Discourse Journal of Agriculture and Food Sciences
www.resjournals.org/JAFS
ISSN: 2346-7002
Vol. 2(5): 160-168, May, 2014

Total Phenolics, Flavonoids, Proanthrocyanidins , Ascorbic Acid Contents and *In-Vitro* Antioxidant Activities of Newly Developed Isolated Soya Protein

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Abstract

Soy protein ingredients have been accredited a number of beneficial effects on preventing obesity, lowering blood cholesterol levels, have evidence for a beneficial role in preventing diseases (cancer, osteoporosis, menopausal disorders and cardiovascular diseases) The aim of the study was to evaluate the quantity of various phenolic compounds, ascorbic acid and in-vitro antioxidant activities of developed isolated soy protein (ISP). *In vitro* antioxidant models like the total antioxidant capacity, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity and reducing power are studied by using UV-VIS spectrophotometer (analytikjena,specord-205). The contents of Phenolics, flavonoids, proanthocyanidins and ascorbic acid are 170±4.68 mg tannic acid equivalent, 135.75±3.98 mg catechin equivalent, 86.06±3.96 mg catechin equivalent and 12.28±2.54 mg in 100 gm ISP respectively. The total antioxidant capacity is 243.02±5.26 mg ascorbic acid equivalent and IC 50 value of ISP in DPPH assay is 35.40 mg/ml whereas IC 50 of standard ascorbic acid in DPPH assay is 3.37 µg/ml. IC 50 value of ISP and ascorbic acid in reducing power assay are 35.40 mg/ml and 31.12 micro gm/ml respectively. The results suggested that besides protein content, the phenolic, flavonoids, proanthocyanidins and ascorbic acid contents should be also considered as an important characteristic features of ISP, and as a potential selection criterion for antioxidant activity in ISP.

Key Words: Isolated soy protein, phenolics, flavonoids, proanthocyanidins and IC 50 value.

INTRODUCTION

Oxidative stress, the consequence of the imbalance between prooxidants and antioxidants in an organism, is considered to play a very important role in the pathogenesis of several degenerative diseases. These diseases include diabetes, aging, cancer, cardiovascular diseases, metabolic syndrome and atherosclerosis. Free radicals, such as hydroxyl, singlet oxygen, nitric oxide, hydrogen peroxide and superoxide radicals, are continuously generated in the cell, as a result of normal human metabolism. However, they can be harmful to the system if not properly regulated and thus may cause variety of pathological effect such as carcinogenesis, aging DNA damage and enzyme inactivation by attacking biological macromolecules. The mechanisms by which free radicals interfere with cellular functions are not yet fully understood, but one of the most important processes seems to be the formation of lipid hydroperoxides (Ilhami Gu¨ lc¸ in *et al*., 2010).

ISP is the major protein-rich food ingredients derived from soybean meals. Soy protein products are excellent sources of high quality protein, are low in saturated fat, and contain dietary fiber and nutraceutical-valued isoflavones. Soy protein ingredients have been attributed a number of beneficial effects on human health such as lowering blood cholesterol levels, preventing obesity, providing nutrition and possibly even play a beneficial role in preventing diseases

(cancer, osteoporosis, menopausal disorders and cardiovascular diseases) (Xiao 2007, Mateos-Aparicio *et al*., . 2008, Takamatsu *et al*., 2003). A rat-feeding study showed that consuming a soy protein diet resulted in 40 to 47% of its iron being converted to hemoglobin iron (Pellett *et al*., 1990). Consumption of soy protein has a beneficial effect on renal function (Anderson *et al*., 2007) and on reducing weight, adiposity (Cope *et al*., 2008) and incidence of breast cancer.

Polyphenolic substances possess many biological effects which are mainly attributed to their antioxidant activities in scavenging free radicals, inhibition of peroxidation and chelating transition metals (Bahman, N *et al*., 2007). For examples, flavonols, cinnamic acids, coumarins and caffeic acids are well known polyphenolic compounds with strong antioxidant properties. Hence play an important role in protecting food, cells and organs from oxidative damage. These compounds (phenolic substances) all share the same chemical patterns, with one or more phenolic groups for hydrogen proton donors and neutralize free radicals. Antioxidants can protect the human body from free radicals and reactive oxygen species (ROS) effects. Antioxidant agents are well known to retard the progress of many chronic diseases as well as lipid peroxidation (Osamuyimen O. Igbinosa *et al*., 2011).

In recent years, one of the areas which attracted the great deal of attention is the possible therapeutic potential of antioxidants in controlling degenerative diseases associated with marked oxidative damage. Several plant extracts and different classes of phytochemicals have been found to have quite prominent antioxidant activity (Bhuiyan *et al*., 2009).The aim of our study is to determine the quantity of total Phenolics, Flavonoids, proanthocyanidins, ascorbic acid and in-vitro anti oxidant activities of developed ISP.

MATERIALS and METHODS

Chemicals and reagents

Folin-Ciocalteau reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric chloride, ascorbic acid , tannic acid and catechin were purchased from Sigma Co. St. Louis, Missouri, USA. Methanol, hydrochloric acid, sodium hydroxide, aluminium chloride, sodium carbonate, sodium hydroxide and potassium ferricyanide were purchased from Merck, Darmstadt, Germany. All the chemicals and reagents were of analytical grade.

Antioxidant activity

Extraction of sample

A total of near about 0.5-1.0 g of ISP was extracted by vortex, mechanical shaking for four hours and finally sonication for 20 minutes with 50 ml methanol. Methanol extract was obtained by filtering the mixture through Whatman No. 1 filter paper and the supernatant was used in the experiment. The extraction repeated three times.

Total phenolics content (TPC)

The amount of total phenol content can be determined by Folin-Ciocateu reagent method (S. Chanda *et al*., 2009) with some modification. 0.5 ml of extract and 0.5 ml of Folin-Ciocalteu reagent (0.5 N) are mixed and incubated at room temperature for 5 min. Then 2.0 ml saturated sodium carbonate is added and further incubated for 30 min at room temperature and absorbance measured at 765 nm. Tannic acid (Wolfe *et al*., 2003) can be used as positive controls. The contents of total Phenolics was determined by using the linear standard equation y=92.92 x-0.780 (R square is 0.999) where x is absorbance and y is concentration of tannic acid.

Total flavanoid determination

Total flavonoid content was determined by aluminium chloride method (Chang C *et al.,* 2002) using catechin as a standard. 1ml of test sample and 4 ml of water were added to a volumetric flask (10 ml volume). 5 min after adding 0.3 ml of 5 % Sodium nitrite, 0.3 ml of 10% aluminium chloride was added. After 6 min incubation at room temperature, 2 ml of 1 M Sodium hydroxide was added to the reaction mixture. Immediately the final volume was made up to 10 ml with distilled water. The absorbance of the reaction mixture was measured at 510 nm against a blank spectrophotometrically (Shimadzu UV-1609, Japan).Total flavonoid content was calculated as catechin (mg/100g) using the following equation

based on the calibration curve:
y = 574.4x - 2.171, R2 = 0.992,
where x was the absorbance and y was the catechin concentration.

Proanthocyanidins content determination

Quantitative estimation of Proanthocyanidins was carried out using the modified vanillin– HCl method (B. H. Abdelseed *et al.,* 2011). Vanillin reagent (0.5%, 5 ml) was added to the extract (1 ml) and the absorbance of the colour developed after 20 minutes at 30oC was read at 500 nm. A standard curve was prepared expressing the results as catechin equivalents, i.e. amount of catechin (mg 100 g-1) which gives a colour intensity equivalent to that given by Proanthocyanidins after correcting for blank. Proanthocyanidins content was calculated as catechin (mg/100g) using the following equation based on the calibration curve: y = 1298x +2.954, R2 = 0.996, where x was the absorbance and y was the catechin concentration.

Determination of ascorbic acid

Ascorbic acid was determined according to the method of Lillian Barros *et al.,* (2007). The dried methanolic extract (100 mg) was extracted with 10 ml of 1% metaphosphoric acid for 45 min at room temperature and filtered through Whatman No. 4 filter paper. The filtrate (1 ml) was mixed with 9 ml of 2,6 dichlorophenolindophenol and the absorbance was measured within 30 min at 515 nm against a blank. Content of ascorbic acid was calculated on the basis of the calibration curve of authentic L-ascorbic acid (0.020– 0.12 mg/ml). The assays were carried out in triplicate; the results were mean values ± standard deviations and expressed as mg of ascorbic acid/100g of ISP.

Antioxidant and radical scavenging assay

Determination of Total antioxidant capacity

The determination of total antioxidant activity was done as per the phosphomolybdenum method with some modifications (Alakh N Sahu *et al.,* 2011). The basic principle of the assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate Mo (V) complex at acidic pH. 0.3 ml extract was combined with a mixture of 3ml of reagent solution (0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes containing the reaction solution were then capped and incubated at 95 °C for 90 minutes. After the samples had cooled to room temperature, the absorbance of the solution was then measured at 695 nm against blank. Methanol (0.3 ml) in the place of extract is used as the blank. The antioxidant activity is expressed as the mg of equivalents of ascorbic acid.

DPPH free radical scavenging assays

The DPPH is a stable free radical and is widely used to assess the radical scavenging activity of
antioxidant compounds. This method is based on the reduction of DPPH in methanol solution in the presence of a hydrogen–donating antioxidant due to the formation of the nonradical form DPPH-H (S. Chanda *et al*., 2009). This transformation results in a color change from purple to yellow, which is measured spectrophotometrically. The disappearance of the purple color is monitored at 517 nm. The free radical scavenging activity can be measured by using 2, 2-diphenyl-1-picryl-hydrazyl or 1, 1- diphenyl-2-picryl-hydrazyl by the method of McCune and Johns (2002) with some modification. The reaction mixture (3.0 ml) consist of 2.0 ml of DPPH in methanol (0.004%) and 1.0 ml of various concentrations of extract and. It is incubated for 10 min in dark, then the absorbance is measured at 517 nm against methanol as a blank and control is prepared by DPPH and methanol in place of sample extract. In this assay, the positive control is ascorbic acid.
The percentage of inhibition can be calculated using the formula:

$$\text{Inhibition (\%)} = (A0 – A1 / A0) \times 100$$

Where; A0 is the absorbance of control and A1 is the absorbance of test. IC50 (mg/mL) was defined as the concentration of an antioxidant extract which was required to quench 50% of the initial DPPH· under the experimental conditions given. It was obtained by interpolation from linear regression analysis.

Reducing power (RP)

The reducing power can be determined accoding to the method described by P. Jayanthi *et al.,* 2011. Various concentrations of the plant extracts in corresponding solvents were mixed with phosphate buffer (2.5 ml) and potassium ferricyanide (2.5 ml). This mixture was kept at 50ºC in water bath for 20 minutes. After cooling, 2.5 ml of 10% trichloro acetic acid was added and centrifuged at 3000 rpm for 10 min whenever necessary. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and a freshly prepared ferric chloride solution (0.5 ml). The absorbance was measured at 700 nm. Control was prepared in similar manner excluding samples. Ascorbic acid at various concentrations was used as standard. Increased absorbance of the reaction mixture indicates increase in reducing power.

Statistical Analysis

All the experiments were carried out in triplicate, and the results were expressed as mean ± SD (Standard deviation). Statistical analysis was performed using Excel 2007.

RESULTS AND DISCUSSION

Contents of total phenolics, flavonoids, proanthocyanidins and ascorbic acid:

The contents of Phenolics, flavonoids, proanthocyanidins and ascorbic acid are 170±4.68 mg tannic acid equivalent, 135.75±3.98 mg catechin equivanet, 86.06±3.96 mg catechin equivanet and 12.28±2.54 mg respectively (Figure: 1) in 100 gm ISP.

Plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators, it was reasonable to determine their total amount in the selected plant extracts (Prakash Veeru *et al*., 2009). Phenolics and polyphenolics (polymeric phenolics) can provide relief from certain physical ailments and degenerative diseases in humans, including the reduction of cardiovascular diseaseand certain cancers (Scalbert *et al*., 2002, Arts and Hollman 2005). Therefore, it is not surprising that the extraction and analysis of phenolics from plants and other food sources have been extensively studied (Naczk *et al*., 2004; Dai *et al*., 2010). The phenols contain hydroxyls that are responsible for the radical scavenging effect mainly due to redox properties (Rice-Evans *et al*., 1997).

Plants consumed by humans may contain thousands of different phenolic compounds. The effects of dietary phenolics are of great current interest, due to their antioxidative and possible

anticarcinogenic activities. A popular belief is that dietary phenolics are anticarcinogens because they are antioxidants, but direct evidence supporting this supposition is lacking. Phenolics may inhibit carcinogenesis by affecting the molecular events in the initiation, promotion, and progression stages (Djordje Malenčić *et al*., 2007). According to our study, the high phenolic content in ISP can explain its high free radical scavenging activity.

Flavonoids present in food of plant origin are also potential antioxidants (D Satheeshkumar *et al*., 2011). ISP is a good source of flavonoid (Figure 1).The antioxidative properties of flavonoids are due to several different mechanisms, such as scavenging of free radicals, chelation of metal ions, such as iron and copper, and inhibition of enzymes responsible for freeradical generation (Benavente-Garcia *et al*., 1997). Depending on their structure, flavonoids are able to scavenge practically all known ROS.The many pharmacological effects of phenolic compounds and flavonoids are linked to theirability to act as strong antioxidants and free radical scavengers, to chelate metals, and to interactwith enzymes, adenosine receptors, and biomembranes. It was reported that the antioxidantactivity of plant materials was well correlated with the content of their phenolic compounds (G. Sathyaprabha *et al*., 2011).

Anthocyanins are probably the largest group of phenolic compounds in the human diet, and their strong antioxidant activities suggest their importance in maintaining health (Velioglu et al., 1998). When consumed regularly, by humans, these flavonoids have been associated with a reduction in the incidence of diseases, such as cancer and heart disease.

Ascorbic acid acting as a chain breaking antioxidant impairs with the formation of free radicals in the process of formation of intracellular substances throughout the body, including collagen, bone matrix and tooth dentine (Aqil et al.,

Table 1. percntage inhibition of DPPH free radical scavenging activity and IC 50 of ascorbic acid and ISP (Fresh weight).

Ascorbic acid			ISP		
Concentration in µg	**% of Inhibition**	**IC 50 in µg/ml**	**Concentration in mg**	**% of Inhibition**	**IC 50 in mg/ml**
5	66.99±2.13		2.48	4.89±0.39	
10	83.52±1.29	3.37	4.97	8.85±0.23	35.40
15	91.83±0.88		7.45	11.48±0.55	
20	97.50±0.73		9.93	15.23±0.19	
25	97.92±0.44		12.42	17.32±0.44	

2006). The quantitative determination of ascorbic acid in ISP shows that this is good source of ascorbic acid (Figure 1).

***In vitro* antioxidant activity results**

Determination of Total antioxidant capacity

The total antioxidant capacity in the methanolic extracts of ISP was determined using the linear regression equation (*y=55.45x+0.636, r2* = 0.997 and where x is absorbance and y is ascorbic acid concentration in microgram) of the calibration curve (Figure 2) and was expressed as ascorbic acid equivalent. The total antioxidant capacity of the ISP is 243.02±5.26 mg Ascorbic acid equivalent/100g.

DPPH Free radical scavenging assay:

Radical scavenging activities are very important due to the deleterious role of free radicals in foods and in biological systems. Chemical assays are based on the ability to scavenge synthetic free radicals, using a variety of radical-generating systems and methods for detection of the oxidation end-point. DPPH radical scavenging methods are common spectrophotometric procedure for determining the antioxidant capacities of components.
DPPH, a pragmatic compound with an odd electron, shows strong absorption band at 517 nm in methanol. The absorbance decreases as the result of color change from purple to yellow due to the scavenging of free radical by antioxidants through donation of hydrogen to form the stable DPPH-H molecule (Mohd Adzim Khalili *et al*., 2010). Table 1 shows IC_{50} value of ISP was determined from the regression line of concentration versus % of inhibition and IC 50 value of ISP is 35.40 mg/ml (fresh weight).

Reduction of DPPH radicals reveals that examined extracts possess radical inhibitors or scavengers with possibility to act as primary antioxidants. They might react with free radicals, particularly with the peroxy radicals, which are the major propagators of the auto-oxidation chain of fat, thus come to an end of the chain reaction. Based on the results obtained, the antioxidative activity of investigated extracts, particularly ISP extracts, could in part be markedly caused by their radical scavenging properties.

Reducing power

The reducing power of methanolic extracts of ISP was found to be correlated with increasing absorbance (at 700 nm) as compared with ascorbic acid, a known antioxidant (Figure 3 and Figure 4). The IC 50 of ascorbic acid and ISP is 31.12 micro gram and 30.41 mg (fresh weight) respectively.
The presence of reductones are responsible for reducing capacity, which involved in prevention of chain initiation, binding of metal ions, decomposition of peroxides and radical scavenging (Yildirm *et al*., 2001). Therefore the reducing power of ISP was relatively more prominent due to the presence of some reductions.
A significant correlation between DPPH scavenging potential vs reducing power was observed in ISP to support the above statement (figure 5)

Antioxidants can be explained as reductants, and inactivation of oxidants by reductants can be described as redox reactions in which one reaction species is reduced at the expense of the oxidation of the other. The presence of reductants such as antioxidant substances in the antioxidant samples causes the reduction of the Fe^{3+}/ferricyanide

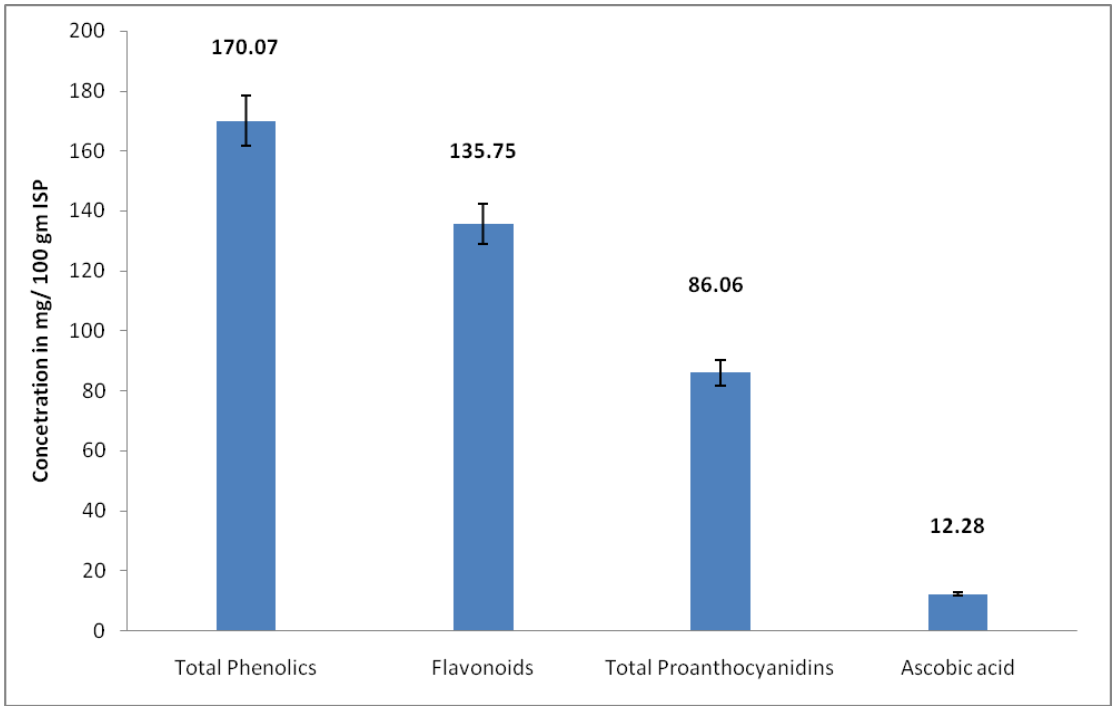

Figure 1. Contents of Phenolics, Flavonoids, proanthocyanidins and ascorbic acid in 100 gm of ISP.

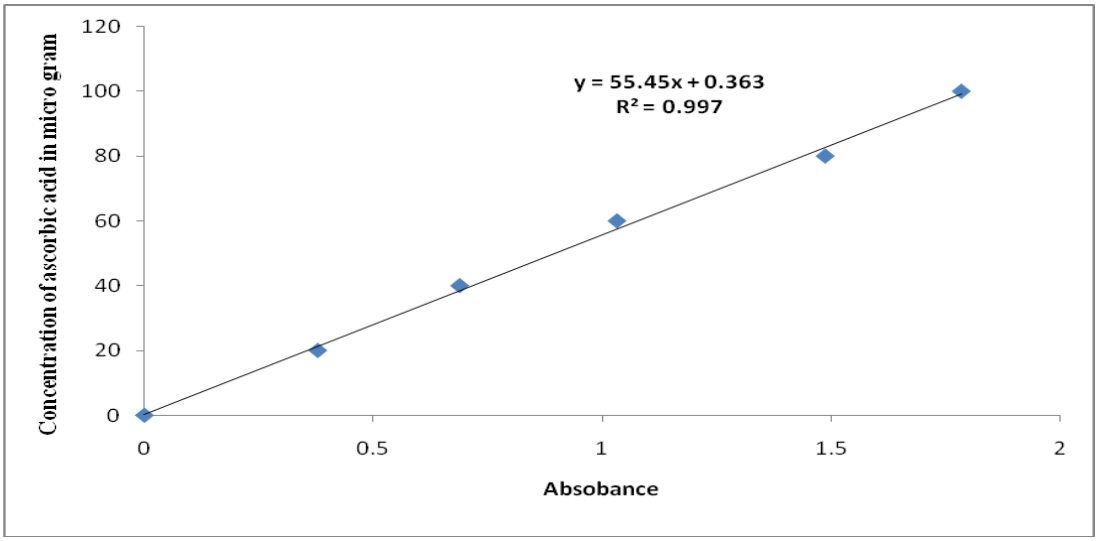

Figure 2. Standard calibration curve of ascorbic acid for total antioxidant determination.

complex to the ferrous form. Therefore, Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Chung *et al*., 2002). The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity.

ISP had effective and powerful reducing power using the potassium ferricyanide reduction method when compared to the standards.

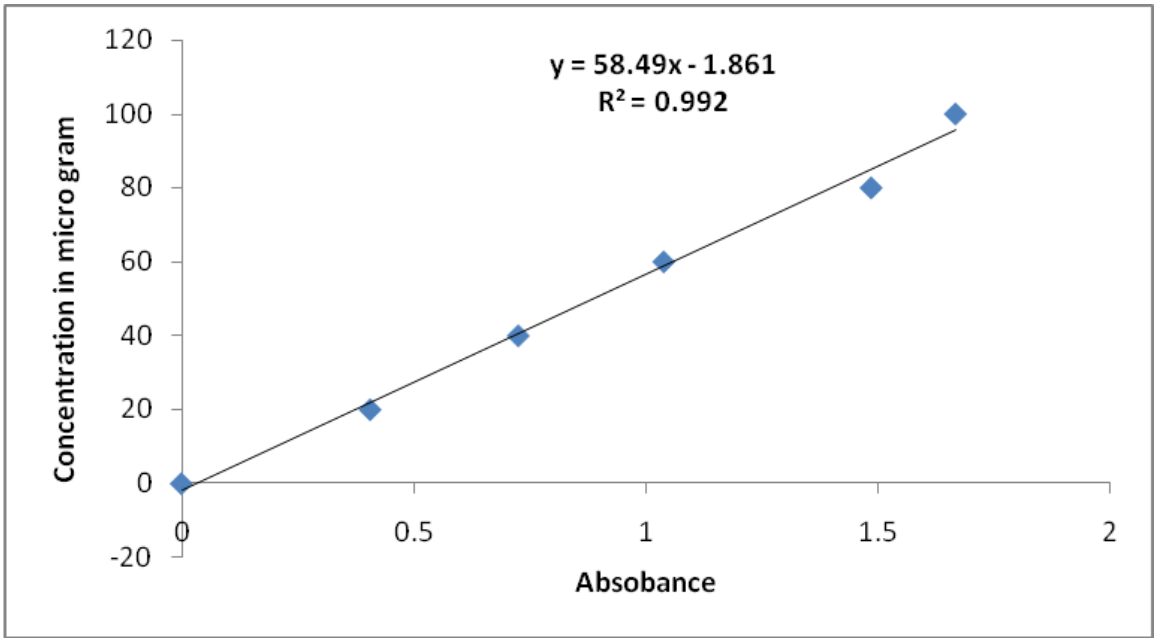

Figure 3. Reducing power of ascorbic acid

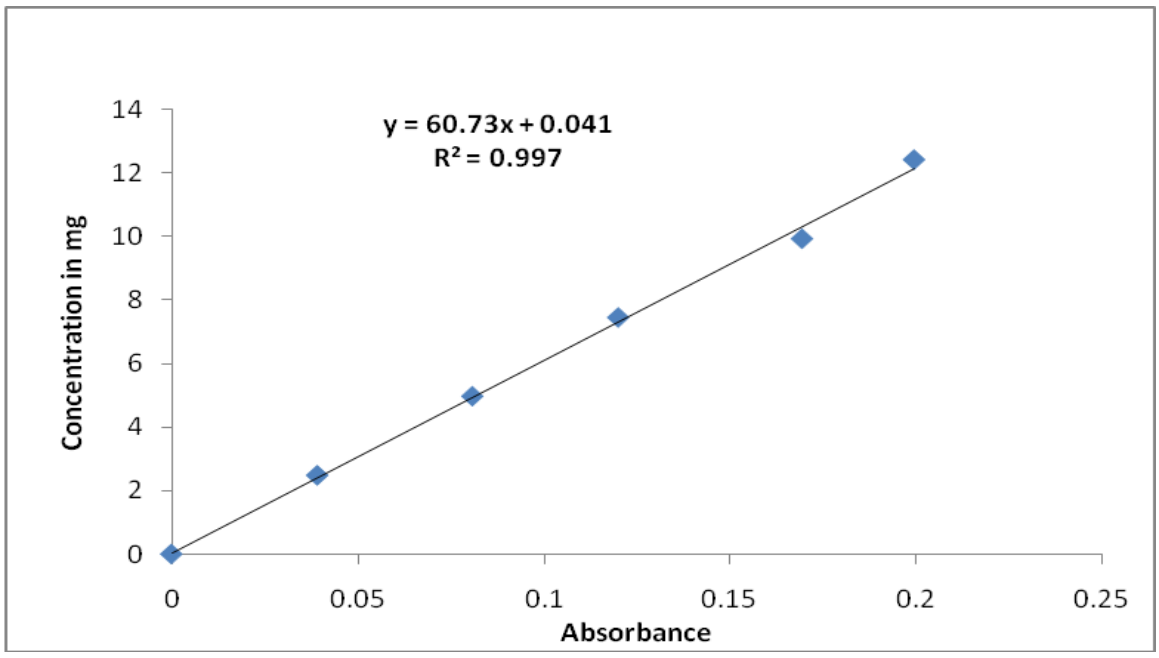

Figure 4. Reducing power of ISP

Reducing power will increase accordingly to the increase in absorbance. As more Fe3+ are reduced to the ferrous form or when more electrons are donated by antioxidant components. Reductants also react with certain precursors of peroxide, thus preventing the formation of peroxide (Janthi P. *et al*., 2001).

The results on reducing power demonstrate the electron donor properties of ISP thereby neutralizing free radicals by forming stable products. The outcome of the reducing reaction is to terminate the radical chain reactions that may otherwise be very damaging.

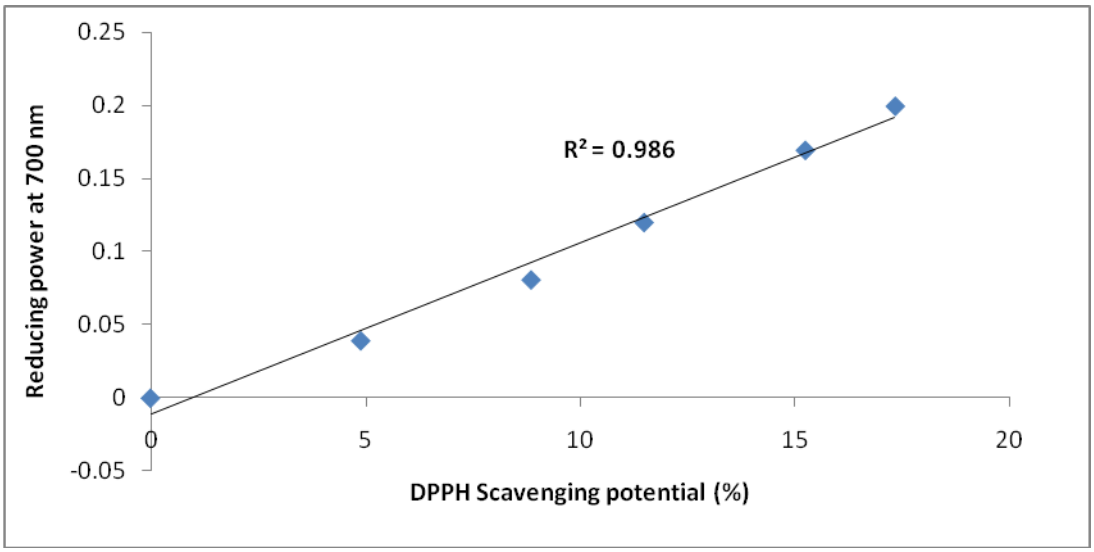

Figure 5. Correlation between DPPH and reducing power in ISP.

CONCLUSION

According to data obtained from the present study, ISP was found to be an effective antioxidant in different in vitro assay including reducing power, DPPH radical and total antioxidant capacity and the content of ascorbic acid, phenolics, flavonoids and proanthocyanidins also present remarkably.

ACKNOWLEDGE

This work was carried under the research and development (RandD) project of the Bangladesh Council of Scientific and Industrial Research (BCSIR), Ministry of Science and Technology, Government of the Peoples' Republic of Bangladesh.

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