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Research Article

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In vitro Free Radical Scavenging, Anti-Hyaluronidase and Anti-Elastase Potential of Flaxseed Lignans Concentrate

Jayarama Naik N.[1] and Basavaraj Madhusudhan[1, 2*]

[1]Department of Food Technology, Davangere University, Davangere – 577 007, Karnataka, India

[2]Research Center for Nanoscience and Technology, Department of Biochemistry and Food Technology, Davangere University, Shivagangothri, Davangere- 577007, Karnataka, India

*Corresponding Author E-mail: prof.basavarajmadhusudhan@gmail.com

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ABSTRACT

The present study was carried out to study the efficiency of flaxseed lignans concentrate (FLC) on hyaluronidase and elastase enzymes inhibition activity. The DPPH and FRAP Assay were carried out using butylated hydroxytoluene and Iron (II) sulfate as standard molecules. The DPPH radical scavenging ability of FLC had the IC_{50} value of 486 µg ml^{-1} and FRAP assay of FLC had IC_{50} value of 74.12 µg ml^{-1}. Furthermore, oleanolic acid was used as reference for enzyme inhibitory assays. The IC_{50} value for hyaluronidase inhibitory activity of FLC was 10.75 µg ml^{-1} whereas oleanolic acid has IC_{50} value of 9.93 µg ml^{-1}. FLC showed elastase inhibition with IC_{50} value of 51.44 µg ml^{-1} whereas standard molecule oleanolic acid had IC_{50} value of 41.72 µg ml^{-1}. It can be summarized from the above observations that flaxseed being a rich source of lignans and rich anti-oxidant properties, the flaxseed lignans concentrate has the potential similar to oleanolic acid and may be utilized as anti-wrinkle agent in cosmetic products.

Key words: *Flaxseed, Anti-wrinkle, Anti-aging, Lignans, Enzymes inhibition.*

INTRODUCTION

When the skin repeatedly exposed to solar radiation it produces excessive free radicals these free radicals causes oxidative stresses, inflammation and destruction of antioxidant system as a result cell membranes, lipids, proteins and DNA get damaged[1]. Increased hyaluronidase and elastase enzymes in skin tissues are responsible for degradation of dermal network which further leads to skin wrinkling and aging. Thus, inhibition of these enzymes is essential in anti-aging prevention[2,3]. Skin contains hyaluronic acid and elastin to maintain elasticity and structure of extracellular matrix of connective tissues. However, the enzyme elastase can degrade it[4]. As a consequence, these substances declines sharply during mature and pre-mature aging[5,6]. Loss of hyaluronic acid and elastin leads to loss of elasticity and water retention capacity of skin[7]. Therefore, to eliminate free radicals from the body through antioxidant defense system and sustaining antioxidant homeostasis always there is an opportunity for new natural antioxidants to prevent skin aging[8].

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Linum usitatissimum L. (family: Linaceae), commonly well-known as 'Flaxseed' or 'Linseed'. Since ages it has been grown for oil and fibre purpose[9]. Major flaxseed growing countries are Canada, China, United States and India[10]. Flaxseed represent richest source of phenolic antioxidant called secoisolariciresinol diglucoside (SDG)[11,12]. SDG without glucose molecule in its structure called as Secoisolariciresinol (SECO) which is insoluble in water[13]. Flaxseed lignans SDG, SECO, ED and EL are found to be equal or somewhat more potent than BHT, vitamin E[14]. Thus, they could have commercial potential as an alternative to these antioxidants[15]. However, so far very limited research attention has been given on flaxseed lignans as an anti-aging agent. Hence flaxseed lignans concentrate was prepared and tested its potential as an ingredient to relieve the skin wrinkling and aging symptoms.

MATERIAL AND METHODS

1. Extraction and preparation of flaxseed lignans concentrate

Briefly, the flaxseed sample (10 kg) was washed and subjected for a dehulling process to obtain hull fraction of flaxseed using Kisan Krishi Yantra Udyog dehuller at Grain Science and Technology Department, CFTRI, Mysore, India. Flaxseeds hull fraction was taken, defatted by extracting with n-hexane. The lignans concentrate was prepared from defatted hull fraction of flaxseed. About 10 g of defatted hull fraction was ground and sieved, mixed with 400 ml of distilled water followed by 500 ml of 2 M aqueous sodium hydroxide. The contents were incubated for 1 h at 20 ºC on a shaking water bath. The hydrolysate was then acidified with dilute sulphuric acid to pH 3.0 and then centrifuged at 5000 rpm for 10 min. The supernatant was centrifuged rapidly to a clear liquid phase and pooled together. The liquid phase (600ml) was mixed with 95% aq. ethanol (900ml), left at room temperature for at least 10 min and total volume was divided into four equal volumes in centrifuge tubes and again centrifuged at 10000 rpm for 5 min to precipitate. Water soluble polysaccharide and proteins were removed carefully. Ethanol extract was evaporated by using rotary evaporator at 40 ºC to obtain FLC. The lyophilized FLC was stored until further analysis[16].

2. DPPH radical scavenging assay

The stable free radical-scavenging activity was determined by the DPPH assay. In this method, the bleaching rate of a stable free radical, DPPH is monitored at a characteristic wavelength in the presence of the various phytochemicals used in the study. In its radical form, DPPH absorbs at 517 nm, but upon reduction by an antioxidant or a radical species, its absorption decreases. Different concentrations of FLC @ 100, 200, 300, 400 and 500 µg (each in three replicates) were added, at an equal volume, to methanolic solution of DPPH (100 M). After 15 min at room temperature, the absorbance was recorded at 517 nm while IC_{50} values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals[17].

3. FRAP assay

The FRAP reagent was prepared from acetate buffer (pH 3.6), 10 mmol 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 mmol HCl and 20 mmol iron (III) chloride solution in proportions of 10:1:1 (v/v/v), respectively. 100 µl, 200 µl and 300 µl of FLC (each in three replicates) were added to 1.5 mL of the FRAP reagent. The absorbance of the reaction mixture was then recorded at 593 nm after 4 min.

The standard curve was constructed using iron (II) sulfate solution (100–2000 g mL^{-1}), and the results were expressed as g ml^{-1} Fe (II) of various concentrations of FLC used in this study[18].

4. Elastase inhibitory activity assay

Elastase inhibitory activity was measured by modified method of Sigma. A mixture of 10 µl to 50 µl of FLC, 5 µl elastase from porcine pancreas [Sigma 45124, USA] (0.5 mU ml^{-1} in the cool aquades) and 125 µL Tris buffer was pre incubated for 15 min at 25°C. Mixed solution was added N-Sucanyl-Ala-Ala-Ala-p-Nitroanilide substrate[19] and then incubated for 15 min at 25°C.

Absorbance was measured at 410 nm wavelength.

% Elastase inhibition = (1-B/A) x 100%

A = sample absorbance

B = control absorbance

5. Hyaluronidase Inhibitory Activity Assay

Hyaluronidase inhibitory of activity was measured by modified method of Sigma Aldrich. A 5 to 25 µL of FLC solution and 3 µL hyaluronidase from bovine testes type I-S [Sigma H3506, USA] was pre-incubated for 10 min at 37 °C and then added 12 µL phosphate buffer (300 mM, pH 5.35) for 10 min at 37 °C. Afterward 10 µL hyaluronic acid substrate [Sigma H5542, USA] was added and incubated for 45 min at 37 °C. Decomposition reaction of hyaluronic acid was stopped by adding 100 µL acidic albumin acid. Mixed solution incubated at room temperature for 10 min, then absorbance was measured at 600 nm wavelength[20].

Quantification of inhibition activity is expressed as:

% Hyaluronidase inhibition= (1-B/A) x 100%

A = sample absorbance

B = control absorbance

STATISTICAL ANALYSIS

All the experiments were repeated at least three times with three replications for each treatments. Therefore, the data represent the means and standard errors (Mean ± SD) and were calculated using Microsoft Excel 2010 software.

RESULTS

1. Extraction of Flaxseed lignans

Several methods have been reported by the earlier researchers for the extraction of lignans. The highest SDG content could be extracted by the method of[21]. On dry matter basis, different samples of flaxseeds varied considerably in their content of SDG (11.9–25.9 mg g^{-1}) along with p-coumaric acid glucoside (1.2–8.5 mg g^{-1}), and ferulic acid glucoside (1.6–5.0 mg g^{-1}). In our study, the SDG content of LC was found to be 23.28 mg g^{-1}.

2. DPPH radical scavenging assay

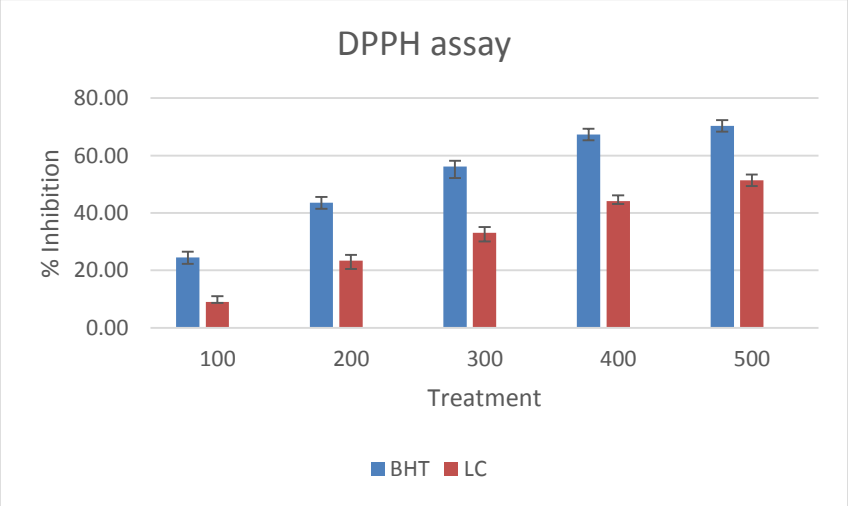

Fig. 5: 1-diphenyl-2-picrylhydrazyl assay of different formulation Each bars point is the Mean ± SD of three replicates from three independent experiments.

The results of the antioxidant, free radical scavenging of lignans concentrate was presented in the Figure 5. The DPPH radical scavenging ability of lignans concentrate has the IC_{50} value of 486 µg ml^{-1} whereas BHT has 267 µg ml^{-1}.

3. FRAP assay

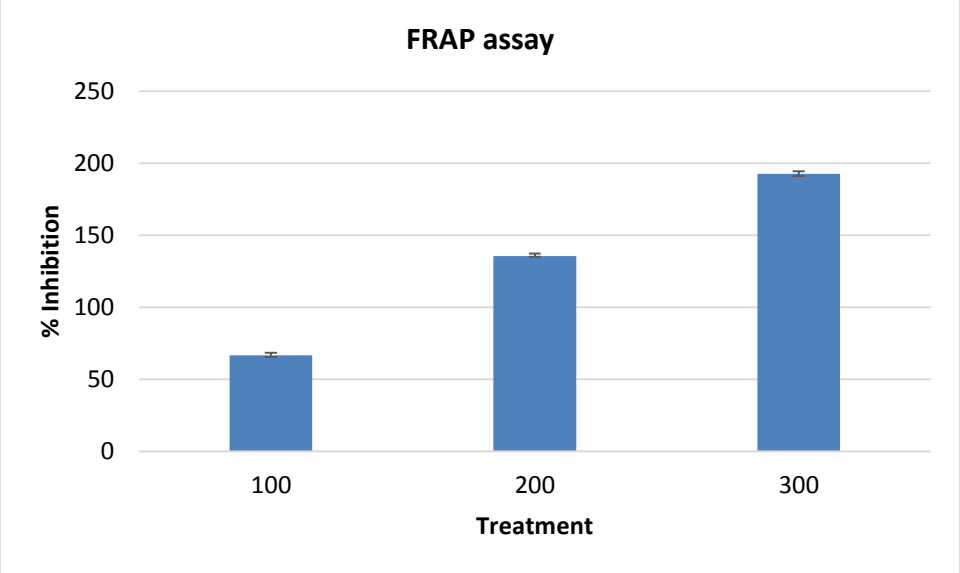

Fig. 6: Ferric reducing antioxidant power assay of different concentration Each bars point is the Mean ±SD of three replicates from three independent experiments

The FRAP assay was carried out according to the procedure Benzie and Strain 1996. The standard curve was constructed using Iron (II) sulfate solution (µg ml^{-1}). The results were expressed as µg ml^{-1} Fe (II) of lignans concentrate used in study. Lignans concentrate has IC_{50} value of 74.12 µg ml^{-1}.

4. Elastase Inhibitory Activity Assay

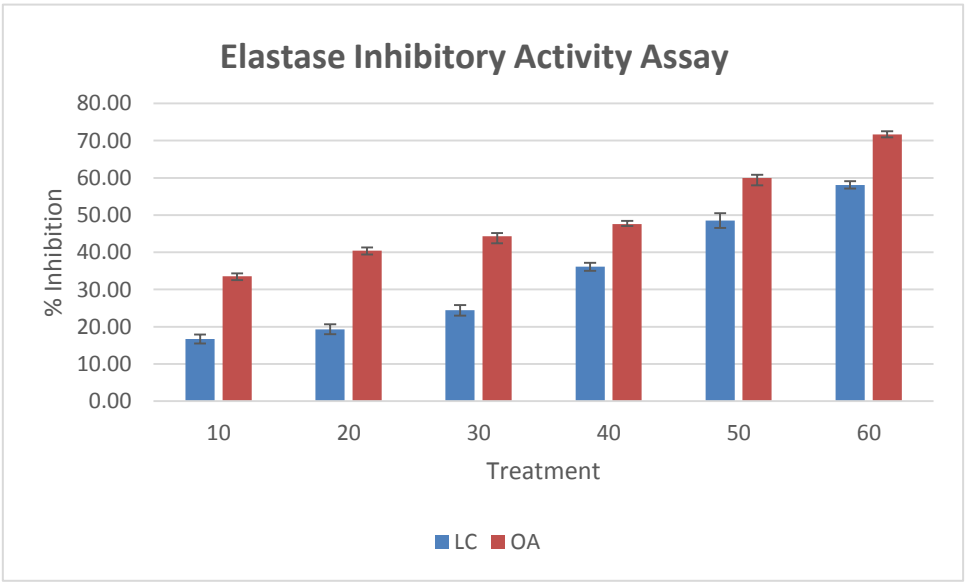

Fig. 7: Elastase inhibitory activity of Lignans Concentrate and Oleanolic Acid Each bars point is the Mean ±SD of three replicates from three independent experiments

The elastase inhibitory activity were measured. Lignans concentrate showed elastase inhibition with IC_{50} value of 51.44 µg ml^{-1} whereas standard molecule oleanolic acid has IC_{50} value of 41.72 µg ml^{-1}.

5. Hyaluronidase Inhibitory Activity Assay

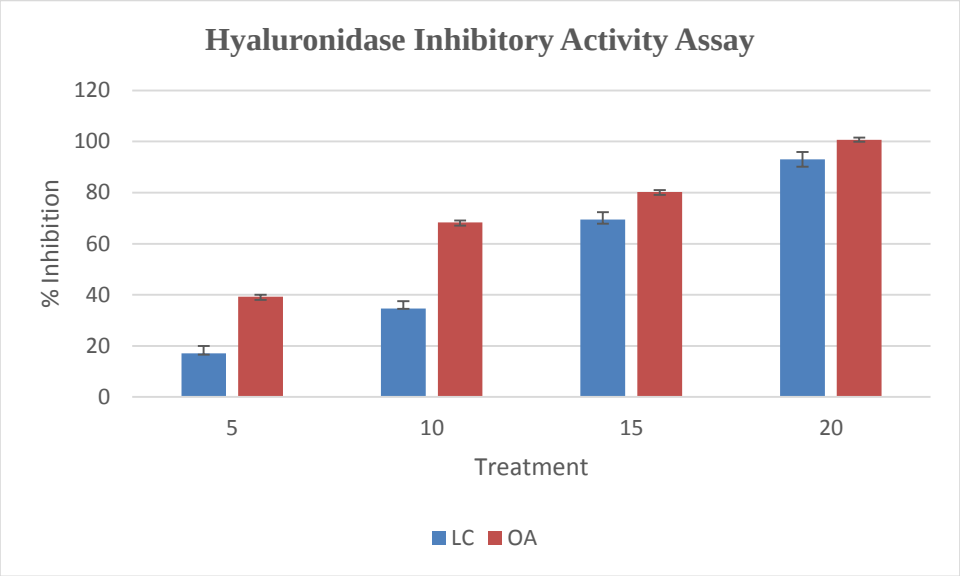

Fig. 8: Hyaluronidase inhibitory activity of Lignans Concentrate and Oleanolic Acid Each bars point is the Mean ± SD of three replicates from three independent experiments

Hyaluronidase was assayed by a highly sensitive spectroscopic method, based on precipitation of hyaluronic acid with cetylpyridinium chloride, which is used for high throughput screening for hyaluronidase inhibitors. The IC_{50} value for hyaluronidase inhibitory activity of lignans concentrate was 10.75 µg ml^{-1} whereas oleanolic acid had IC_{50} value of 9.93 µg ml^{-1}.

DISCUSSION

The DPPH method is commonly used worldwide for *in vitro* quantification of free radical scavenging activity[22]. In the present study, the investigation of total antioxidant capacity was measured as the capacity of the flaxseed lignans concentrate to scavenge stable organic free radicals with colour development, which gave the absorbance maxima at 517nm. As this method is sensitive to light, oxygen, pH and type of solvent used[23] extra care was taken to overcome these. It has been proved that phenolic, flavonoids compounds and other secondary metabolites present in the plants are mainly responsible for antioxidant activity[24]. From the above results, it can be confirmed that lignans, flavanoids and polyphenolics proved their higher efficiency as an antioxidant. For further confirmation of antioxidant potential of Flaxseed lignans concentrate FRAP assay was carried out. The phenolic phytochemicals exhibits oxidation-reduction properties, which play a crucial role in determining the total antioxidant properties[25]. In the present study, the reducing ability of FLC was strongly correlated with the percent inhibition by standard molecule. It has been established that elastin, collagen and hyaluronic acid are the main components of skin which have an important role in maintaining skin structure and hydration[26]. Moreover, elastin is a fibrose protein that compose 2–4% of the ECM and involved in the hydration of the skin[29]. On the other hand, collagenase, hyaluronidase and elastase cause repetitive collagen fibers breakdown and responsible for structural defect in dermis and wrinkle development[27]. Collagenase and elastase contribute in production and degradation of fibers, which is also influenced by free radical oxidative stress[28]. The enzyme elastase is responsible for increased tissue permeability, inflammation progress and delayed wound healing[30]. Moreover, elastase is also the key enzyme that attacks all the major connective tissue matrix protein[31]. While, the enzyme hyaluronidase selectively degrade hyaluronic acid by lowering its viscosity and

increasing the permeability[32]. Which is an acidic structural polysaccharide found exclusively in the extracellular matrix (ECM). The HPLC analysis of flaxseed lignans concentrate revealed that it includes major fraction as SDG along with p-coumaric acid glucoside and ferulic acid glucoside[21]. Moreover, a smaller quantities of other type lignans such as matairesinol, isolariciresinol, lariciresinol and pinoresinol have also been identified in the flaxseed[9]. Further, it has been proved that phenolic, flavonoids compounds and other secondary metabolites present in the plants are known to inhibit extracellular matrix degradative enzymes. Hyaluronidase inhibitors are thus potent regulators that maintain hyaluronic acid homeostasis and they might serve as anti-inflammatory, anti-aging, antimicrobial, anti-cancer and antitoxin and contraceptive agents[33].

CONCLUSION

From the present study, it was found that lignans extracted from defatted flaxseed hull potentially inhibited the hyaluronidase and elastase enzyme activity. It also showed free radical scavenging activity. Thus, it can be concluded that Flaxseed Lignans may have potential role on skin care, which has very good economic potential and being cultivated in more than 50 countries. In addition to SDG, smaller quantities of other type lignans such as matairesinol, isolariciresinol, lariciresinol and pinoresinol have also been identifid in the flaxseed. SDG is the converted into mammalian lignans, enterodiol (ED) and enterolactone (EL) by colon bacteria can be explored further for its use in cosmetics for its enzyme inhibition activity.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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