARS) and compared with L-ascorbic acid. After hydrolysis, the scavenging ability and inhibition of lipid per oxide was doubled, and a ~ 7 fold increase in reducing power activity was observed. The small molecular weight fractions (F-II) of hydrolyzate obtained after gel filtration showed further increase in the activity. These results demonstrate that hydrolysis produces better antioxidants that could be used as an alternative to synthetic antioxidants.

Keywords: Antioxidant activity; DPPH; Nori; Porphyra; Pepsin

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1. Introduction

In recent years, it has been determined that degenerative diseases result from an increase in oxidative stress. An imbalance in formation of pro-oxidants and antioxidants results in accumulation of free radicals and ROS. Consequently, they become stable by binding with macromolecules like DNA, proteins or lipids, resulting in damages to cells. This damage contributes to degenerative diseases, such as cancer, atherosclerosis, cardiovascular, and inflammatory diseases. Enzymes that occur naturally in the cells protect against this damage. At times, these protective mechanisms are disrupted by various means. Therefore, supplementation of antioxidants becomes vital. In general, many synthetic antioxidants that are currently in use are suspected to be toxic (Ito et al., 1986; Safer and Al- Nughamish, 1999), mutagenic (Devi et al., 2008), neurotoxic (Vijayabhaskar and Shiyamala, 2012), or carcinogenic (Branen et al.,

1975). Hence, the search for natural antioxidants has increased enormously because of their lack of side effects. This has paved the way to exploring a wide variety of phytochemicals from various terrestrial resources. In this regard, seaweeds can be considered as a moderately exploited resource, as they are mostly cultivated for industrial applications (Ganesan et al., 2008). The total global seaweed production was above 15 million metric tons in 2004 (FAO, 2006), which shows their abundance worldwide. India remains as one of the top countries in the production of seaweeds.

A comparative study of the nutritional properties of edible seaweeds with that of currently consumable whole foods showed that seaweeds contain higher amounts of total dietary fiber, mineral salts, polyunsaturated fatty acids (PUFA), proteins, and vitamins. In addition, seaweeds also possess other bioactive components, namely alginic acid, fucoidan, mannitol, porphyran, etc. (Macartain et al., 2007). Various types of edible seaweeds are consumed as food stuffs. Certain purple laver (*Porphyra sp*.) are most widely consumed by most easterners especially Japanese, and many forms of purple laver products are available in the markets (eg. dried, seasoned, and toasted); those that are made from Susabi- nori (*P. yezoensis*), Asakusa-nori (*P. tenera*), and Iwa-nori (*Porphyra sp*.) are common (Yabuta et al., 2010). Dried purple lavers are rich in various nutrients, and some studies also prove their therapeutic properties (Takenaka et al., 2001; Tamura et al., 1998; Fujiwara 1961; Soni et al., 2008, 2009).

Seaweeds can serve as a better antioxidant defense system if they are farmed in an environment where they are exposed to various combinations of light and oxygen. This results in the production of free radicals and strong oxidizing agents that induce seaweeds to synthesize

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antioxidant components in order to resist these damages (Matsukawa et al., 1997). Their stability during storage proves their antioxidative potential (Ramarathnam et al., 1995).

In recent years, several antioxidant activities have been reported from crude extracts of seaweeds (Kuda et al., 2005; Chandini et al., 2008; Keyrouz et al., 2011; Wang et al., 2009; Ganesan et al., 2011; Bhatia et al., 2011; Balboa et al., 2013). Although many bioactivities have been studied using crude extracts, only a few have been reported after enzymatic hydrolysis. Yabuta et al. (2010) showed that phycoerythrobilin compound released from phycoerythrin on in vitro digestion produced higher antioxidant activity in comparison to the crude extract of purple laver.

The present study has been carried out in order to find more potent, non-toxic natural antioxidant from aqueous extract of Nori. The antioxidant activity of aqueous extract of algae (AEA) and the pepsin hydrolyzate (PHA) was compared by studying radical scavenging activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH), reducing power assay, total antioxidant capacity (TAC), and lipid per oxidation. To simulate the physiological condition, pepsin (the first proteolytic enzyme involved in the digestion of food) was used for the hydrolysis. This form of study might be helpful to further understand the exertion of bioactivities by the compounds after in vivo mammalian digestion.

2. Materials and Methods

Nori sheets were purchased from Japan. Pepsin, DPPH, and thiobarbituric acid (TBA) were purchased from Sigma-Aldrich, Canada. L-ascorbic acid, potassium chloride, SDS and the egg yolk homogenate was purchased from HiMedia (India). Methanol, hydrochloric acid, and

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sulfuric acid are of LR Grade purchased from RANKEM (India). Ammonium molybdate, potassium ferricyanide, ferric chloride, sodium phosphate, and sodium hydroxide were of analytical grade and purchased from SRL (India).

2.1.1. Preparation of AEA and Enzymatic Hydrolyzate (PHA)

The dried Nori sheets were pulverized to a fine powder using pestle and mortar. The algal powder was then weighed (1.0 g) and soaked in 25 ml of 0.2 mM KCl-HCl buffer (4% w/v), kept under stirring overnight at 4°C.

2.1.2. Enzymatic hydrolysis of crude

The enzymatic hydrolysis was performed according to Suetsuna and Nakano (2000). The pH of above processed crude was adjusted to 2.0 with 1.0 M HCl and pre-incubated at 45°C for 10 min. Pepsin was added to the above mixture in the ratio of 40:1 (w/w) and digested for 5 hrs at 45° C. After digestion, the enzyme was inactivated by adjusting the pH to 7.0 and then denaturing at 80° C for 10 min. The supernatant was collected by centrifuging for 20 min, 5000 rpm at 4° C, and stored at -86° C until further analysis. An equivalent amount of sample (0.4 mg dry weight) was used in all the experiments.

2.1.3. Fractionation of pepsin hydrolyzate and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

The pepsin hydrolyzate was fractionated using gel permeation chromatography. A 1.0 ml (4.0 mg) sample of pepsin hydrolyzate was loaded onto a Sephadex G-100 (Sigma-Aldrich) gel filtration column (1.4 × 20 cm), equilibrated with 50 mM tris buffer (pH 8.0). A total of 50

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fractions (3 ml each) were collected at a flow rate of 0.5 ml/min, and the absorbance was measured at 220 nm. All the fractions were screened for antioxidant activity by DPPH scavenging assay. The active fractions were pooled, partially lyophilized in a Micromodulyo freeze dryer (Thermo Scientific, Waltham, MA, USA). The lyophilized fractions were run on SDS-PAGE (10% gel). Medium range molecular markers (BSA-66 Kda; Ovalbumin- 45 Kda; Lysozyme-14 Kda; Cytochrome C- 12 Kda and Insulin- 3 Kda) were run also.

2.2. Antioxidant assays

2.2.1. DPPH radical scavenging activity

The DPPH scavenging activity was evaluated according to Mensor et al. (2001). Briefly, equivalent amount of AEA and PHA was made up to 0.5 ml with methanol and incubated at room temperature for 10 min. Then, 0.5 ml of 0.4% DPPH solution (dissolved in methanol) was added and incubated at room temperature for 20 min in dark. The absorbance was recorded at 517 nm by UV spectrophotometer (UV 1800, Shimadzu, Kyoto, Japan). L-ascorbic acid (25 ppm) was used as standard. The same amount of pepsin, incubated along with test sample in the same condition, was used as a negative control. The % scavenging activity was calculated using the formula:

$$\% \text{ scavenging} = (\text{control} - \text{test}) / \text{control} \times 100$$

2.2.2. Reducing power assay

Reducing power activity was assayed by the method described by Oyaizu (1986). L-ascorbic acid served as standard. Absorbance was read at 700 nm. Increased absorbance

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indicates increased reducing power. Reducing activities were expressed as ascorbic acid equivalents obtained from ascorbic acid calibration curve.

2.2.3. Determination of total antioxidant capacity

The antioxidant capacity was estimated using the method given by Priesto et al. (1999). The assay involves the reduction of Mo (VI) to Mo (V) by the samples and formation of green Phosphate/Mo (V) complex. Equivalent amount of samples were made up to 0.3 ml with distilled water. The above was mixed with 3 ml of reagent mixture containing 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. In addition, 0.3 ml of distilled water along with reaction mixture served as blank. The tubes were incubated at 95°C for 90 min. After cooling the tubes, the absorbance was measured at 695 nm. The antioxidant activity is expressed as the number of gram equivalents of ascorbic acid.

2.2.4. Lipid per oxidation by TBARS

The assay was performed as described by Ohkowa et al. (1979). It measures the lipid per oxide formed from egg yolk homogenate (Ruberto et al., 2000). To 0.5 ml of egg yolk homogenate, 0.1 ml of sample was added and made up to 1.0 ml with distilled water. Fifty micro liter of ferrous sulfate (70 mM) was added to induce per oxidation and incubated for 30 min. Then, 1.5 ml of 20% acetic acid and 1.5 ml of 0.8% TBA (w/v) in 1% SDS were added. The above mixture was mixed well and heated at 95° C for 60 min. Then, 5.0 ml of butanol was added after cooling and centrifuged at 3,000 rpm for 10 min. The organic layer was withdrawn, and absorbance was measured at 532 nm. The percent lipid per oxide inhibition was calculated

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using the formula %= (1— E) / C ×100, where C represents the absorbance of fully oxidized control, and E represents the absorbance in presence of sample.

2.3. Statistical analysis

All tests were performed in triplicate. The average and standard deviation were calculated using Excel 2007. Analysis of variance (ANOVA) was used to evaluate the significant difference, with the criterion of $P < 0.05$.

3. Results and discussion

3.1. Antioxidant activities of PHA and its fractions

As the antioxidant activity is influenced by various factors in a test, the activity is characterized in a better way by employing different assays. Thus, the antioxidant activity of PHA and AEA was studied using DPPH scavenging assay, reducing power, TAC, and lipid per oxidation by TBARS.

3.1.1. DPPH assay

DPPH is a free radical donor, which has been used widely in studying the scavenging effects of various compounds (Matsukawa et al., 1997; Jao and Ko, 2002). The method is based on the reduction in absorbance at 517 nm in presence of proton-donating substance, accompanied by a sharp color change from purple to yellow. The pepsin hydrolyzate and the fractions showed a significant decrease in both absorbance and the color change. The scavenging ability of PHA increased up to 60 %, whereas AEA showed ~ 30 % (Fig 1). A similar result was reported for phycoerythrin pigment protein hydrolyzate from *Porphyra*

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(Yabuta et al., 2010). From the above result, it is obvious that PHA has the ability to quench DPPH radical more effectively than AEA. This might be due to production of small peptides during digestion that contribute to the activity. These substrates formed after digestion could scavenge free radicals in a much better way and convert them to more stable products and thus terminate the reaction.

3.1.2. Reducing power

Reducing power is used to evaluate the potential of an antioxidant to donate hydrogen or electrons. It is widely accepted that an increase in absorbance over 700 nm results in greater reducing power (Duh, 1998). The reducing power of AEA was enhanced 7 times by digestion with pepsin. The reducing power activity was expressed in terms of ascorbic acid equivalents (Table 1). A study conducted by Moure et al. (2006) revealed that peptides of 0.7 Kda from soy protein hydrolyzate showed maximal reducing power, and the activity was greatly influenced by protein size (especially by low molecular weight fractions) and concentration. Carnosine and anserine, peptides obtained from mackerel protein hydrolysis, revealed higher reducing power activity (Wu et al., 2003). These studies reveal that after hydrolysis, the effectiveness of the antioxidant property could be greatly enhanced.

3.1.3. Total antioxidant capacity

TAC determines the efficacy of an antioxidant to donate its electrons. TAC was expressed in terms of ascorbic acid equivalents (Table 2). After hydrolysis, the TAC increased greatly. This increase indicates that peptides formed after hydrolysis must be responsible for the

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enhanced activity. Thus, naturally formed peptides can be used as antioxidants instead of chemically synthesized antioxidants as therapeutics and preservatives.

3.1.4. Lipid per oxidation by TBARS

Food may undergo rancidity during storage and might end up in the production of primary and secondary lipid per oxidation products, which ultimately results in the loss of flavor, texture, and nutrients of stored food. In living systems, per oxidation leads to formation of ROS, malonodialdehyde (MDA), a cytotoxic product that influences many cellular functions and causes chronic disease (Sevanian and Ursini, 2000). Natural antioxidants were found to be much safer and more effective than synthetic antioxidants (Elias et al., 2008). In the present study, the PHA showed 52% inhibition and AEA showed 24% inhibition. Pepsin, used for hydrolysis, did not show any inhibition of lipid per oxidation; on the contrary, it showed higher absorbance than the control, which was a mixture of egg yolk homogenate along with other reagents and considered fully oxidized. Hence, while calculating the percent inhibition of lipid per oxidation, the pepsin showed negative enhances per oxidation (Fig 2). Therefore, these results indicate that PHA seemed to contain some antioxidant peptides that were responsible for enhanced activity. Hagen and Sandnes (2004) also showed reduction in lipid per oxidation in an experiment conducted by injecting brine solution containing salmon fish protein hydrolyzate into smoked salmon fish fillets.

3.2. Radical scavenging activity of fractions of PHA

PHA was fractionated by size exclusion chromatography on Sephadex G-100, and two major peaks showing higher absorbance at 220nm were designated as F-I and F-II (Fig 3).

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DPPH assay was performed for all the fractions (Fig 3). The peak F- I showed little activity, whereas the peak F- II showed a maximum activity of 75 %, which is higher than the aqueous extract (30%) as well as the pepsin hydrolyzate (60%). The electrophoresis pattern on SDS-PAGE revealed the peak F-II to be a low molecular weight peptide of lower than 3 Kda (Fig 4).

From this study, it is evident that antioxidant property can be enhanced by means of enzymatic hydrolysis. In a similar study, Yabuta et al. (2010) showed an increase in antioxidant activity after digestion with proteases, and this activity attributed to colored compound phycoerythrobilin having absorbance at 493 nm. In the present study, the enhanced antioxidant activity is mainly due to small molecular weight (< 3 Kda) peptides formed by pepsin digestion (Fig 4) having colorless appearance showing maximum absorbance at 220 nm (Fig 3). Although AEA and PHA showed absorbance at 492 nm, the Peak F-II did not show any absorbance at this wavelength (Fig 5), which ruled out the presence of phycoerythrobilin in the sample. Many studies have reported that after enzymatic hydrolysis, the antioxidant activity significantly increased (Lin et al, 2012; Samaranayaka et al., 2011; Yabuta et al, 2010). Various food sources like milk, fish, egg, soya beans, and several others have been exploited to produce antioxidant protein hydrolyzate and peptides using various proteases (Samaranayaka et al., 2011). Further, in most of the studies, fresh and raw materials of algae (Ganesan et al., 2008, 2011; Tamura et al., 1998) have been used, but dried, commercially available seaweed can be employed in order to know about the changes in antioxidant property. Thus, the present study was carried out using commercially available seaweed, and we found that antioxidant activity has tremendously increased when hydrolyzed using enzymes. As most of the food based industry employs enzymes for hydrolysis, this method is cost-effective, as well.

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4. Conclusion

Further studies have to be carried out in order to isolate and identify the active component responsible for antioxidant activity. Present studies indicate that seaweeds can serve as a significant source of natural antioxidant, which might be helpful in preventing age related chronic diseases along with other beneficial effects to human health. This hydrolyzate can also be used as food preservatives to prevent the oxidative damages without any side effects.

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Fig 1: Radical scavenging ability of aqueous extract of dried nori sheets (AEA) and its pepsin hydrolyzate (PHA). The assay was performed in triplicate (n=3). Values are represented as mean and found to be significant with p-value<0.05.

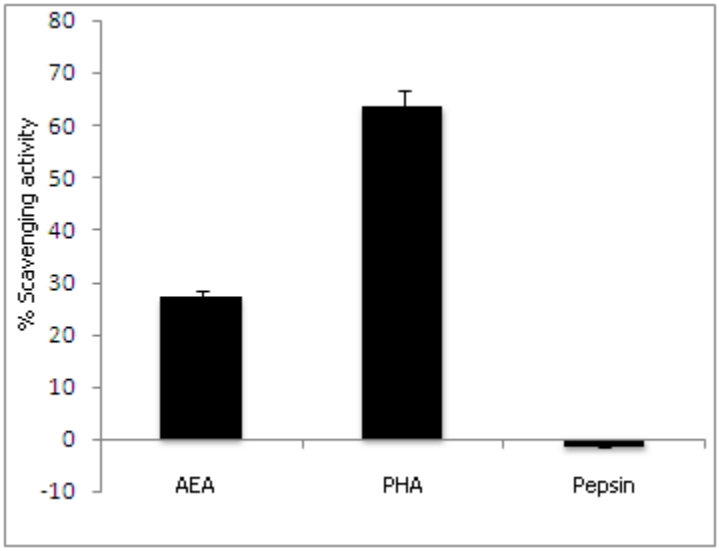

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Fig 2: Inhibition of lipid per oxidation by AEA, PHA, and pepsin.

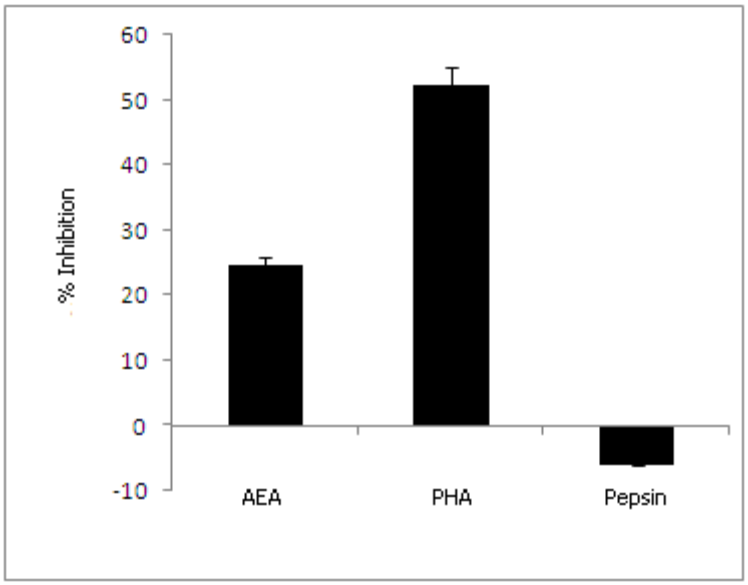

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Fig 3: Elution profile of pepsin hydrolyzate by size exclusion chromatography (dark line) and DPPH scavenging activities of fractions (dotted line).

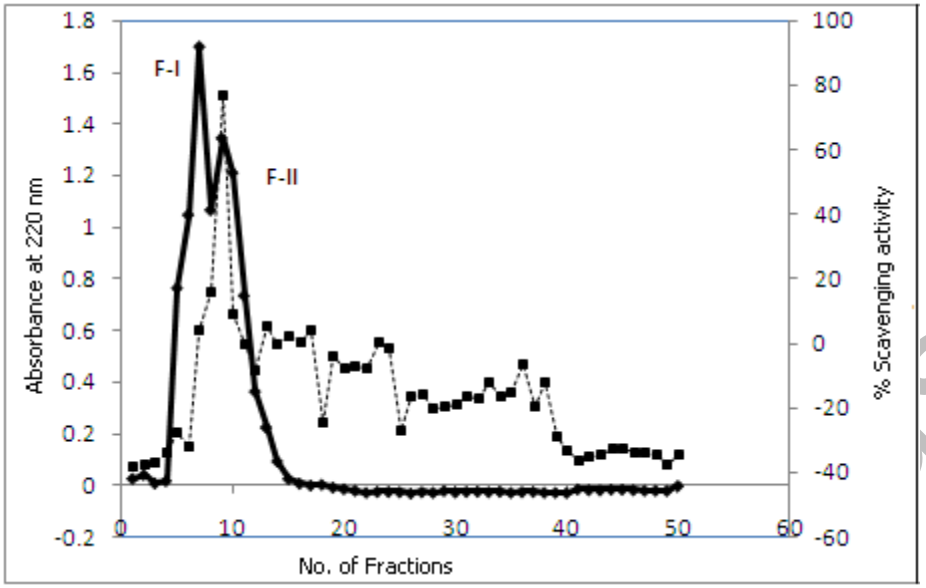

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Fig 4: SDS-PAGE of aqueous extract of nori sheets along with pepsin hydrolyzate and active fraction F-II. Lane *1*- Peak F- II, Lane *2*- PHA, Lane *3*- ACA, and Lane *4*- Medium range molecular weight markers. The black arrow shows band of low molecular weight peptides of < 3.0 Kda in Lane 1 and 2.

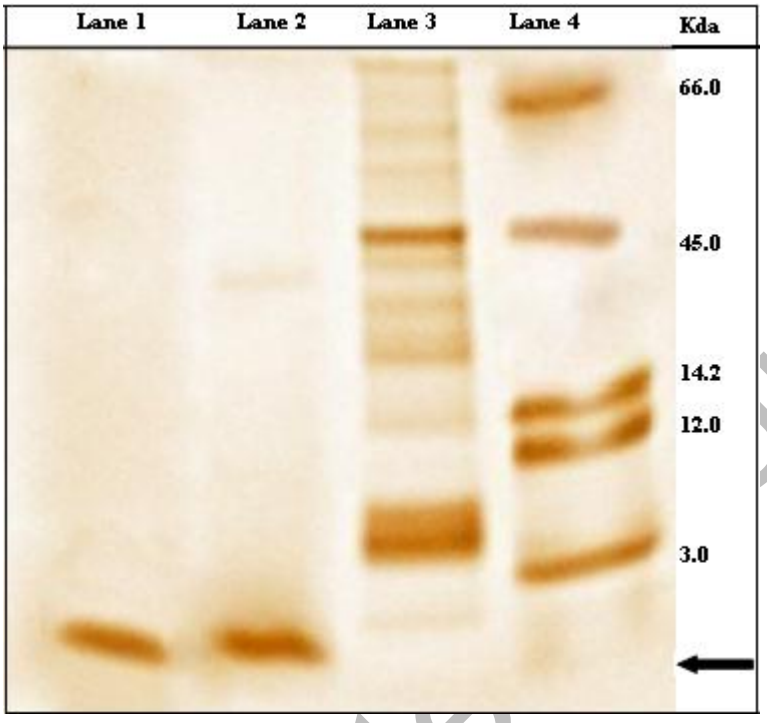

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Fig 5: Comparison of UV-visible absorption spectrum of aqueous extract of nori powder (AEA), pepsin hydrolyzate (PHA), and the low molecular weight fraction obtained after gel filtration (F-II).

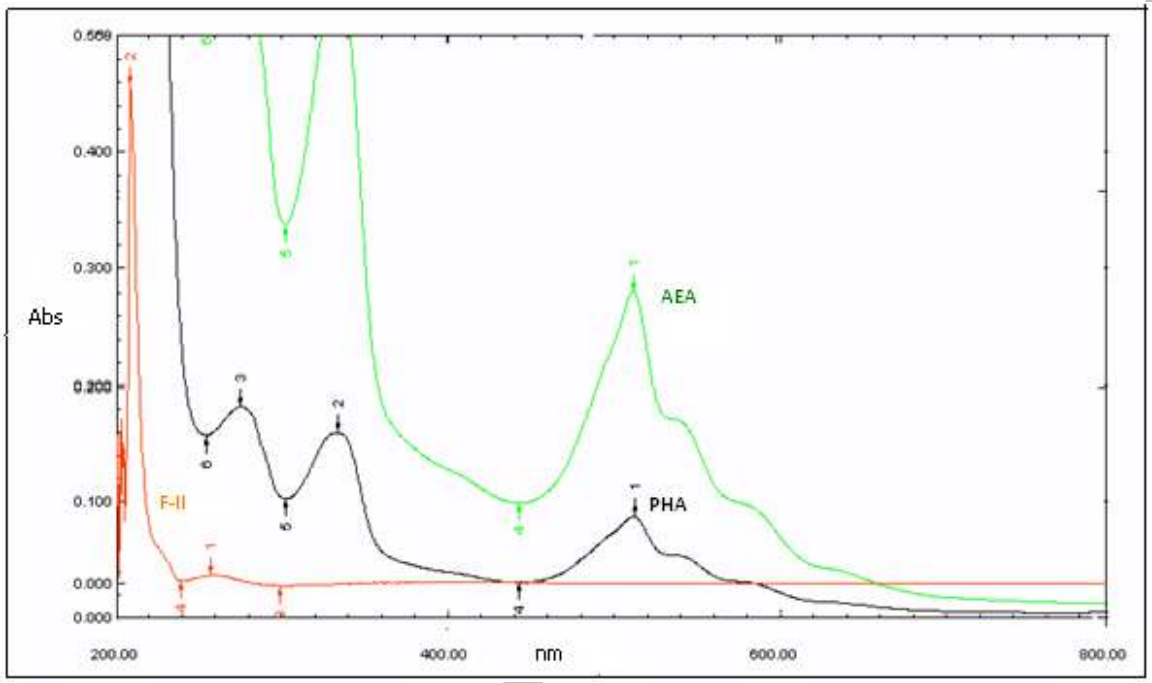

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Table 1: Reducing power capacity (µg ascorbic acid equivalents) of AEA and PHA[1]

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Sample	Average absorbance at 700nm	Ascorbic acid equivalents(µg)
AEA	0.102±0.005	8.5
PHA	0.708±0.008	59

[1]All the values are mean (n=3) ± SD: Standard Deviation. As pepsin does not show any significant activity, it was excluded from the table.

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Table 2: Total antioxidant capacity (µg ascorbic acid equivalents) of AEA and PHA[1]

Sample	Average absorbance at 695nm	Ascorbic acid equivalents(µg)
AEA	0.302±0.01	37.8
PHA	0.807±0.02	101

[1]All the values are mean (n=3) ± SD: Standard Deviation. As pepsin does not show any significant activity, it was excluded from the table.

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