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A STUDY ON *INVITRO* ANTIOXIDANT ACTIVITY OF FERMENTED AND UNFERMENTED FLAXSEED (*Linum usitatissimum* L.)

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ABSTRACT

In India several plants possessing pharmaceutical and curative properties are considered to be the richest bio-resources of drugs used in traditional medicinal systems, modern medicines, food supplements and pharmaceuticals. Research indicates a strong relationship between the dietary intake of foods rich in antioxidants and incidence of chronic long term diseases. Flaxseed (*Linum usitatissimum* L.) has been popularly used as a food supplement since ancient time. The present study was carried out to exploit the antioxidant activity of flaxseed fermented with *Lactobacillus acidophilus* and to compare it with unfermented flaxseed extract. *In vitro* antioxidant activity were assessed using DPPH and FRAP assay. The results indicated that the ability to scavenge free radicals depends upon concentration and increased with increase in concentration. Hence, the results point out that flaxseed represents a valuable source of antioxidant due to the presence of various bioactive phytochemical metabolites such as flavonoids, phenolic acid, phenylpropanoids and lignans.

KEYWORDS: free radicals, antioxidants, flaxseed, food supplement.

1. INTRODUCTION

Oxidation is an essential biological process for energy production in living organisms which involves the production of free radicals or reactive oxygen species. Reactive oxygen species

(ROS) is a collective term used for oxygen-centered radicals such as superoxide, hydroxyl and non-radical oxygen derivatives, namely hydrogen peroxide and singlet oxygen.[1]

Antioxidants are substances that prevent or retard the oxidation of easily oxidizable biomolecules such as lipids, proteins and DNA, thus preventing oxidative damage.[2] Antioxidants exert their action either by scavenging the reactive oxygen species or by protecting the antioxidant defense mechanisms. Although the human body possesses an inherent antioxidant defense system to protect against oxidative damage, the over-production of reactive oxygen species or lack of sufficient antioxidants to scavenge the free radicals can result in tissue injury and in development of a variety of physiological conditions including cellular aging, mutagenesis, carcinogenesis, coronary heart disease, diabetes and neuro degeneration.[3]

Fermentation which is generally used to enhance food quality features such as shelf life, nutritional value, and organoleptic properties is now applied to increase the production and extraction yields of bioactive compounds in the food and pharmaceutical industries.[4, 5] Fermentation by *Bacillus subtilis* increased the antioxidant and anticancer activity of black rice bran.[6] Lactic acid bacteria strains are also reported to have antioxidative effects.[7]

Recently, there have been increasing reports which indicates that edible plants provide protection against some chronic diseases. Flaxseed (*Linum usitatissimum* L.) a blue flowered annual oilseed crop, which belongs to the family, *Linaceae* is cultivated worldwide and has been used for its oil, seed and fiber since ancient times. Humans have consumed flaxseed since the beginnings of the earliest civilizations. Flaxseed has received attention among consumers and health care professionals due to reported health benefits such as cardio protective effects, anticancer effects, antiviral and bactericidal effects, anti-inflammatory effect, ion reduction, laxative effects, beneficial effects on renal function in patients with lupus nephritis, impact on bone health, management of diabetes.[8,9,10]

Accumulating evidences suggest that flaxseed is an excellent source of natural antioxidants. Flaxseed is a rich source of different types of phenolics such as lignans, phenolic acids, flavonoids, phenylpropanoids and tannins. Phenolic compounds seem to be the main component responsible for the observed antioxidant activity.[11] Since there was no report documented on the antioxidant activity of fermented and unfermented flaxseed, the purpose

of the present study was designed to investigate such information for potential application of flaxseed in the food and pharmaceutical industry.

2. METHODOLOGY

2.1 Plant Material

Flaxseed procured from an organic store was cleaned to remove the impurities present in them. The seeds were grounded to a fine powder using a mixer. The resulting flax seed powder was then stored in an air tight container and utilized.

2.2 Preparation of extracts

Unfermented/Aqueous extract of flaxseed

Five gram of flaxseed powder was mixed with 100ml of distilled water which was boiled for a period of 5-10 minutes in a boiling water bath. This was then filtered through Whatman filter paper No. 1 in a Buchner funnel. The solution was stored at 18°C until use.

Fermented extract of flaxseed

Fermentation process was carried out in a sterilized conical flask by mixing 5 grams of flax seed powder with 100 ml of distilled water. This was then subjected to autoclaving. After autoclaving, it was allowed to cool to room temperature and was inoculated with *Lactobacillus acidophilus* to initiate the process of fermentation. The fermentation process was carried out for a period of 48 hours at 37°C.It was then blended with (PBS solution) phosphate buffer solution. This was then subjected to centrifugation at 5,000 rpm for 15 minutes. The residue was discarded and the resulting supernatant was again mixed with trichloroacetic acid and centrifuged again at 5,000 rpm for a period of 20 minutes. The resulting pellet was then freeze dried and stored at 4°C until use.

2.3 Qualitative screening of antioxidant activity by DPPH assay on TLC (Thin Layer Chromatography)

1: 10 dilutions of the sample extract and ascorbic acid as standard was made in methanol. 5 microliter of dilution was applied on TLC plate. The plate was developed by methanol, ethyl acetate and water in the ratio 7:3:1. The plate was sprayed with 0.2% DPPH reagent in methanol and left for 30 minutes at room temperature. Formation of yellow spot indicated the presence of antioxidant activity.

2.4 Quantitative analysis of antioxidant activity by DPPH assay

The free radical scavenging activity was determined by using DPPH assay.[12] The decrease of the absorption at 517nm of the DPPH solution after the addition of the antioxidant was measured in a cuvette containing 1000µl of 0.1mM ethanolic DPPH solution mixed with DMSO at varying concentrations (200 to 1000µg/mL) of test samples and vortexed thoroughly. The setup was left at dark in room temperature and the absorption was monitored after 20minutes. The ability of the test sample to scavenge DPPH radical was calculated by the following equation.

$$\frac{\text{Absorbance in control} - \text{Absorbance in sample}}{\text{Absorbance in} - \text{control}} \text{ x } 100$$

Absorbance control was the absorbance of DPPH and ethanol.

Absorbance sample was the absorbance of DPPH radical and the test sample.

2.5 Determination of antioxidant activity by FRAP assay (Ferric Reducing Ability of Plasma)

FRAP assay (ferric reducing ability of plasma) evaluates total antioxidant power and was chosen to assess the presumable effects of flaxseed. FRAP assay depends upon the ferric tripyridyltriazine (Fe (III)-TPTZ) complex to the ferrous tripyridyltriazine (Fe(II)-TPTZ) by a reductant at low pH.Fe(II)-TPTZ has an intensive blue color and can be monitored at 593 nm.[13] Different concentrations (200 - 1000µg/ml) of the test samples were perched in methanol solvent (ranging from 1000µl, 995µl, 990µl, 985µl, 980µl and 975µl) and diversed with 1 ml of 1% Potassium ferricyanide and 1 ml of 0.2 M phosphate buffer (pH 6.6). This mixture was incubated at 50ºC for 20 min, 1 ml of 10% TCA was added and the mixture was assorted with distilled water (1 ml) and $FeCl_3$ (0.5ml, 0.1%) and the absorbance was measured at 700 nm.[14]

% of Ferric Reducing Potential (% FRP)

$$= \frac{\text{Absorbance of sample} - \text{Absorbance of control}}{\text{Absorbance of sample}} \text{ x } 100$$

3. RESULTS AND DISCUSSION

Scavenging activity of free radicals such as 1.1-diphenyl-2-picrylhydrazyl (DPPH) has been widely used to evaluate the antioxidant activity of natural products from plant sources.

Qualitative analysis of antioxidant by DPPH assay (Table 1) clearly indicate that both unfermented and fermented flaxseed had the ability of scavenging free radicals which was evidenced by change of DPPH colour from purple to yellow and indicating its anti-oxidant property.

Table 1: Qualitative analysis of antioxidant activity using DPPH Assay.

Sample	Presence of Antioxidant activity
Aqueous extract of flaxseed	+
Fermented flaxseed extract	+

Determination of quantitative analysis of antioxidant activity by DPPH assay

The result pertaining to antioxidant activity using DPPH assay is shown in **Fig1.** DPPH is a stable radical and has been widely used for studying the free radical scavenging activity of different kinds of antioxidants. It is evident that the scavenging activity of free radicals increased with increase in concentration. Fermented flaxseed exhibited greater scavenging activity (65.25% at a concentration of 1000µg/ml) followed by unfermented/aqueous extract of flaxseed which had the ability to scavenge only 51.35% of free radicals.

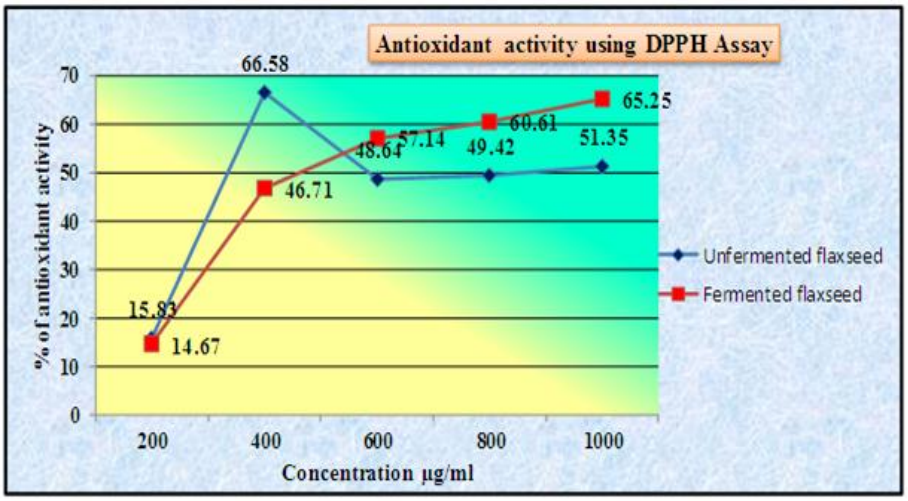

Fig.1. Antioxidant activity using DPPH Assay

Antioxidants can be classified as primary (chain-breaking) antioxidants or secondary (preventive).[15] Primary antioxidants most often act by donating a hydrogen atom, while secondary antioxidants may act by binding metal ions able to catalyze oxidative processes, by scavenging oxygen, by absorbing UV radiation, by inhibiting enzymes or by decomposing hydroperoxides.[16]

Fermentation improves antioxidative activity by increasing the release of Phytonutrients from plant-based foods, which can be a useful method for increasing the supply of natural antioxidative materials. For example, fermentation-induces structural breakdown of the cell walls of plants may release bioactive compounds and/or induce the synthesis of various other compounds.[17, 18] Fermentation increases the total phenolic contents in plant and the observed antioxidative activity may be due to the increase in the total phenolic compounds.[19]

Antioxidant activity by FRAP assay

The result pertaining to antioxidant activity by FRAP assay is presented in **Fig .2**. It is evident that the reducing power of both the fermented and unfermented extract of flaxseed increased in a concentration dependent manner as evident from **Fig .2.** Among the extracts analyzed, aqueous extract of flaxseed possessed stronger ferric reducing assay power than fermented flaxseed.

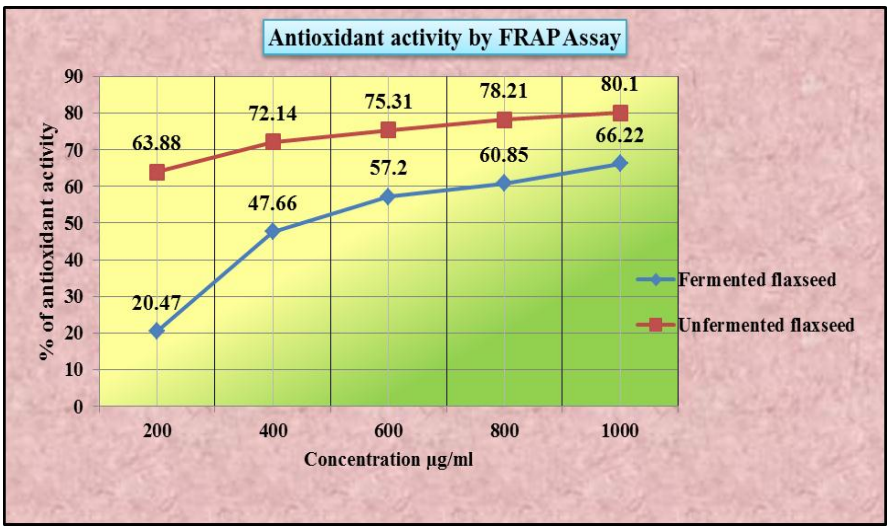

Fig.2. Antioxidant activity by FRAP Assay

There has been a global trend toward the use of natural substance present in medicinal plants and as therapeutic antioxidants as there is an inverse relationship between the dietary intake of antioxidant-rich foods and incidence of human diseases. Nowadays there is a growing interest in finding new and safe antioxidants from natural sources as it would be a promising alternative for synthetic antioxidants in respect of cost, highly compatible with dietary intake and no harmful effects inside the human body. Many antioxidant compounds, naturally occurring in plant sources have been identified as free radical or active oxygen scavengers.[20]

Plant lignans are the biologically important class of phenolic compounds and the richest source being flaxseed. The prevailing lignan in flaxseed is secoisolariciresinoldiglucoside (SDG).[21] Among the phenolic acids, ferulic and p-coumaric acid glucosides are present in high concentrations in flaxseed all contributing to its antioxidant property.[22] In addition, phenolic acid like caffiec acid and their glucosides were also reported in flaxseed.[23] Flaxseed was reported to contain 8-10 g/kg total phenolic acids, about 5 g/kg of esterified phenolic acids and 3-5 g/kg of etherified phenolic acids.[24] Ethanol extract of defatted flaxseed meal were fractionated into four major fractions, according to their maximum UV absorption and concluded that out of four major fractions, fraction I of phenolic compounds with maximum UV absorption at 290 nm was found to be most active.[25] Dietary flaxseed supplementation increases antioxidant defenses through both reduced reactive oxygen species generation and increased reactive oxygen species detoxification.[26]

4. CONCLUSION

Free radicals are responsible for the development of many chronic long term illnesses such as cardiovascular and inflammatory disease, cataract, and cancer. The need for effective, potent natural compounds having antioxidant activity has been intensified in recent years as synthetic antioxidants are reported to be dangerous to human health. Thus this study clearly highlights that the health benefits of flaxseed reside in their antioxidant capacity by acting as sequestrants of free radicals, therefore enabling flaxseed to be used as a natural functional food supplement.

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