Available online at www.scholarsresearchlibrary.com

Scholars Research Library

Journal of Natural Product and Plant Resources, 2023, 13 (1): 1-13
(http://scholarsresearchlibrary.com/archive.html)

ISSN: 2231-3184

Total Phenolic Content, Flavonoid Content and Antioxidant Activities of Sprouted and Fermented Soybean (*Glycine max*) Seed Extracts

Shivani Chapagain*

Department of Pharmacy, Universal College of Medical Sciences, Tribhuwan University, Rupandehi, Nepal

***Corresponding Author:** Shivani Chapagain, Department of Pharmacy, Universal College of Medical Sciences, Tribhuwan University, Rupandehi, Nepal; E-mail: Cwanichapagain17@gmail.com*

***Received:** 17 September, 2020, Manuscript no. jnppr-23-19335; **Editor assigned:** 22 September, 2020, Pre QC no. jnppr-23-19335 (PQ); **Reviewed:** 06 October, 2020, QC no. jnppr-23-19335 (Q); **Revised:** 02 June, 2023, Manuscript no. jnppr-23-19335 (R); **Published:** 30 June, 2023*

ABSTRACT

Soybeans (Glycine max L.) are a species of legumes which is nutritionally rich source of dietary fibers and phytochemicals such as flavonoids phenolic, and isoflavones. Germination and fermentation is the most effective process to improve the quality of legumes. In this study, Total Phenolic Content (TPC), Flavonoid Content (TFC) and Antioxidant activity of germinated and fermented two different varieties of soybean seed was evaluated. Sprouting of seeds, fermentation, evaluation of TPC, TFC and antioxidant activity of the extract were done by using the standard method as described. The methanolic extracts of black soybean seeds and sprouts have higher antioxidant activity than the yellow soybean seeds and sprouts. Fermentation significantly increased the TPC, TPC and antioxidant activity of soybean seed and sprouts. The highest antioxidant activity was observed in fermented black soybean sprouts extract 88.02% DPPH, 88.54% H_2O_2, 6.56 eq to ascorbic acid FRAP value and 70.03% of lipid peroxidation inhibition activity. The TPC and TFC were noted 13.18 ± 0.04 mg GAE/gm of extract and 66.9 ± 0.68 mg QE/gm of extract, respectively. A strong positive correlation was observed between phenolic content with antioxidant activity with DPPH (R^2=0.8124), H_2O_2 (R^2=0.8092) and FRAP (R^2=0.9806), respectively. This study showed that the effect of germination and fermentation enhances Phenolic, flavonoid and antioxidant activity. Thus, germination and fermentation proved to be desirable bioprocess for producing enriched ingredient with health promoting antioxidant compound in a natural way.

Keywords: Legumes, Sprouting, Fermentation, Anti-oxidant, Correlation, Food nutrition

INTRODUCTION

Legumes belonging to *Leguminosae (Fabaceae)* family are the world's second most important food source after cereals. Grains of legumes are the good source of micro-nutrients, carbohydrates dietary, fibers, vitamins, minerals, etc. In addition to it nutritional components, legumes also incorporates bioactive additives along with polyphenols, flavonoids, saponins, alkaloids and antioxidants that exerts physiological results. Many studies have shown that most of the bioactive chemical compounds on legumes play an essential position in stopping persistent illnesses including stroke, type as stroke, kind diabetes, cardiovascular risks and cancer. Oxidative stress is a major chance factor inside the pathogenesis of severa continual illnesses. Free radicals and other reactive oxygen species are the sellers involved in the pathogenesis of illness such as allergies, inflammatory, most cancers human aging, atherosclerosis. Antioxidants can also help to prevent oxidative harm within the human body. As an end result, scientific interest has expanded inside the degrees of antioxidant components in meals [1].

The expanding enthusiasm towards more beneficial food and ways of life has guided the scientific network to give incredible consideration to the field of free radicals and cancer prevention agent compounds. It is believed that significant antioxidant activity of food is due to the presence of high phenolic and flavonoid content. The antioxidant activity of legumes can be performed by various mechanisms: (i) Eliminate species that perform peroxidation, (ii) provide a hydrogen or an electron, (iii) chelate metal ions preventing the generation of reactive species or lipid peroxides de-composition, (iv) reduce the radical O_2- preventing peroxides formation, (v) breaking anti oxidative chain reaction, (vi) prevent pro-oxidative enzymes and/or (vii) reduce localized O_2 concentrations.

Soybean or Soy is a legume of the family Fabaceae. Soy (*Glycine max (L.)* Merrill) is a quickly developing herbaceous yearly plant up to 1 m high. It is local to south East Asia however as of now becomes around the world. Soy beans are extremely rich in isoflavones and soy protein, are exceptionally famous food in japan (Figure 1).

Taxonomical classification

Kingdom: Plantae

Sub-kingdom: Tracheobionta

Phylum: Tracheophyta

Super-division: Spermatophyta

Division: Mangoliophyta

Class: *Mangoliopsida*

Sub-class: *Rosidae*

Order: *Fabales*

Family: *Fabaceae*

Genus: *Glycine*

Species: *max*

Common names

Hindi name: Vattmas

Nepali name: Bhatmas

English name: Soybean, coffee bean

Variety of soybean: Black, yellow, Brown

Figure 1. Variety of soybeans

Geographical distribution

Soybean is local to Eastern Asia, basically China, Korea and Japan, from where it spread to Europe and America and others parts of the world in the 18th century.The plant is categorized as an oilseed rather than a pulse. It is a yearly plant that has been utilized in china for a long time as a food and a segment of medications. Cultivation is effective in tropical climate with optimum growing conditions in mean temperatures of 20°C to 30°C (68°F to 86°F), temperatures of below 20°C and over 40°C (68°F, 104°F) stunt growth fundamentally. They can develop in a wide scope of soils, with ideal development in sodden alluvial soils with a decent natural content [2].

Botanical description

The tallness of the plant ranges from less than 0.2 m to 2.0 m. The different parts of plants are enclosed with fine brown or gray hairs. The leaves are trifoliate, having three to four leaflets are 6 cm-15 cm (2.4-5.9 in) long and 2 cm-7 cm (0.79-2.76 in) broad. The fruit is a hairy shell that grows in clusters of three to five; each is 3-8 cm long (1-3 in) and usually contains two to four (rarely more) seeds which is 5 mm-11 mm in diameter.

Chemical constituents

Soybean comprises significant amount of all the essential amino acids. It is a good source of protein and vegetable oils, too. The Phytochemicals present in the glycine max are: Ferritin Saponins (2%) Phospholipids (1%-3%): Phospatidyl choline, phosphatidyl ethanolamine, phospatidyl inositol, phospatidic acid Isoflavones: Daidzin, genistein, glycitein vitamins and minerals: Tocopherols (α-tocopherols, β-Tocopherols, γ-tocopherols, δ-Tocopherols), vitamin-B, but it lacks vitamin-B12 and vitamin-C. Minerals presents are K, P, Mg, Ca and Fe. Oils (19%): Linoleic acid, α-linoleic acid. Proteins: Storage globulins and there is presence of biologically active protein components such as; Haemagglitinin, Trysin inhibitors, α-amylase and lipoxygenases.

Nowadays, there is a wide interest in the effects of processing on the nutritional value, especially on antioxidant compounds of legumes. Indeed, soybean seeds contain excessive protein and lipid in addition to many bioactive compounds with antioxidant activity that can make a contribution to fitness advertising inside the prevention of cancers together with breast and prostate cancers, cardiovascular illnesses, bone fitness, and diabetes. For these reasons, soybeans are widely used in food industry and occupy an important place in human nutrition worldwide. There are different processes such as breeding, cooking, soaking sprouting or fermenting, that dramatically influence the content of bioactive compounds in legumes.

Germination and fermentation is the most effective process to improve the quality of legumes. Germination is a simple and inexpensive process and increases the beneficial effects by reducing anti-nutritional and indigestible factors. During germination process, end enzymes are activated to produce bioactive compounds by hydrolyzing starches, protein and lipids. While, the Fermentation process was carried out naturally by the microorganism presents in the seeds. It enhances the release of bound phenolic compounds and also converts phenolic compounds into different metabolites which increases their bioactivity and improve their anti- oxidative activity. The both bioprocess increases the anti-oxidant property of different types of soybeans seeds. On the basis of this consideration, this study was carried out to evaluate total phenolic content, flavonoid content and antioxidant activities of different types of soybean (*G. max*) seed and sprouts extracts. Sunlight is the only energy source used in the majority of crop agriculture for photosynthesis. The amount of sunlight that is avail-able March not always be enough to support year-round, vigorous crop production as we transfer agricultural production indoors. Therefore, supplementary lighting is frequently needed for crop cultivation indoors. When we shift crop production into completely enclosed structures, a so-called sole-source lighting system must supply all of the energy needed for photosynthesis. Since robust plant development requires very high light intensities, the electricity consumption connected with plant lighting. Systems are often con-siderable and will have a significant impact on the cost of manufacturing. reported on the important performance parameters, such as power consumption and efficacy, of the various lamp types utilized in horticulture applications. Controlling temperature and humidity is another significant expense for vertical farming operations in addition to personnel costs. Additionally, the expense of continuously pumping water can be fairly high when crops are cultivated on numerous floors [3].

Similar to other types of agriculture, vertical farming techniques are frequently improved through trial and error. This process is time- and money-consuming, and the systems it produces are frequently site-specific due to regional factors and limitations. Addition-ally, it makes the creators more defensive of their methods and solutions. Although sensible, this mindset has precluded widespread col-laborations with academic researchers and forced start-up businesses to frequently reinvent the wheel. We think that this situation has slowed down progress, and we urge the vertical farming sector to come to agreements that protect important production components while also fostering more cooperation and information sharing. This essay's goal is to offer suggestions for anyone organizing or running a vertical farm. We are aware of the potential that verti-cal farming has to expand the availability of wholesome food, particularly in areas with high population densities, and tomake some components of our food supply chain more efficient and resilient. On the other hand, we want to draw attention to a few problems that, in our opinion, call for deeper analysis and study.

Constructing a vertical farm

Operating and capital costs for vertical farming systems can be high. Venture capital or investment businesses frequently need tomake investments in the larger vertical farms. Finding the right spot can be difficult, especially in urban regions where the cost of land and buildings is high. In order to cultivate, harvest and marche store the crop, resources including labour, energy, and supplies are required. Energy is often the second-highest operational expense after labour, but additional inputs are also required. It is anticipated that production prices would decrease as the vertical farming sector grows and production techniques and methodsgain popularity. According to, there are numerous locations in most cities that are suited for vertical farming and with the right planning, busi- nesses can operate profitably while offering crucial services to the surrounding community. However, institutional, monetary, and technological assistance are still lacking in many places. Additionally, it is not always expected that planners and decision- makers will make supporting decisions since they lack appropriate expertise about commercial indoor crop-production techniques. Therefore, explaining the suggested designs and persuading decision-makers that vertical farming can have demonstrable advantages

for regional communities march need a lot of work. Urban sites such as roofs, warehouses, and other vacant or abandoned areas can be effectively utilized for vertical farming. There are many such under-utilized structures and sites in the United States. Some businesses intend to build their operations nearby urban areas to cut costs while yet requiring short travel distances. The suitability ofproperties and struc-tures for use as vertical farming operations depends on the length of time a property is available, the amount of space available, and the site's zoning. While some localities, districts, counties, or states March have laws, rules, or regulations that support agriculture and the development of facilities related to it such laws often do not take into account the particular problems posed by vertical farming. Due to the rarity of vertical farms, zoning officials March need to be informed about the proposed use andany potential effects, and a zoning variance March need to be obtained. As a result, more time March be needed to obtain all the licenses needed to run a verti-cal farm. When developers suggest new vertical farming operations, a thorough feasibility assessment might help allay any worries.

Technical difficulties and marketability should be taken into account while picking a crop for a vertical farm. The capacity to create and manage a vertical farming system that cultivates the chosen crop is one of the grow-technical difficulties. Marketability is a term used to describe how competitive the items produced in such a system are. Any crop March theoretically be cultivated in a vertical farm, although the majority would present significant technical and growing difficulties. For instance, because of their tiny size, quick growing cycles, and generally minimal energy requirements, many vertical farming systems produce leafy greens. However, huge, energy-intensive crops like tree fruit or heavy vining crops March need a specific design that is not typical of a vertical farming system. As a result, vertical farms rarely grow these types of crops. Companies specializing in designing vertical farming technology are in charge of creating unique designs for those systems.

Operators must think about a number of issues relating to how marketable their product is in addition to what is grow-technicallypossible. Because they require more work and energy than conventionally grown crops, vertical farm produce typically cannot com- pete on price. Therefore, in order to compete with crops farmed in a traditional manner, their goods must have some added value.The main objective of the majority of firms is to increase profits for the owners or other stakeholders. Following that, most companies will concentrate on their clients, the neighborhood, and society as a whole. The existential problems that our communities are currently facing can be solved by vertical farming operations that encourage transparency and are eager to interact with their clients and local communities. Vertical farming operations allow for a high level of control over crop production methods, enabling growers to optimize resource use efficiency while minimizing detrimental effects on the environment and society (Figure 2) [4].

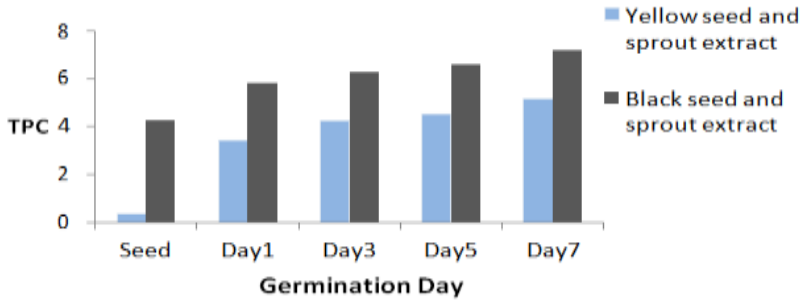

Figure 2. Total phenolic content of methanolic extract of black and yellow seed and sprout

Management of a vertical farm

To successfully operate a vertical farm, a range of problems must be understood. The viability of a vertical farm depends critically on choosing a crop that can be cultivated both financially and safely. Due to the system's intrinsic complexity, a cohesive team and work delegation are necessary. Vertical farms require ongoing maintenance much like typical farms do during the growing season; with the primary distinction being that a vertical farm's growing season lasts all year. To maintain a crop, one must control pests, crop growth, and the growing system. To continuously monitor production and assess additional farm optimization strategies, a systematic approach to data collection, processing, and analysis is required. Operators of vertical farms should typically concentrate on a cultiva-tion method that has been proven effective in other contexts akin to their own and leave the creation of novel methods to specialist businesses. There are numerous systems to pick from depending on the crop being raised. The most widely used systems make use of hydroponics in some way to reduce weight and increase the effectiveness of the usage of nutrients and water. This system can be di-vided into two major categories: vertical columns and stacked horizontal layers. Standard hydroponic systems are divided into several layers and stacked vertically in stacked horizontal layer systems. Vertical columns, on the other hand, make use of towering columns that drip or spray nutritional solution onto the plants' suspended roots. Systems with stacked horizontal layers are substantially more difficult and expensive to develop than systems with vertical columns (Figure 3) [5].

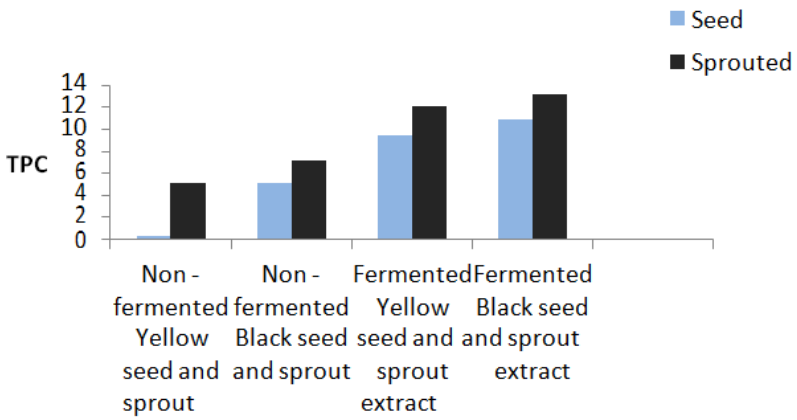

Figure 3. Total phenolic content of methanolic extract of fermented and non-fermented black and yellow soybean seed and sprout

After choosing a crop and growing method, pest management must be carefully considered. A vertical farm without an integrated pest management programme will almost probably experience crop failure due to pests. Since vertical farms function in confined spaces, pests almost always enter from the outside, whether they are on people, seeds, in the air, or in the water. Fungal or arthropod pests are the most common pests seen in controlled environments. Pests can be kept out of the facility by using air showers, coveralls,seed sterilization, air filtering, and water treatment. The first line of defence should be the exclusion of pests through various decontami- nation methods, but it cannot be the sole one. Pests will eventually infiltrate the building, and effective treatment. In order to prevent humidity buildup and condensation, which can encourage fungal growth, proper environmental control is necessary. Arthropod pest outbreaks March be managed with the aid of bio-control agents. However, their introduction in response to an outbreak will often not be swift enough to prevent crop damage, thus they must be employed as a preventative measure. For greatest efficiency, beneficialinsects must be introduced before outbreaks and their populations [6].

MATERIALS AND METHODS

Sample collection

Materials: The yellow and black seeds of soybean were collected locally and germination and fermentation process was carried out.

Seed germination: Yellow and white soybean seeds were wiped clean for 1min by using immersion in ethanol. By then, the seeds turned into lowered in deionized water in quantity 1:10 (m/v) for 12 hour (hr). By then, the sprinkling water changed into poured off and, the seeds had been spread on clean stackable plate and washed a couple of times every day using deionized water to keep away from microbial turn of activities. Germination of the seeds was surpassed on at room temperature within the volume of 22°C ± 1°C (12/multi day/night time). Sprouts seeds amassed after 3, 5, 7 days of development and dry, ground in plant and set aside in haziness till further assessments (Table 7) [7].

Fermentation process: Seeds were finely grounded into flour in an electric blender and passed through a 0.5 mm mesh standard sieve. A total of 25 gm of sample was taken in a sterilized container and distilled water added in the ration of 1:2 samples were kept in an incubator set at 37°C for time interval upto 40 hr and allowed to naturally ferment. Then, the fermented seed was taken out in a sterilized petridish and dried in the hot air oven at 50°C and powdered by using blender for further analysis. Hexane defatting for lipid removal: Lipids were extracted from the sample seeds using n-hexane as a solvent, add solvent to sample ratio of 1:4. Samples were stirred and stored for 24 hrs and then hexane was removed by filtering. The obtained defatted sample was kept in hot air oven (Figure 4).

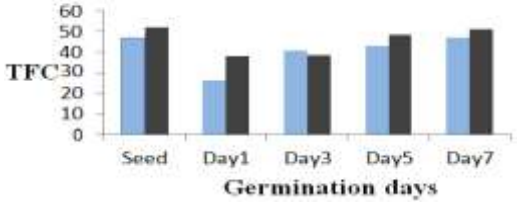

Figure 4. TFC of methanolic extract of black and yellow soybean seed and sprout

Extraction procedure: First, all of samples had been dried in hot air oven at 60°C for twenty-four hr and pulverized to powder using blender. The ground powder turned into extracted with 80% methanol (1:20 w/v) for 1 hr at room temperature with sonicating. The extraction method was repeated twice, and the extracts had been pooled collectively and filtered with the aid of the use of filterpaper. The filtrates had been dried at 45°C below vacuum and re-dissolved with 80% methanol to final concentration at 2 hundred mg/ml. The extracts were stored in screw-capped amber glass bottle at 20°C till used [8].

Determination of Total Phenolic Content (TPC)

The standard curve of gallic acid was plotted by making the concentration of 1100 µgm/ml solutions of gallic acid. The total phenolic content of the samples was estimated by the method described by singleton and rossi. A 0.1 ml of methanol extract diluted with 0.4 ml of deionized water, and then the obtained solution was mixed with 2.5 ml of 0.2 M Folin-ciocalteau reagent solution and 2 ml of 7.5% (m/v) sodium carbonate solution. After 2 hrs of incubation, the absorbance was measured against a blank, using UV Spectrophotometer (760 nm). Total phenolic content was expressed as mg of Gallic Acid Equivalents (GAE) per gm. dry matter of fermented and non-fermented seeds and sprouts (Tables 1-2).

Table 1. Mean TPC of Methanolic extract of yellow soybean seed and sprouts ranged (0.35 ± 0.02 mgGAE/gm to 5.15 ± 0.02 mgGAE/gm). Each value was expressed in Mean ± std. (n=3)

S. no	Mean abs (760 nm)	Conc. eq to GA (mg/ml)	SD	TPC
Seed	0.033	0.349206	0.022676	0.35 ± 0.02
Day 1	0.169	3.433107	0.022676	3.43 ± 0.02
Day 3	0.20567	4.26455	0.022676	4.26 ± 0.013
Day 5	0.21633	4.506425	0.013092	4.51 ± 0.013
Day 7	0.24467	5.148904	0.026184	5.15 ± 0.02

Table 2. Mean TPC extract of black soybean seed and sprouts ranged from (4.26 ± 0.03 mg GAE/gm. to7.17 ± 0.07 mg GAE/gm) respectively. Each value is expressed in mean ± std. (n=3)

S. no	Mean abs (760 nm)	Conc. eq to ga (mg/ml)	SD	TPC
Seed	0.20567	4.26455	0.026184	4.26 ± 0.03
Day 1	0.27533	5.844293	0.047203	5.84 ± 0.05
Day 3	0.29367	6.260015	0.034638	6.26 ± 0.03
Day 5	0.309	6.60771	0.022676	6.61 ± 0.02
Day 7	0.33367	7.167045	0.072892	7.17 ± 0.07

Determination of Total Flavonoid Content (TFC)

This technique is primarily based on the reaction among the flavonoids and aluminium chloride, forming a yellow complex. 4 ml of deionized water and 0.3 ml of sodium nitrate solution (15 gm/100 ml) became brought to 1 ml of appropriately diluted methanol extract. After that, 3 ml of aluminum chloride methanol answer (10 gm/100 ml) and 4 ml of sodium hydroxide solution with concentration (conc) (4 gm/100 ml) have been introduced to the ensuing solution after which the complete sample was diluted with deionized water to a final quantity of 10 ml. The mixture turned into stirred and left to a very last volume of 10 ml and left to face for 15 mins. Finally, absorbance changed into measured at 415 nm. The total flavonoid content in the extracts become in comparison to the usual curve for quercetin answer and expressed as mg of quercetin equivalents consistent with gm of fermented and non- fermented extracts of seeds and sprouts (Figure 5 and Tables 3-4) [9].

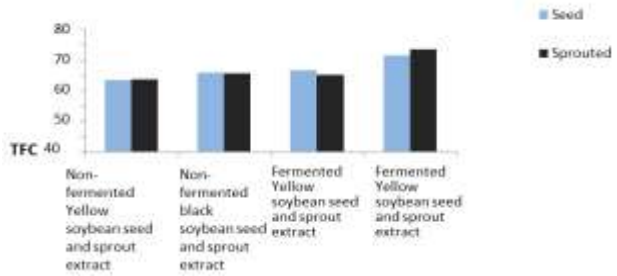

Figure 5. TFC of methanolic extract of fermented and non-fermented Black and yellow soybean seed and sprout extract

Table 3. Total phenolic content of methanolic extract of fermented and non-fermented yellow and black soybean seed and sprout. Each value is expressed in mean ± std

S. no	F.BSE	F.YSE	NF.BSE	NF.YSE
Seed	10.88 ± 0.05	9.4 ± 0.25	4.26 ± 0.03	0.35 ± 0.02
Sprout	13.18 ± 0.04	12.09 ± 0.0	7.17 ± 0.07	5.15 ± 0.02

Table 4. Mean TFC of methanolic extract of non-fermented yellow soybean seed and sprout each value is expressed in mean ± std (n=3)

S. no	Mean ab (415 nm)	Conc eq to quercetin (mg/m l)	SD	TFC (Eqtoquercetin/gm of dry extract)
Seed	0.17167	46.90991	0.156041	46.91 ± 0.15
Day 1	0.09533	26.27928	0.156041	26.28 ± 0.15
Day 3	0.149	40.78378	0.27027	40.78 ± 0.27
Day 5	0.15733	43.03604	0.412845	43.04 ± 0.41
Day 7	0.17233	47.09009	0.156041	47.1 ± 0.15

DPPH radical scavenging activity

The free radical scavenging activity of the extracts was measured using DPPH radical (1, 1-diphenyl 2-picrylhydrazyl). A total of 10 mg of sample was mixed with 10 ml of acidified methanol and heated at 40°C in water bath for 20 min. On the whole, 100 µl of sample extract prepared and put in a test tube and then diluted with 2.9 ml of pure methanol. The resultant sample was mixed with 150 µl of DPPH solution (4.3 mg in 3.3 ml methanol) which also serve as a control with same concentration .It was then incubated for 15 min in dark, and the decrease in absorbance was measured at 515 nm with the help of UV-visible spectrophotometer. Ascorbic acid was used as standard/positive control. The percentage radical scavenging activity was calculated by using the following formula (Table 5).

%DPPH radical scavenging=$(A_o-A_s /A_o) \times 100$ Where, A_o=Absorbance of control

A_S=Absorbance of sample

Table 5. Mean TFC of methanolic extract of non-fermented black soybean seed and sprout. Each value is expressed in mean ± std. (n=3)

S. no	Mean absorbance (41 5 nm)	Conc eq to quercetin (mg/ml)	SD	TFC (EQtoquercetin/gm of dry extract)
Seed	0.18667	50.96396	0.312081	51.96 ± 0.31
Day 1	0.139	38.08108	0.468122	38.1 ± 0.46
Day 3	0.14033	38.44144	0.156041	38.44 ± 0.15
Day 5	0.17767	48.53153	0.412845	48.53 ± 0.41
Day 7	0.18833	51.41441	0.156041	51.42 ± 0.15

Ferric Reducing Antioxidant Power (FRAP)

The reducing power of the individuals extracts reflecting its antioxidant activity was determined using modified Fe^{3+} to Fe^{2+} reduction assay Briefly, 1ml of extracts solution (10 µg/ml-200 µg/ml) in methanol became introduced to 2.5 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% w/v Potassium ferricyanide [$K_3Fe(CN)_6$] Solution. The combination changed into vortexed and incubated at 50°C for 20min assisted with vortex shaker followed by addition of 2.5 ml of 10% w/v Trichloroacetic acid and centrifugation at 3,000 RPM. Finally, 2.5 ml of supernatant turned into mixed with 2.5 ml of deionized water and 0.5 ml of 0.1% w/v ferric chloride and perl's prussian blue coloration was measured at 700 nm towards blank. Ascorbic acid turned into used as the standard and the end result became expressed as mg ascorbic equivalents in keeping with gram seeds (Figure 6 and Table 6) [10].

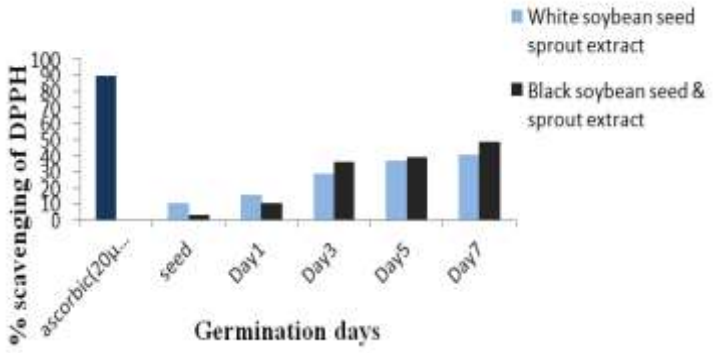

Figure 6. % DPPH scavenging activity of black and yellow soybean seed and sprout extract with ascorbic acid at equal concentration of 20 µgm/ml

Table 6. TFC of methanolic extract of fermented and non-fermented Black and yellow soybean seed and sprouts. Each value is expressed in mean ± std.

S. no	F.BSE	F.YSE	NF.BSE	NF.YSE
Seed	63.22 ± 0.54	53.04 ± 0.56	51.96 ± 0.31	46.91 ± 0.15
Sprout	66.91 ± 0.68	50.1 ± 0.56	51.42 ± 0.15	47.1 ± 0.15

Hydrogen peroxide scavenging activity

The radical scavenging activity of individuals extracts was determined using the H_2O_2 method. Briefly, 2 ml of extracts solution (10 µg/ml-100 µg/ml) in methanol was added to 4 ml of H_2O_2 (20 mM) solution in phosphate buffer (PH 7.4). After 10 min, the absorbance was measured at 230 nm against the phosphate buffer blank solution. The percentage scavenging of H_2O_2 was calculated by using formula

% Scavenging of H_2O_2= $[(A_o-A_s)/A_o] \times 100$ Where,

A_o= Absorbance of the control (Phosphate buffer with H_2O_2) and A_1=Absorbance of the test extracts or standard

Inhibition of lipid peroxidation activity using ferric thiocyanate method

Antioxidant activity of the extract against lipid peroxidation was measured by ammonium thiocyanate assay method. A sample solution containing 0.2 ml of extract, 0.2 ml of linoleic acid emulsion (25 mg/ml in 99% of ethanol) and 0.4 ml of 50 mM phosphate buffer (pH 7.4) was mixed and incubated in the dark at 40°C Then, 3 ml of 70% (v/v) Ethanol and 0.1 ml of 30% Ammonium thiocyanate was added to 0.1 ml of sample solution. Then, 0.1 ml of 20 mM ferrous chloride in 3.5 % (v/v) hydrochloric acid was also added to the sample solution. After the 3 min of addition, the absorbance of the resulting red color was measured at 500 nm. The sample solution was assayed every 24 hr until absorbance of the control solution (same solvent except extract) were maximum than absorbance of sample solution. Butylated Hydroxytoulene (BHT) was used as positive control. All determinations were performed in triplicate. % Inhibition of lipid peroxidation was calculated by the following equations (Table 7).

% Inhibition=(A_c-A_s) $A_c \times 100$

Where A_c=Absorbance of control A_s=Absorbance of sample or standard

Table 7. % Free radical scavenging activity of black and yellow soybean seed and sprout extract with standard ascorbic acid at concentration of (20 µgm/ml)

S. no	Black soybean seed and sprout extract	Yellow soybean seed and sprout extract	Ascorbic acid (20 µmg/ml)
Seed	3.17	10.77	89.34
Day 1	10.89	15.83	
Day 3	35.81	28.83	
Day 5	39.42	36.81	
Day 7	40.47	48.34	

Statistical analysis

The data were reported as the mean ± standard deviation. Data analysis for phenolic and flavonoid content with antioxidant activity was done by using Microsoft excel 2010 and linear regression coefficient (R^2) for phenolic and flavonoid content with antioxidant activity was analyzed by graph pad prism for windows, Version 7 (graph pad software). A p-value<0.05 was considered significant (Table 8).

Table 8. %DPPH scavenging activity of fermented and non-fermented black and yellow soybean seed and sprout extract with ascorbic acis as standard at eq conc of 20 µmg/ml

S. no	Fermented		Non-fermented		Ascorbic
	Black	Yellow	Black	Yellow	89.34
Seed	78.72	54.01	3.18	10.77	
Sprouted	88.02	80.98	40.47	48.33	

RESULT AND DISCUSSION

Total phenolic content

The Total phenolic content of methanolic extracts of soybean seed and sprout was measured by using the folin ciocalteu reagent in each extract. The results were derived from a standard curve (y=0.0441x+0.0176, R^2=0.991) of gallic acid (0-100 µg/mL) and expressed in Gallic Acid Equivalents (GAE) per gram dry extract weight. The content of phenolic compounds in methanol extracts of fermented and non-fermented soybean seed and sprout ranged from 0.35 to 13.18 GAE/gm dry mass of extract. Total phenolic content was found to be highest in Fermented black soybean sprouts and lowest was found to be in non-fermented yellow soybean seed extract. Phenolic compounds are important plant constituents with redox properties responsible for antioxidant activity.The hydroxyl groups in legume extracts are responsible for facilitating free radical scavenging. According to the data, The TPC of black soybean seed and sprouts is higher than the yellow soybean seed and sprouts. This result is steady with the finding of. It is due to the larger content of the Phenolic compounds present in black seed coat (Table 9) [11].

Table 9. FRAP activity of black and yellow soybean seed and sprout extract

S. no	FRAP (eq to ascorbic acid) of black seed and sprout	FRAP (eq to ascorbic acid) of Yellow seed and sprout
Seed	0.94	1.02
Day 1	1.73	2.09
Day 3	1.96	2.24
Day 5	2.03	2.46
Day 7	2.39	3.09

Similarly, the germination process causes a steady increase in TPC of soybean seed. The TPC of yellow soybean seed increased from (0.35 ± 0.02, 3.43 ± 0.02, 4.26 ± 0.013, 4.51 ± 0.13 and 5.51 ± 0.02) mg GAE/gm of extract while black soybean seed increased from (4.26 ± 0.03, 5.84 ± 0.05, 6.26 ± 0.03, 6.61 ± 0.02 and 7.17 ± 0.07) mg GAE/gm of extract within 0, 1, 3, 5 and 7 days of germination. Germination ended in widespread changes inside the phenolic composition, because of activation of endogenous enzymes and the complicated biochemical metabolism of seeds throughout this process. Therefore, The TPC of black and yellow sprouts increased 2 times greater than the TPC of soybean seeds (Figure 7).

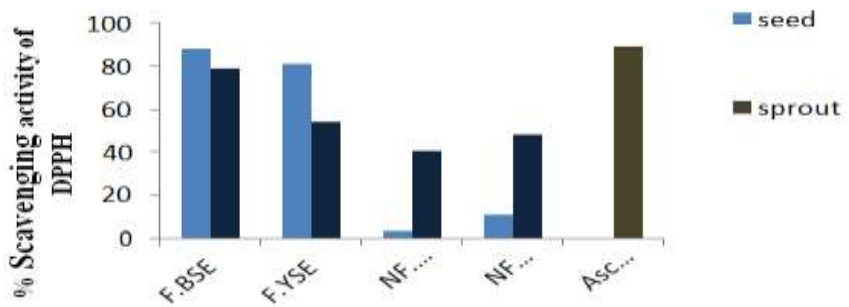

Figure 7. % DPPH scavenging activity of fermented and non-fermented black and yellow soybean seed and sprout extract

After the fermentation, the content of phenolic compounds of fermented soybean seed and sprouts increased by 4 times greater than the non-fermented soybean seed and sprouts. There is an evidence of widespread better attention of phenolics turned into received after fermentation while in comparison to unfermented soybeans. These results can be explained by the fact that amounts of bioactive compounds can be modified during fermentation by the metabolic activity of microorganisms. Particularly, numerous microbial enzymes would possibly result in structural breakdown of legumes cell walls and/or hydrolyse esterified and insoluble-certain phenolics, facilitating their liberation prior to extraction (Table 10).

Table 10. FRAP activity of fermented and non-fermented black and yellow soybean seed and sprout extract

S. no.	F.BSE	F.YSE	NF.BSE	NF.YSE
Seed	4.58	4.25	1.02	0.94
Sprout	6.56	6.39	3.09	2.39

Overall, the total phenolic content of black soybean seed and sprouts is higher than the yellow one. The effect of germination and fermentation process significantly increases the total phenolic content of soybean seed and sprouts. Hence, the highest total phenolic content is obtained in fermented black soybean sprout and the least in non-fermented yellow soybean seed (Table 11).

Table 11. % Hydrogen scavenging activity of methanolic extract of yellow and black soybean seed and sprouts

S. no	control	Blackseed and sprouts	Yellowseed and sprouts	Ascorbic
seed	9.947	48.392	43.222	89.97
Day1	9.947	53.389	50.779	
Day3	9.947	55.568	52.163	
Day5	9.947	60.232	57.987	
Day7	9.947	69.663	66.415	

Total flavonoid content

Total Flavonoids content was calculated by using the quercetin as standard, and plotting the calibration curve as a reference to find out the concentration of an unknown sample of different extracts. Total Flavonoids Content (TFC) was found to be ranged between (26.28 ± 0.15 to 66.91 ± 0.68 mg EQ to Quercetin. TFC was found to be highest in Fermented black soybean sprout while least was found in non-fermented yellow soybean seed (Figure 8 and Table 12) [12].

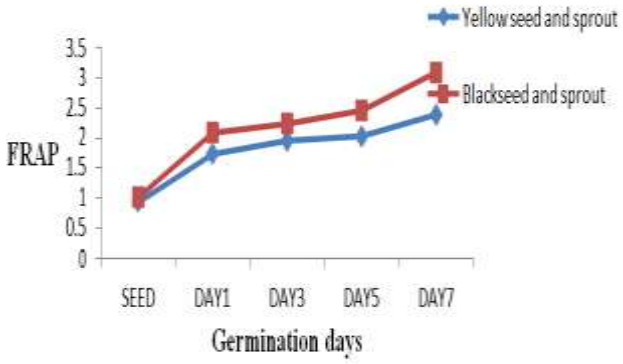

Figure 8. FRAP activity of fermented and non-fermented black and yellow soybean seed and sprout extract

Table 12. %Hydrogen scavenging activity of methanolic extract of fermented and non-fermented black and yellow soybean seed and sprouts

S. no.	F.BSE	F.YSE	NF.BSE	NF.YSE	Ascorbic
Seed	69.64	58.91	48.39	43.22	89.97
Sprout	88.54	78.8	69.66	66.41	

Flavonoids are secondary metabolites with antioxidant activity in which the number and position of free OH groups determine their potency. The content of flavonoids was found to be ranged between (26.28 ± 0.15 to 66.91 ± 0.68) mg Eq to Quercetin/gm of extract. The maximum TFC is present in Fermented black soybean sprout extract and minimum content in the non-fermented yellow soybean seed extract. The content of flavonoids in soybean extracts varied between 46.91 ± 0.15 and 51.96 ± 0.31 mg CE/g extract. TFC of black soybean exhibited 10% higher content of total flavonoids than that of yellow soybean seed.

As compared to the original soybean seeds, germination caused significant increases in the total Flavonoid content. In a survey of past literature reports, it was found that germination of soybean increased (nearly seven times) the flavonoid level, compared to the dry seeds, and this increment depended on the soybean variety. These reports agree with increased value of TFC in soybean sprouts than the soybean seeds. It was found that TFC of fermented sprouts was higher than non-fermented sprouts. These results can be explained by the fact that levels of bioactive compounds can be modified during fermentation by the metabolic activity of microorganisms. Particularly, several microbial enzymes might induce structural breakdown of legume cell walls and/or hydrolyse esterified and insoluble-bound phenolics, facilitating their liberation prior to extraction. Thus, the higher TFC was found to be in fermented black soybean sprouts and least TFC in non-fermented yellow soybean seeds (Table 13).

Table 13. %Lipid inhibition activity of methanolic extract of black and yellow soybean seed and sprout with BHT as standard at a concentration of 20 µgm/ml

S.no	Black seed and sprout	Yellow seed and sprout	BHT
Seed	17.98	8.77	81.86
Day 1	21.71	9.78	
Day 3	27.06	10.67	
Day 5	30.67	15.29	
Day 7	34.58	17.67	

Anti-oxidant activity

Free radical scavenging activity of methanolic extract of soybean seed and sprout were estimated by using ascorbic acid as a standard. The obtained % scavenging activity of DPPH of methanolic extract of soybean seed and sprouts was ranged between (3.09% to 88.09%). Maximum DPPH Free radical Scavenging activity was shown by Fermented black soybean sprout extract. The ferric reducing antioxidant activity and hydrogen Scavenging activity was calculated by using ascorbic acid as standard. The FRAP value was ranged from 0.94 mg to 3.09 mg equivalent to ascorbic acid while hydrogen scavenging activity was ranged from 43.222% to 66.41%. The lipid peroxidation activity was calculated by using butylated hydroxyl toulene as a standard. The % lipid inhibition of methanolic extract of yellow and black soybean seed and sprout was ranged from 8.77% to 70.30%.The maximum % lipid inhibition was found to be in fermented black soybean sprout extract (Figure 9 and Table 14)) [13].

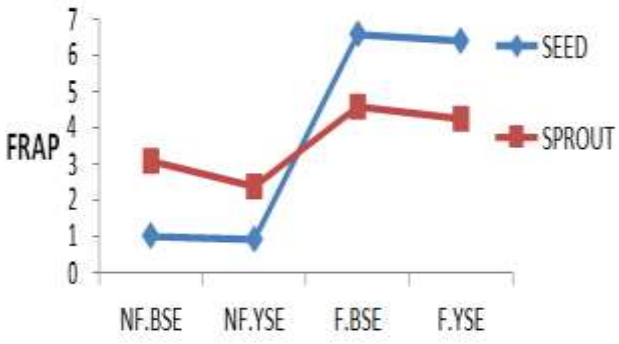

Figure 9. FRAP activity of fermented and non-fermented black and yellow soybean seed and sprouts extract.

Table 14. % Lipid inhibition activity of fermented and non-fermented black and yellow soybean seed and sprouts extract with BHT as a standard

S.no	F.BSE	F.YSE	NF.BSE	NF.YSE	BHT
Seed	50.15	40.88	17.98	8.77	81.86
Sprout	70.3	61.15	34.58	17.67	

It was observed that the DPPH free radical scavenging activity of black soybean seed was higher than the yellow soybean seed. This results is agree with various other studies. Indicated that black soybeans incorporate a excessive attention of anthocyanins, belong to the flavonoid family and act as natural colorants, positioned mostly in its seed coat. Anthocyanins have been stated to possess the potential to scavenge unfastened radical. Similarly, it was also found that germination process increases the anti-DPPH radical activity of soybean seed. The % DPPH scavenging activity increased by 12 folds during germination process of black and yellow soybean seed extract. It is observed that the fermentation process enhanced the % DDPH Scavenging activity of soybean seed and sprouts extracts. The fermented products have been reported on their higher antioxidant activity via microbial fermentation due to the break down of the glycosilated forms and releasing the free aglicon. Like the free radical scavenging activity, the FRAP activity of black soybean seed was higher than the yellow soybean seed.

The germination and fermentation process enhanced the antioxidant activity. The H_2O_2 scavenging potency of the methanolic extract of black and yellow soybean seed and sprouts was found that fermented black soybean sprouts have higher peroxide scavening activity than yellow soybean seed and sprout. Hydrogen peroxide (H_2O_2) is a strong oxidizing agent, that could set off the signalling pathway to stimulate cellular proliferation or differentiation. It is generated in a biological system with the aid of many oxidizing enzymes including superoxide dismutase. However, anomalous accumulation of H_2O_2 is responsible for oxidative stress and inflammation reactions, which might be correlated with pathological conditions like cancer, diabetes, and cardiovascular illnesses. This is due to rapid decomposition of H_2O_2 and next technology of the hydroxyl radical (OH) that initiates lipid peroxidation and damage of cellular components. Regulation of H_2O_2 generation with the aid of herbal antioxidants is of high interest in biological research (Figure 10).

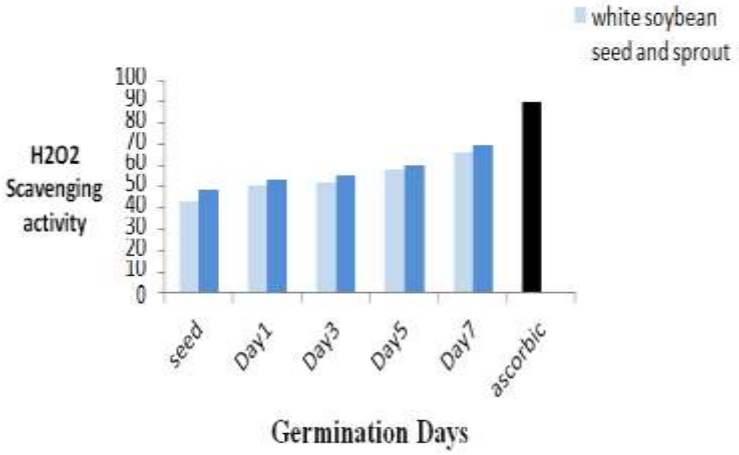

Figure 10. %Hydrogen scavenging activity of methanolic extract of black and yellow soybean seed and sprouts.

Lipid peroxidation inhibition activity measures the ability of a sample to inhibit peroxidation that can arise in lipids because of free radicals. Peroxidation of linoleic acid produces peroxyl free radicals, which might be used to oxidize ferrous ions into ferric ions. The ferric ions formed coloured complex, ferric thiocyanate, with thiocyanate ions present in the medium. The formation of the complex is measured spectrophotometrically at 500 nm. Antioxidants will slow down the peroxidation in lipids resulting in low production of peroxides. As a result, there will be less oxidation of ferrous into ferric and therefore less amount of the complex will be formed. Thus, the stronger the antioxidant, the less is the formation of ferric thiocyanate and lower will be the absorbance. The %lipid peroxidation inhibition activity of methanolic extract of black and yellow soybean seed extract with the BHT (Butylated Hydroxyl Toulene) of equal concentration as standard. From the data, it was noted that the black soybean seed and sprouts have greater lipid peroxidation inhibition activity than the yellow soybean seed and sprout extract. This is similar with the finding of others reports (Figure 11) [14].

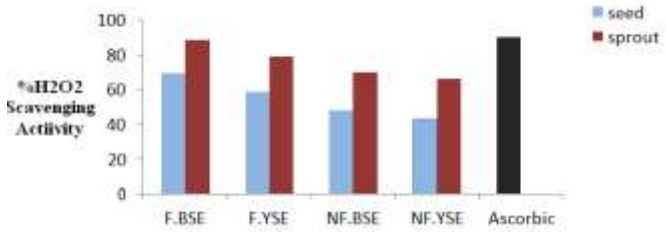

Figure 11. %Hydrogen scavenging activity of methanolic extract of fermented and non-fermented black and yellow soybean seed and sprouts with ascorbic acid as standard at eq conc of 20 µgm/ml

One possible mechanism for the mounting of the antioxidant activity of soybean seeds processed with the aid of germination and fermentation. They determined that the enzymatic hydrolysis of proteins by way of microbial protease expose the active radicals of amino acids and the peptides resulted from that hydrolysis may exert an antioxidant activity greater intense than proteins. Isoflavones are presented in soybeans in four chemical forms: Aglicon, glycoside, acetilglycoside and malonil-glycoside. Germination and fermentation decide the increasing of free aglicon that are better absorbed in organism and have a very good antioxidant activity, by protecting the LDL, cholesterol from oxidation and preventing the peroxidation chain. Therfore, the maximum %lipid peroxidation inhibition activity is found in Fermented black soybean sprouts extracts and plays a crucial role in inhibition of lipid peroxidation (Figure 12 and Table 15).

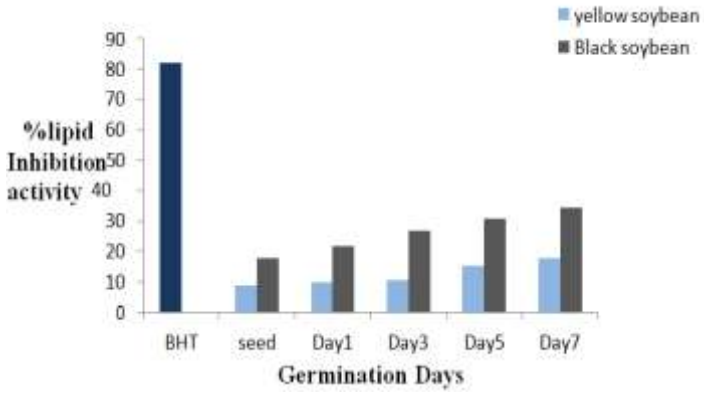

Figure 12. %Lipid Inhibition activity of methanolic extract of black and yellow soybean seed and sprouts with BHT as standard at eq conc of 20 µgm/ml

Table 15. Correlation between TPC and TFC with antioxidant activity

TPC	TFC	%DPPH	$\%H_2O_2$	FRAP
4.26	25	3.17	48.39	1.02
5.84	38.1	10.89	53.38	2.09
6.26	38.44	35.81	55.56	2.24
6.61	48.53	39.41	60.23	2.46
7.17	51.42	40.47	69.66	3.09

Correlation coefficient of TPC and TFC with antioxidant activity

The Linear regression co-efficient (R^2) of TPC and TFC with Antioxidant activity of black soybean seed and sprout was analysed by graphpad prism software. Phenolic and flavonoid molecules are vital antioxidant additives which are responsible for deactivating free radicals primarily based on their ability to donate hydrogen atoms to free radicals. They additionally have best structural characteristics for free radical scavenging. Different literature reviews indicate a strong linear correlation of total phenolic content with antioxidant ability. The correlation of total phenolic and flavonoid content with antioxidant capacity of black soybean seed and sprout extract. There was significant results of correlations between total phenolic content with DPPH (R^2=0.8124), H_2O_2 (R^2=0.8092) and FRAP (R^2=0.9806) at a 95% confidence level which is consistent with the above literature reports (Figures 13 and 14).

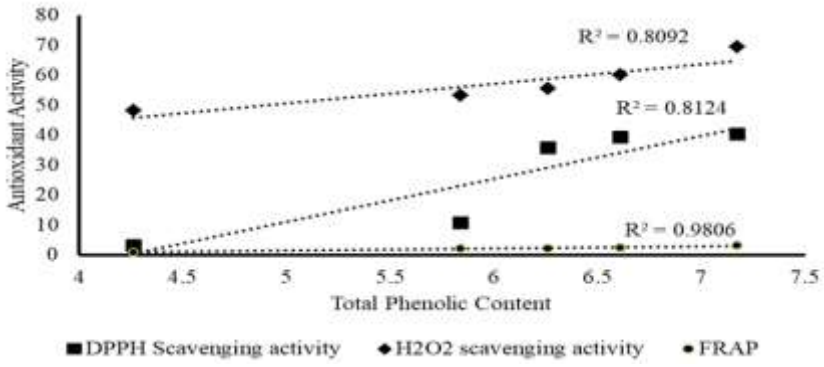

Figure 13. %Lipid inhibition activity of fermented and non-fermented black and yellow soybean seed and sprout extract with BHT as standard

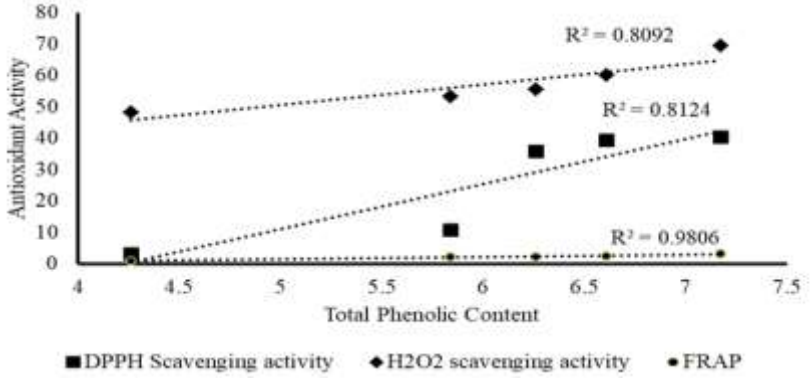

Figure 14. Correlations of TPC with Antioxidant activity where linear regression co-efficient of DPPH (R^2=0.8124), H_2O_2 (R^2=0.8092) and FRAP (R^2=0.9806) respectively

A negative correlation is observed between total flavonoid content with DPPH at a 95% confidence level, which is probably caused by the antioxidants depending now not only on the concentration, however additionally on the structure and the interaction between the flavonoids. By comparing the correlation coefficients (R-values), it is possible to suggest that phenolic groups are highly responsible for the antioxidant activity of black soybean seed and sprout extract. The correlation of TPC with FRAP suggest that total phenolics in soybean are the potential electron donors and reduce the oxidized intermediates of lipid peroxidation processes (Figure 15) [15].

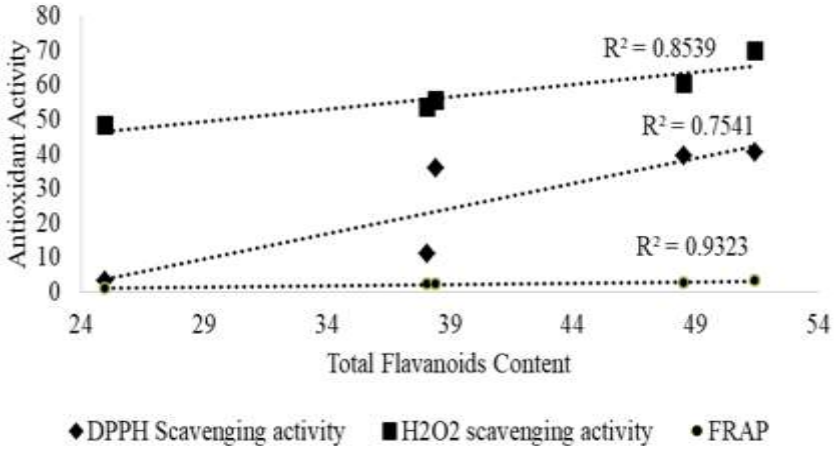

Figure 15. Correlations of TFC with antioxidant activity where linear regression co-efficient of DPPH (R^2=0.7541), H_2O_2 (R^2=0.8539) and FRAP (R^2=0.9323)

CONCLUSION

Results of our study suggest that the soybean seeds have higher phenolic and flavonoids which could be a significant source of natural antioxidants.The concentration of phenolic compounds are higher in black soybean seed coat than the yellow soybean seedcoat. Based on this information, it can be concluded that the TPC, TFC and anti-oxidant activity of black soybean seed is higher than the yellow soybean seed. From this study ,it was also found that the application of germination and fermentation techniques on soybean seed significantly increased the antioxidant properties of soybean seeds and sprouts.It enhances the release of bound phenolic compounds and also convert phenolic compounds into different metabolites which increases their bioactivity and improve their antioxidative activity. Moreover, The stastistical analysis showed strong positive correlation between total phenolic content with their antioxidant activities. Thus, germination and fermentation process could therefore offer an excellent strategy to produce ingredients enriched with health-promoting compounds in a natural way that can be used in nutraceuticals. Further research is required to evaluate the qualities of anthocyanins and isoflavones that would be masked in the present study.

ACKNOWLEDGMENT

My study owes its existence to the help, support and inspiration of several people. I would like to thank all those people who made this thesis possible and an unforgettable experience for me. First of all, I would like to express my very sincere gratitude to Prof. Dr. Anand Kumar, (the principal of universal college of medical sciences, ranigaun bhairahawa) for the support to make this thesis possible. Then, I am thankful to Mr. Sushant Aryal, Mr. Chhitij Thapa and all other lectures of department of pharmacy for the support and encouragement whenever I was in need. I am thankful to my parent's colleagues, senior, junior and lab staff for their help and support during my research work.

REFERENCES

1. Kushi, L.H., et al. *Am J clin nutr*, **1999**, 70(3):451s-458s.
2. Mahdi-Pour, B., et al. *Asian Paci J Trop Biomed*. **2012**, 2(12):960-965.
3. Ames, B.N., et al. *Proceed Nat Acad Sc*i. **1993**, 90(17):7915-7922.
4. Cos, P., et al. *J pharm pharmacol*. **2003**, 55(9):1291-1297.
5. Verni, M., et al. *Foods.* **2019**, 8(9):362.
6. Takahashi, R., et al. *J Agri Food Chem*. **2005**, 53(11):4578-4582.
7. Kanchana, P., et al. *World J Pharm Pharmaceut Sci*. **2015**, 5(1):356-371.
8. Lien, D.T.P., et al. *Science.* **2012**, 6: 93-101.
9. Walker, A.F., et al. *Proceed Nutri Soc*. **1982**, 41(1):41-51.
10. Victoria, B.G., et al. *Rom Biotechnol Lett***. 2015**, 20(2):10212-10220.
11. Pajak, P., et al. *Food chem*. **2014**, 143:300-306.
12. Carciochi, R.A., et al. *Plant Food Hum Nut*. **2016**, 71(4): 361-7.
13. Lopez-Amoros, M., *J Food Compo Anal*. **2006**, 19(4):277-283.
14. Kumaran, T., et al. *Screening*. **2015**, 9:10-16.
15. Yadav, N., et al. *Int J Food Propert*. **2018**, 21(1):437-451.