EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 8
Issue: 1
49-56
Year: 2021

TOTAL PHENOLICS, FLAVONOID, ASCORBIC ACID, AND IN-VITRO ANTIOXIDANT ACTIVITY OF SOYA CHEESE

Ummey Hafsa Bithi, M. N. Uddin and Kanika Mitra*

Institute of Food Science and Technology (IFST), Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhanmondi, Dhaka-1205, Bangladesh.

***Corresponding Author: Kanika Mitra**
Institute of Food Science and Technology (IFST), Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhanmondi, Dhaka-1205, Bangladesh. **DOI: https://doi.org/10.17605/OSF.IO/YNXRA**

Article Received on 27/10/2020 **Article Revised on 17/11/2020** **Article Accepted on 07/12/2020**

ABSTRACT
In this work, the contents and antioxidant ability of various classes of phenolic compounds present in the Cheese of soybean were evaluated. Total phenolics and flavonoids were determined spectrophotometrically by using tannic acid and catechin standards, respectively after extraction of Cheese with methanol. Also, the ascorbic acid contents were determined by UPLC-MS-MS.The in vitro antioxidant models studied are the total antioxidant capacity by phosphomolybdenum method, the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity assay, and reducing power by using standard ascorbic acid. The methanol solvent extract of cheese contains significant amounts of phytochemicals including total phenolic (123.3 mg gallic acid equivalent/100g), and flavonoids (78.28 mg catechin equivalents/g) compared to the raw soy extract. Similarly, in the cheese extract of methanol solvent, has high level of total antioxidant activity (about 472.07 mg ascorbic acid equivalent/100g) and reducing power (11.76±0.09 mg ascorbic acid equivalent per 100 gm) than raw soy. The quantity of ascorbic acid was found to be 119.03±4.07µg/100g in Cheese. The free radical scavenging activity of the Cheese was measured and the results are shown that the IC_{50} is 138.73±3.00mg (fresh basis) and 43.01±0.92 mg (dry basis). The results of the present investigation strongly indicate the importance of this soy product for human health.

KEYWORDS: UPLC-MS-MS, ascorbic acid, total phenols; flavonoids; total antioxidant activity; DPPH assay; reducing power.

INTRODUCTION
Plant and plant products are being used as a source of medicine for a long time. Among the most important constituent of edible plant produce, low molecular weight antioxidants are the most important species.[1] Phenolic compounds are abundantly present in the human diet and act as antioxidants and are widespread constituents of fruit, vegetables, cereals, olive oil, dry legumes, chocolate, and beverages.[2] Also, they are found in both edible and non-edible plants. They may exert antioxidant effects as free radical scavengers, as hydrogen donating sources, or as singlet oxygen quenchers and metal ion chelators.[3] Phenolic compounds are known to counteract oxidative stress in the human body by helping to maintain a balance between oxidant and antioxidant substances.[4,5]

Antioxidants have been shown to play an important role in preventing many diseases like cancer, inflammation, and brain dysfunction. The interests in phenolic compounds, particularly flavonoids have considerably increased in recent years because of their broad spectrum of chemical and diverse biological properties. Flavanoids have been associated with a possible role in the prevention of several chronic diseases involving oxidative stress as well as their protective effect against low-density lipoprotein (LDL) oxidation.[6]

Soybean seed extracts suggest that phenolic content should be considered as an important feature of soybeans, besides protein and oil contents. Soybeans are widely accepted as a "healthy food" and some of their pharmacological effects could be attributed to the presence of these valuable constituents.[7] Consumption of soybeans and soy products has been associated with reducing the risks of various cancers, such as prostate and mammary, and several other chronic inflammatory diseases. The health-promoting activity associated with soy consumption is attributed to the presence of isoflavone. The structural similarities of isoflavones to naturally occurring estrogens may protect hormone-dependent cancer by modulating the activity of estrogen cholesterol levels. Soybean isoflavones have reportedly increased HDL cholesterol and lowered LDL cholesterol.[8]

The present study aimed to carry out the determination of total phenolic, flavonoid, and vitamin-C content in the Soy Cheese and its in-vitro antioxidant activity.

MATERIALS AND METHOD

Sample preparation and extraction

Soya bean was collected from the local market of the capital city Dhaka, Bangladesh. The cheese was produced by coagulating the soya milk. After production of the soya Cheese, it was preserved by using the solution of citric acid, sodium chloride, and heating in the oven for the appropriate time. After these steps, the Cheese was preserved in the refrigerator until analysis. For desired chemical analysis, about 2-3 gm of the Cheese sample was taken and homogenized in the mortar with a pestle, shaken mechanically, and finally sonicated. Then, the sample was filtered and the filtrate solution (extract) was stored in a freeze for analysis.

Total phenolics content (TPC)

The amount of total phenol content was determined by the Folin-Ciocalteu reagent method[9] with some modification. 0.5 mL of the extracted sample and 0.5 mL of Folin-Ciocalteu reagent (0.5 N) were mixed and incubated at room temperature for 5 min. Then, 2.0 mL saturated solution of sodium carbonate was added to the mixture and further incubated for 30 min at room temperature, and the absorbance of the solution was measured at 765 nm. Tannic acid was used as positive controls.[10] The total phenolic content was expressed in terms of standard equivalent (mg^{-1} of the extracted compound).

Total flavanoid determination

Total flavonoid content was determined by the Aluminium chloride method[11] using catechin as a standard. 1 mL of the test sample and 4 mL of water were taken in a volumetric flask (10 mL volume) and after 5 min, 0.3 mL of 5 % Sodium nitrite, 0.3 mL of 10% Aluminium chloride were added. After 6 min incubation at room temperature, 2 mL of 1 M Sodium hydroxide was added to the mixture. Immediately the final volume was made up to 10 mL with deionized water. The absorbance of the reaction mixture was measured at 510 nm against a blank sample spectrophotometrically (UV-1609, Shimadzu, Japan). Results were expressed as catechin equivalents (mg catechin/g dried extract).

Determination of ascorbic acid by UPLC-MS-MS

Ascorbic acid was determined according to Laloo and Sahu[12] with some modification.

Chemicals, solvents and experimental conditions

Formic acid (BDH, AnalaR), methanol (BDH, AnalaR), acetic acid (BDH, AnalaR), HCl (reagent grade), and deionized water were used in the experiment. Ascorbic acid was purchased from Sigma-Aldrich. The standard solution was freshly prepared.

UPLC-MS-MS Condition

LC Condition

LC System: Acquity UPLC system, Column: Acquity UPLC C18, 2.1X50 MM, 1.8 micrometer, Column temperature: 40 ℃, Sample temperature: 4 ℃, Flow rate: 0.4 mL/min. Mobile Phase A: 0.1% formic acid in the water, Mobile Phase B: 0.1% formic acid in CAN, Total runtime: 5.0 min, Injection Volume: 10.0 microliters, full loop.

MS Condition

MS System: Xevo TQ MS, Ionization: ESI Positive, Capillary Voltage: 1.0 KV, Source Temperature: 130℃, Desolvation Temperature: 450 ℃, Desolvation gas: 900L/hr, Acquisition: Multiple reaction monitoring (MRM) with RADAR full scan, Collision gas: Argon at $3.5x10^{-3}$ mhb.

Preparation of standard solution

The standard samples were accurately weighed and transferred into three 100 mL volumetric flask separately. Initially, 7 mL of acetic acid and 50 mL of methanol were added to each flask; the contents were dissolved by sonication for 10 min and allowed to cool to ambient temperature. The contents were diluted to volume with water and thoroughly mixed. These solutions were used as reference working standard solution. Before injecting into the liquid chromatography, the solution was filtered through a 0.45-micrometer membrane filter. The samples were quite stable at room temperature. The stock solutions of standards were kept in a refrigerator for further use and remain unchanged for a period of a month.

Preparation of sample solution

15.07 g of neutraceutical enriched with vitamins was accurately weighed and transferred into a 250 mL round bottom flask. Initially, about 10 mL of 0.1 N HCl and 80 mL water were added and then reflux on boiling water bath for 15 min. After completion of the refluxing period, the flask was cooled and volume made up to 100 mL in a volumetric flask. The content was centrifuged (1400 rpm) to remove suspended material. The supernatant solution was first filtered through a Whatman qualitative 1 filter paper and the resulting filtrate was again filtered through a 0.45-micrometer membrane filter before injection into the LC system. The stock solutions of the sample were kept in a refrigerator for further use and remain unchanged for a period of a month.

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.[13] 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 of the extract was mixed with a mixture of 3 mL of reagent solution (0.6 M

sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes containing the reaction solution were then capped and incubated at 95 °C for 90 min. After the samples had cooled to room temperature, the absorbance of the solution was then measured at 695 nm against the blank. Methanol (0.3 mL) in the place of the extract was used as the blank. The antioxidant activity is expressed as the number of equivalents of ascorbic acid.

Free radical scavenging assays

The DPPH is a stable free radical and 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 hydrogen–donating antioxidant due to the formation of the non-radical form DPPH-H.[14] 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 was measured by using 2, 2-diphenyl-1-picryl-hydrazyl or 1, 1-diphenyl-2-picryl-hydrazyl by the method of McCune and Johns[15] 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. The mixture was incubated for 10 min in dark, and then the absorbance was measured at 517 nm against methanol as a blank, and control was prepared by DPPH and methanol in place of sample extract. In this assay, the positive control was ascorbic acid. The percentage of inhibition was calculated using the following formula.

Inhibition (%) = (A0 – A1 / A0) × 100

Where A0 is the absorbance of the control and A1 is the absorbance of the test.

Reducing power (RP)

The reducing power was determined by following Jayanthi et al.[16] 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 50 ºC in a water bath for 20 min. After cooling, 2.5 mL of 10% trichloroacetic 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 similarly excluding samples. Ascorbic acid at various concentrations was used as a standard. Increased absorbance of the reaction mixture indicates an 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 2013.

RESULTS AND DISCUSSION

The antioxidant activity of phenols is mainly due to their redox properties which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quencher. Besides, they have a metal chelating potential. Flavanoids and polyphenolic compounds are responsible for health care because of their antioxidant and anticancer properties. Bio-active compounds found in plants are known to possess antioxidant activity.[17]

Total phenolics

The total phenolic content of the Cheese was measured using the Folin-Ciocalteu method (Standard curve shown in figure 1A), and the results are shown in Table 1. As seen from Table 1, the total phenolic content is 123.3±2.4 mgTAE/100g (dry basis) and 37.66±0.60 mgTAE/100g (Fresh basis). Plant polyphenols, a diverse group of phenolic compounds (flavanols, flavonols, anthocyanins, phenolic acids, etc.) possess ideal structural chemistry for free radical scavenging activity. Antioxidative properties of polyphenols arise from their high reactivity as hydrogen or electron donors from the ability of the polyphenol derived radical to stabilize and delocalize the unpaired electron (chain-breaking function) and from their potential to chelate metal ions (termination of the Fenton reaction).[18]

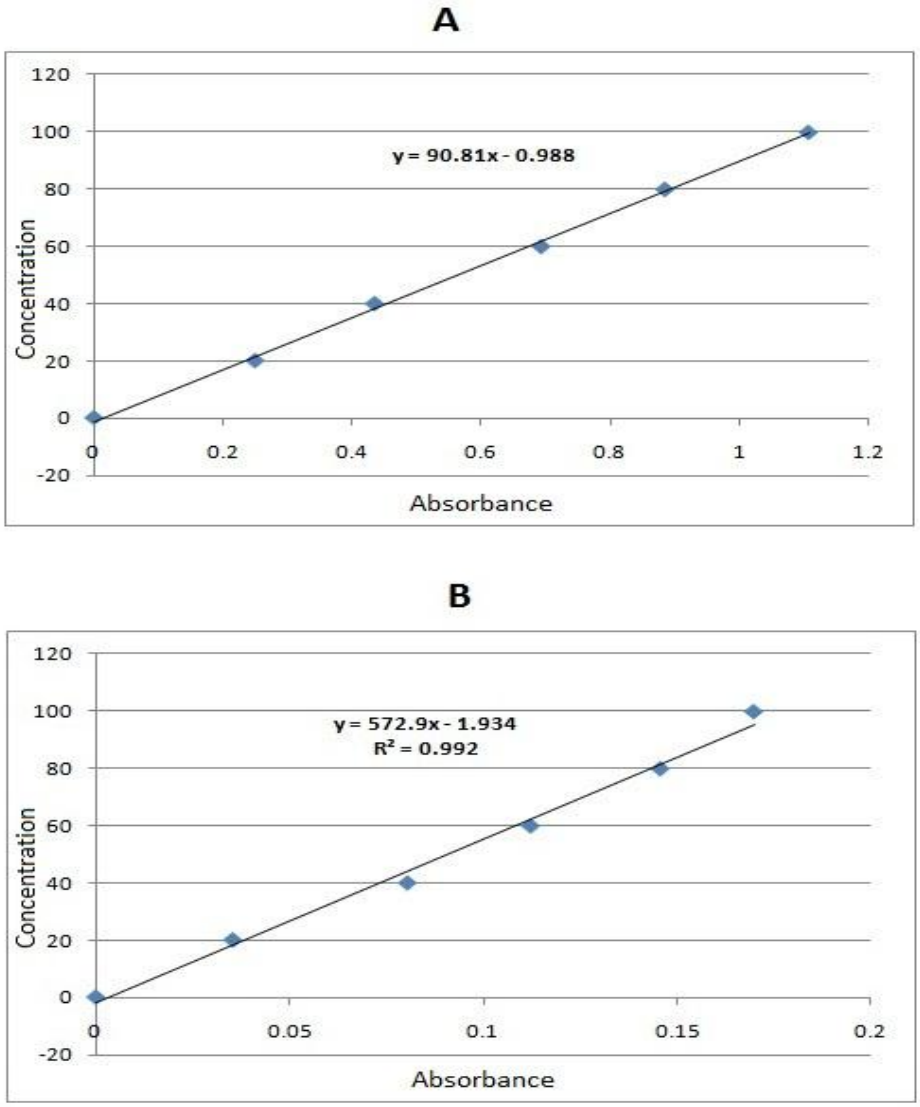

Figure 1: A. Standard curve of tannic acid for determining total phenolics, B. Standard curve of catechin for determining flavonoids.

Table1: Total amount of phenolic, flavonoid, and ascorbic acid content of the methanolic extract Cheese (Mean ± S. dev., N=3).

Parameters	Soy Cheese (Fresh)	Soy Cheese (dry)
Total phenolics mg/100 g Cheese (in TAE)	37.66±0.60	123.3±2.4
Total flavonoid mg/100 g Cheese (in CE)	23.98±3.59	78.28±11.72
Ascorbic acid µg/100 g Cheese	36.47±1.24	119.03±4.07

Flavonoids

Flavonoid content of the Cheese was measured (Standard curve shown in figure 1B) and the results are shown in Table 1. As seen from Table 1, the total phenolic content is 78.28±11.72mgCE/100g (dry basis) and 23.98±3.59mgCE/100g (Fresh basis). 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 the free radical generation.[19] Depending on their structure, flavonoids can scavenge practically all known ROS.

Ascorbic acid determination

Ascorbic acid acts as a chain-breaking antioxidant impairs the formation of free radicals in the process of formation of intracellular substances throughout the body, including collagen, bone matrix, and tooth dentine.[20] The quantitative determination of ascorbic acid in Cheese shows that they are a good source of

ascorbic acid. The quantity of ascorbic acid was found to be 119.03±4.07µg/100g in Cheese. A striking pathological change resulting from ascorbic acid deficiency is the weakening of the endothelial wall of the capillaries due to a reduction in the amount of intercellular substance. Therefore, a clinical manifestation of scurvy hemorrhage from the mucous membrane of the mouth and gastrointestinal tract, anemia, pains in the joints can be related to the association of ascorbic acid and normal connective tissue metabolism.[21]

Total antioxidant capacity

The total antioxidant capacity in all the three Cheese methanolic extracts was determined using the linear regression equation of the calibration curve (Standard curve shown in figure 2A) and was expressed as ascorbic acid equivalent (Table 2). The total antioxidant capacity of the Cheese was measured and the results are shown in Table 2. As seen from Table 2, the total antioxidant capacity is 472.07±12.86 mg ascorbic acid equivalent/100g (dry basis) and 144.64±3.94 mg ascorbic acid equivalent /100g (fresh basis).

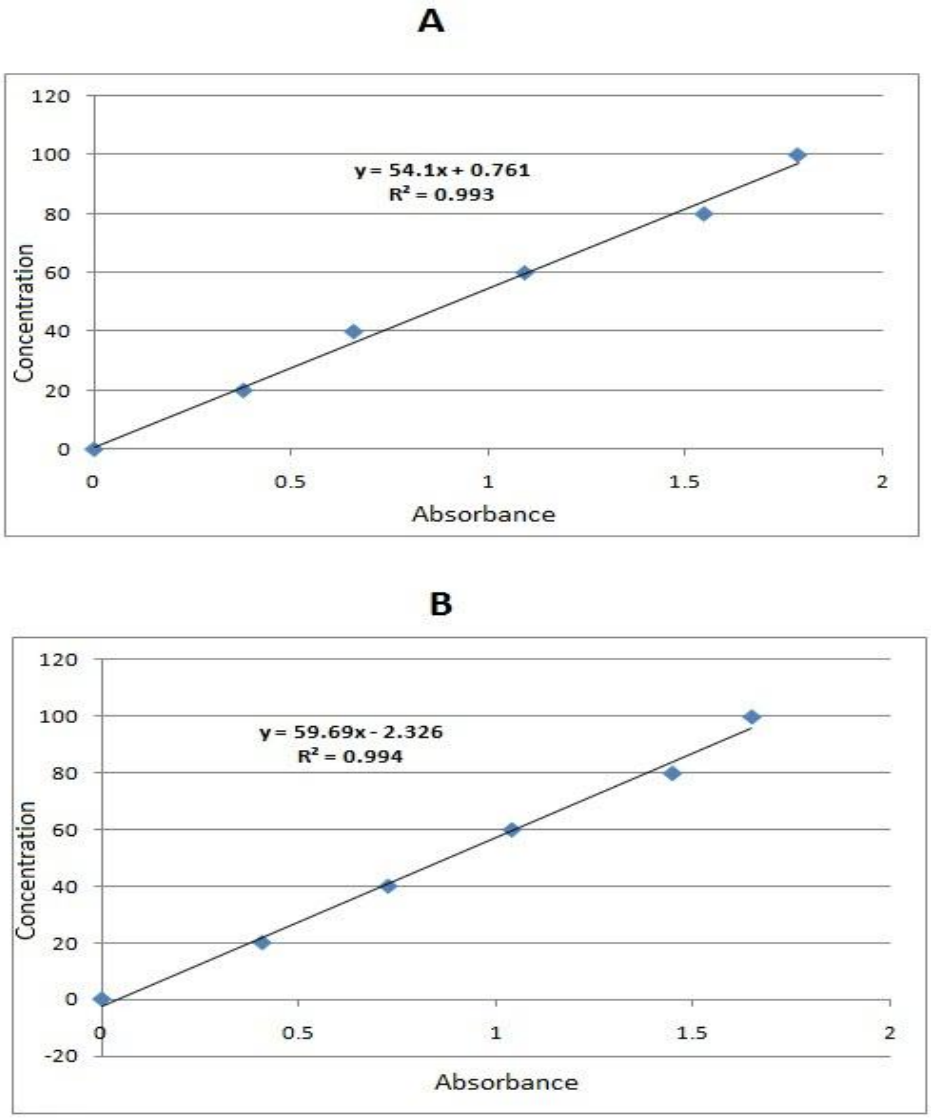

Figure 2: A. Standard curve of ascorbic acid for determining total antioxidant, B. Standard curve of ascorbic acid for determining reducing power.

Table 2: Total antioxidant and radical scavenging activity of standard and 100 gm Cheese methanolic extracts (IC50 expressed as Mean ± S. dev.).

Parameters	Soy Cheese (fresh)	Soy Cheese (dry)
Total antioxidant capacity Equivalent to Ascorbic acid	144.64±3.94	472.07±12.86
educing power (RP) Equivalent to Ascorbic acid	3.60±0.03	11.76±0.09

DPPH Assay

The free radical scavenging activity of the Cheese was measured and the results are shown in Table 3. As seen from Table 3, the IC 50 is 138.73±3.00mg(fresh basis) and 43.01±0.92 mg(dry basis).The free radical scavenging activity of the ethyl acetate extract was also evaluated through its ability to quench the synthetic radical 1,1-diphenyl-2-picryl-hydrazyl(DPPH). The phenolic compounds or secondary metabolites, constitute a wide and complex assay of phytochemicals that exhibit antioxidant action and consequently, a beneficial physiological effect.[22]

The key role of phenolic compounds scavenger of free radicals is emphasized in several reports. Phenols are important components of plants. They were reported to eliminate radicals due to their hydroxyl groups, and they contribute directly to the antioxidant effect of the system. Polyphenolic compounds have an important role in stabilizing lipid oxidation and are associated with antioxidant activity.[23]

DPPH is a reactively stable free radical. The assay is based on the measurement of the scavenging ability of antioxidants towards the stable free radical DPPH. From the present results, it may be postulated the soya Cheese reduces the radical to the corresponding hydrazine when it reacts with the hydrogen donors in the antioxidant principle.

Table 3: DPPH free radical scavenging activity of soy Cheese and ascorbic acid (IC50 expressed as Mean ± S. D.).

Concentration in µg	% of Inhibition	IC 50in µg	Concentration in mg	% of Inhibition	IC 50 Fresh basis in mg	IC 50 Dry basis in mg
5	66.99	3.37±0.02	5.30	4.87	138.73±3.00	43.01±0.92
10	83.52		10.60	6.58		
15	91.83		15.90	9.10		
20	97.50		21.20	10.78		
25	97.92		26.50	11.70		

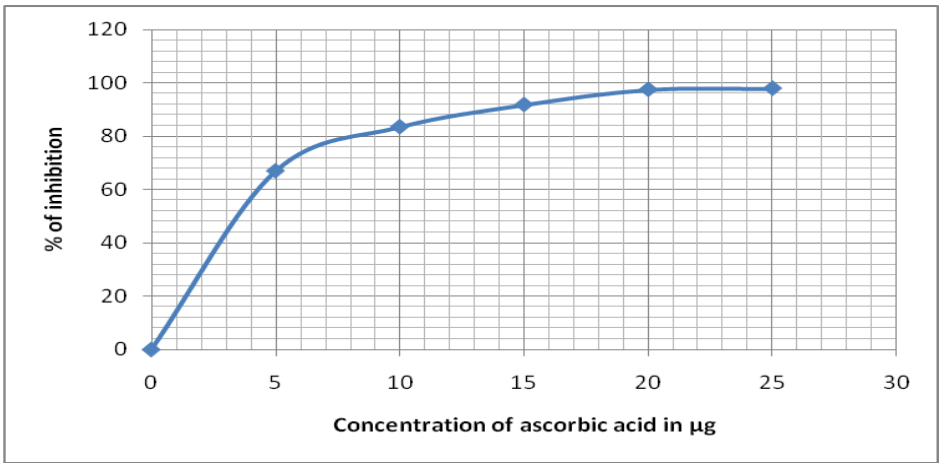

Figure 3: DPPH radical scavenging activity of ascorbic acid.

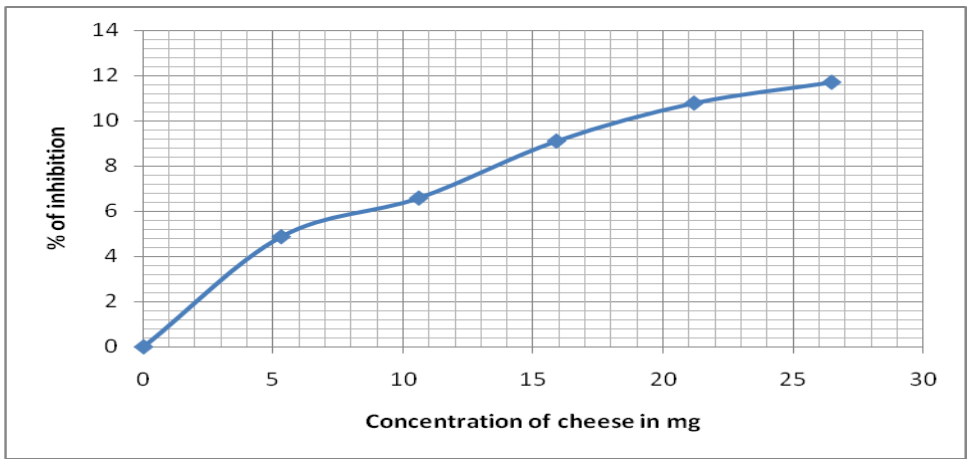

Figure 4: DPPH radical scavenging activity of Soy Cheese.

Reducing power assay

Table 2 shows the reducing power of the extracts using the potassium ferricyanide reduction method Standard curve shown in figure 2B). The reducing capacity is 11.76±0.09 mg ascorbic acid equivalent per 100 gm Cheese**.** It has been reported that the reducing power was associated with the antioxidant activity and its relationship of phenolic constituents has been well established in several plant sources including vegetables.[24] The yellow color of the test solution changes to various shades of green and blue depending upon the reducing power of each extract. The presence of reductant (Antioxidants) in the Cheese extracts causes the reduction of Ferric (Fe^{3+}) cyanide complex to ferrous (Fe^{2+}) form. Therefore, Fe^{2+}-complex can be monitored by measuring the formation of Perl's Prussian blue at 700 nm.

Polyphenols in the Cheese extracts appear to function as good electron and hydrogen atom donors and therefore should be capable of converting free radicals to more stable products. Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity.[25] Cheese with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes so that they can act as primary and secondary antioxidants.[26]

CONCLUSIONS

In our present investigation, we found that soy Cheese has varying antioxidant activity. Moreover, the isolation, identification, and antioxidant studies of different Phenolic phytoconstituents may be carried out to identify the specific phenolic phytoconstituents having the major role in antioxidant activity. The results of the present investigation strongly indicate the importance of this soy product, and this product could serve as a very useful source for antioxidant.

Funding

This work was carried out under the research and development (R and D) project ("Development of Nutritive food products using gluten free raw materials", Project duration was July 2018-June 2020) of the Bangladesh Council of Scientific and Industrial Research (BCSIR).

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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