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Antioxidant Potentials of Flaxseed by in Vivo Model

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The present study reports the antioxidant activity of flaxseed as measured by feeding weanling albino rats with 5.0% and 10.0% of flaxseed (constituting approximately 0.75 and 1.5 g kg^{-1}) for 14 days followed by challenging animals with 2.0 g kg^{-1} b.w. CCl_4 as toxin. Activity was assessed by measuring hepatic marker enzymes like catalase, superoxide dismutase (SOD), and peroxidase and comparing with those from the normal group and from a group receiving toxin without flaxseed. Treatment of CCl_4 at dose of 2.0 g kg^{-1} b.w. decreased the activities of various antioxidant enzymes such as catalase, superoxide dismutase (SOD), and peroxidase by 35.6%, 47.76%, and 53.0%, respectively, compared to the control group, and the lipid peroxidation value increased nearly 1.2-fold compared to that of the group treated with toxin without flaxseed. Pretreatment of rats with 5.0% flaxseed followed by CCl_4 treatment caused restoration of catalase, SOD, and peroxidase by 39.7%, 181.42%, and 123.7%, respectively, as compared to control. The group treated with 10.0% flaxseed has shown the restoration of 95.02%, 182.31%, and 136.0% of catalase, SOD, and peroxidase. In the case of the group treated with toxin without flaxseed, the level of superoxide dismutase and the catalse value decreased 91.4% and 55.33%, respectively, in comparison with the control group. These results clearly indicate the beneficial effect of flaxseed components as an antioxidant as seen by restoration of hepatic enzymes, which were varied from normal to one due to toxicity induced by toxin (CCl_4). Owing to this property, the flaxseed known for its functional properties can be further extended to exploit its possible application for various health benefits as nutraceuticals and food ingredient.

KEYWORDS: Flaxseed; antioxidant activity; carbon tetrachloride; secoisolariciresinol diglucoside; α-linolenic acid; LDL

INTRODUCTION

Currently, there is great interest in functional components of food ingredients. This is mainly due to the fact that many studies have shown that reactive oxygen species (ROS), including oxygen free radicals (O•), are part of the etiology of degenerative disorders including some hepatopathies and other serious organ damage (*1*−*4*). ROS have also been shown to modify damage proteins, carbohydrates, and DNA using both in vitro and in vivo models (*5*). These free radicals attack unsaturated fatty acids of biomembrane, which results in lipid peroxidation as well as desaturation of proteins and DNA. This was observed in whole blood cells by measuring 7-hydroxy-8-oxo-2′-deoxyguanosine and the comet assay. Research has also shown that both single and a combination of antioxidants are capable of protecting DNA as seen in the case of effects in mononuclear blood cells, lymphocytes, or leukocytes (*6*). DNA damages may concern strand breaks, cross-links (to proteins, lipids), base/sugar alterations, and formation of DNA adducts. Much circumstantial evidence suggests that free radical biochemistry must be considered to have major significance in mutagenesis, carcinogenesis, aging, and neurodegenerative diseases, such as Parkinson's and Alzheimer's diseases (*6*). This causes a series of deteriorative changes in the biological systems leading to cell inactivation, as in Alzheimer's, coronary heart diseases, and diabetes, which are being continuously studied even today. ROS is also utilized as defense against many of the pathogens especially by white blood cells (*7*). Thus identification of antioxidants, which can retard the process of lipid peroxidation by blocking the generation of free radical chain reaction, has gained attention in recent years (*8*, *9*). The antioxidants may also act by raising the levels of endogenous defense by up-regulating the expression of genes encoding the enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase, or lipid peroxidase (*10*, *11*). The level of antioxidant activity in nutraceutical and pharmaceutical preparations is quantified by in vitro and in vivo studies where the focus is mainly on the role in scavenging reactive oxygen species, since ROS

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is a well-known contributor to the aging process and pathogenesis (*5*). According to in vitro and in vivo studies, several classical antioxidants have been shown to protect various cells such as hepatocytes and nephrocytes against lipid peroxidation or inflammation, thereby preventing the occurrence of hepatic neurosis, kidney damage, and other radical associated activities (*12*−*14*).

Flaxseed is obtained from *Linum usitatissimum* belonging to family linaceae, commonly known as linseed. Flaxseed is an important oilseed crop grown around the world for its oil and fiber. Flaxseed has been consumed as an ingredient in various food formulations and currently has a high demand in food industries. Flaxseed has been playing a major role in the field of diet and disease research due to its potential health benefits associated with α-linolenic acid (57%) and a major lignan, secoisolariciresinol diglucoside (SDG) (*15*, *16*), which is 600−700 times higher than in other edible sources of plant (*17*). Flaxseed is rich in α-linolenic acid and soluble and insoluble fiber; it is also the richest source of mammalian lignan precursors ranging from 100 to 800 times higher than in any other vegetable source (*17*−*19*). The lignan SDG is converted to mammalian lignans enterodiol and enterolactone by the action of gut microflora (*20*). The other lignans such as matairesinol, isolariciresinol, and pinoresinol are also present in much lower amounts in flaxseed (*15*, *16*, *21*, *22*). There are reports on anticarcinogenic and antiestrogenic properties of flaxseed, which are attributed to the presence of lignans (*23*). In addition, the antioxidant activity of the flaxseed lignan secoisolariciresinol diglucoside and its mammalian lignans enterodiol and enterolactine were also reported (*24*, *25*). SDG is implicated in the suppression of antioxidant conditions of ROS, such as hydroxyl radical, hydrogen peroxide superoxide anion, etc., in the chronic diseased body. The other plant lignans such as sesamin and sesamolin of sesame seed have been reported for both their in vivo and in vitro antioxidant activity (*26*, *27*). The mice treated with lignan schisandrin B have shown the increased activity of the antioxidant enzymes when compared to carbon tetrachloride treated mice and control group mice (*26*). The lignan genistein has been shown to be a potential antioxidant in vitro (*28*). Therefore, the incorporation of flaxseed in food and in animal diet has great advantages, helping in the inhibition of diseases and the promotion of health.

Research evidence has shown that SDG prevents/inhibits mammary carcinogenesis in rats (*29*−*31*). Other than antioxidant property, flaxseed lignans also cause changes in the menstrual cycle by prolonging the luteal phase and hormone progesterone (*32*).

In the present study, we reported possible antioxidant potentials of flaxseed using an in vivo model, which was done in order to enhance the functional attributes of the well-known traditional food ingredient flaxseed. The experiment was performed using rats by measuring some of the hepatic biochemical markers after treating with carbon tetrachloride as toxin. This study provides, for the first time, direct evidence for the antioxidant role played by SDG and phenolic compounds rich flaxseed.

MATERIALS AND METHODS

All the solvents and chemicals used were of analytical/HPLC grade obtained from Merck (Mumbai, India). The UV−visible spectrum measurements were carried out using a 160A spectrophotometer, Shimadzu Instrumentation Co., USA. Standard SDG was a generous gift sample from Saskatoon Research Center, Saskatoon, Canada (which was 98% pure and was extracted according to the Klosterman method described by Rickard et al. (*33*).

A. Flaxseed Material. The flaxseed (*L. usitatissimum*) grown in North Karnataka, India, was purchased from the local market. It was authenticated at University of Agricultural Sciences, Hebbal, Bangalore, India, and the seed variety was identified as LVF-01. The samples (1.0 kg) were cleaned and subjected for size reduction to a coarse powder in a domestic mixer grinder and used for study.

B. Experimental Design. Albino rats of both sexes of the Wister strain weighing 180−220 g (24 in total) were used for the studies. The animals were grouped into four groups ($N = 6$): The first group served as normal and received normal diet without treatment of toxin and flaxseed. The second group was named the control and received a regular commercial diet (containing crude protein 21%, crude fiber of 4.8%, fat 4.0%, and 3.0% of added minerals; average consumption of diet by an animal was 12 ± 2.0 g). The third and fourth groups were fed with normal diet and supplemented with flaxseed (5% and 10% which was mixed in the concentration with commercial diet replacing the quantity of the same to make a pellet) for 14 days. The animals of the second, third, and fourth group were given a single oral dose of CCl_4 at a dose of 2.0 g kg^{-1} b.w. (in 1:1 olive oil) 6 h after the last day feeding of diet on the fourteenth day. After 24 h the animals were sacrificed, and the liver from each animal was isolated to prepare the liver homogenate. Liver homogenate 5.0% (w/v) was prepared with 0.15 M KCl and centrifuged at 800 g for 10 min. The cell-free supernatant was used for the estimation of lipid peroxidation, catalase, and peroxidase. The other 5.0% (w/v) homogenate prepared using phosphate buffer (5.0 M) containing 0.25% sucrose (w/v) was used for SOD assay (*34*). All the protocols were followed as per ethical committee guidelines after clearance for experiment.

C. Analysis of Catalase. The catalase assay was carried out by the method of Aebi (*35*). One milliliter of liver homogenate from groups 1−4 was taken with 1.9 mL of phosphate buffer in different test tubes (125 mM, pH 7.4). The reaction was initiated by the addition of 1 mL of hydrogen peroxide (30 mM). Blank without liver homogenate was prepared with 2.9 mL of phosphate buffer and 1 mL of hydrogen peroxide. The decrease in optical density due to decomposition of hydrogen peroxide was measured at the end of 1 min against the blank at 240 nm. Units of catalase were expressed as the amount of enzyme that decomposed 1 μM H_2O_2 per minute at 25 °C. The specific activity was expressed in terms of units per milligram of protein.

D. Analysis of SOD. The assay of SOD was based on the reduction of nitroblue tetrazolium (NBT) to water insoluble blue formazan, as described by Beuchamp and Fedovich (*36*). Liver homogenate (0.5 mL) was taken, and 1 mL of 125 mM sodium carbonate, 0.4 mL of 24 μM NBT, and 0.2 mL of 0.1 mM EDTA were added. The reaction was initiated by adding 0.4 mL of 1 mM hydroxylamine hydrochloride. Zero time absorbance was taken at 560 nm followed by recording the absorbance after 5 min at 25 °C. The control was simultaneously run without liver homogenate. Units of SOD activity were expressed as the amount of enzyme required inhibiting the reduction of NBT by 50.0%. The specific activity was expressed in terms of units per milligram of protein.

E. Analysis of Peroxidase. The peroxidase assay was carried out as per the method of Nicholas (*37*). Liver homogenate (0.5 mL) was taken, and to this were added 1 mL of 10 mM KI solution and 1 mL of 40 mM sodium acetate solution. The absorbance of potassium periodide was read at 353 nm, which indicates the amount of peroxidase. Twenty microliters of hydrogen peroxide (15 mM) was added, and the change in the absorbance in 5 min was recorded. Units of peroxidase activity were expressed as the amount of enzyme required to change 1 unit OD per minute. The specific activity was expressed in terms of units per milligram of protein.

F. Lipid Peroxidation Activity. Thiobarbituric acid (TBA) reacts with malondialdehyde (MDA) to form a diadduct, a pink chromogen, which can be detected spectrophotometrically at 532 nm (*38*). Liver homogenate (0.5 mL) and 1 mL of 0.15 M KCl were taken. Peroxidation was initiated by adding 250 μL of 0.2 mM ferric chloride. The reaction was run at 37 °C for 30 min. The reaction was stopped by adding 2 mL of an ice-cold mixture of 0.25 N HCl containing 15% trichloroacetic acid, 0.30% TBA, and 0.05% BHT and was heated at 80 °C for 60 min. The samples were cooled, and results were expressed as MDA an equivalent, which was calculated by using an extinction coefficient of

Table 1. Analysis of Variance (ANOVA) for Different Treatments[a]

		SOD		POD		catalase		ALP	
source of variation	Df	MSS	F-value	MSS	F-value	MSS	F-value	MSS	F-value
replication	5	1.3639	0.6011	12.782	3.7623	238.6	1.0525	7.5719	0.9736
treatment	3	80.841 6	35.6285**	545.69 62	160.6238 **	99588. 38	439.316 1**	110.0933	14.1564 **
error	15	2.269	1	3.3974	1	226.68 96	1	7.7769	1

[a] Df: Degrees of freedom. MSS: Mean sum of squares. F-value: Fishers value. **Significant at 1.0% level of significance.

1.56×10^5 M^{-1} cm^{-1}. One unit of lipid peroxidation activity was defined as the amount of TBA that converts to TBARS. The specific activity was expressed in terms of units per milligram of protein.

G. Determination of Proteins. Protein was determined using the method of Lowry et al. (*39*).

H. HPLC of SDG. The SDG concentrate was prepared from flaxseed fractions by the Klosterman method described by Rickard et al. (*33*). About 100 g of hull, endosperm, and flour fractions obtained from flaxseeds were taken, defatted by extracting with petroleum ether (1:6 w/v) and $CHCl_3$ (1: 6 w/v). The defatted flaxseed fractions were extracted with 10 mL of 1,4-dioxane/95% ethanol (1:1, v/v) in screw-capped test tubes for 16 h at 60 °C in a circulating water bath. The supernatant was separated from the residue by centrifugation at 2000 rpm for 30 min. After separation, solvent was gently evaporated under vacuum at 40 °C using Rotavapor-laborata-4000 (Heidolph, Heizbad, WB, Germany). Then, the concentrate were treated with 0.3 M aqueous sodium methoxide in anhydrous CH_3OH and concentrated. The concentrate was acidified to pH 3.5 by adding H_2SO_4 and further eluted in silica column with $CHCl_3$:CH_3OH:H_2O in the ratio of 65:35:10 (v/v/v) prior to HPLC analysis.

High performance liquid chromatographic analyses were carried out using Shimadzu model LC-10A (Shimadzu, LC-10A, Japan) fitted with solvent delivery system, guard cartridge column, photodiode array detector, and integrator. The column was a Shimpack RP-C18 column with 5 μm particle size, 4.6 mm inside diameter, and 250 mm lengths, and the detector was a SPD 10M AVP photodiode array detector and C-R7A integrator with class-10A software real time analyzer. The linear gradient mobile phase was 1% (v/v) acetic acid in water (solvent A) and methanol (solvent B). The following linear gradient profile was run at 1 mL min^{-1} over 55 min. At 0 min A = 100%, A = 40% and B = 60% at 44 min, and A = 100% and B = 0% at 55min. The solvent (mobile phase) was allowed to run for 3−5 min at initial gradient before injecting the next sample. The column thermostat was set to 40 °C, and the absorbance at 280 nm is used to detect migrating organic compounds. A series of five different standard solutions of SDG ranging from 0.0625 to 1.0 mg mL^{-1} was used to obtain the linearity curve. The SDG peaks of samples were identified by comparison with those of standard SDG solutions obtained from the linearity curve for standard SDG prepared by injecting 10 μL of standard solutions and quantified by spiking with a known amount of standard and also by comparing the area under curve. The repeatability of the method was evaluated by injecting standard solutions of SDG six times, and the relative standard deviation (RSD) percentage was calculated.

I. Statistical Analysis. Each biochemical estimation was done in triplicate, and the results were calculated separately for each set of estimations and expressed as mean ± standard deviation. The significance ($p < 0.05$) of the variables studied was assessed by a one-way analysis of variance (ANOVA) test using Microsoft Excel XP. The mean separations were performed by Tukee'y multiple range test for segregating means where the level of significance was set at $p \leq 0.05$.

RESULTS AND DISCUSSION

The total SDG content was found to be 2.4% w/w in the seeds of *L. usitatissimum*. The HPLC chromatogram showed the presence of SDG as one of the major components among the organic molecules of seed extracts, which has shown maximum absorbance at 280.0 nm along with other constituents (**Figure 1**). The retention time for SDG was 31.12 min. The amount of SDG present in flaxseed was calculated.

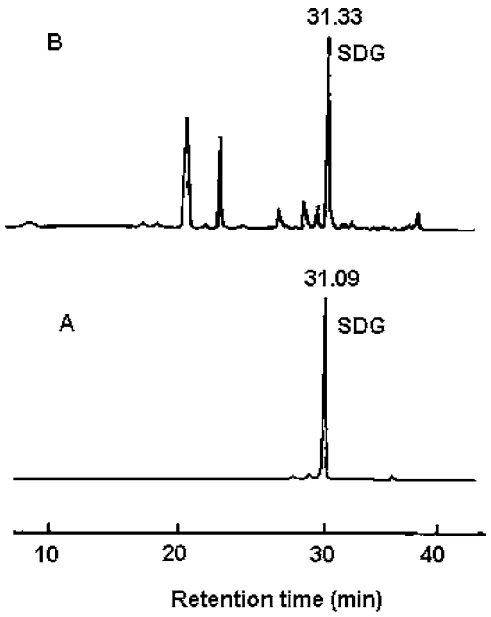

Figure 1. HPLC chromatograms of SDG. (**A**) Standard SDG (t_R, 31.09 min). (**B**) Flaxseed extract showing other components along with SDG (t_R, 31.33 min).

Among the animal groups, the ones which were treated with flaxseed at the concentrations of 5.0% and 10.0% showed significant antioxidant activity, i.e., protection against oxidative stress when compared to control group. Treatment of toxin to flaxseed treated animals was assessed for protection in terms of hepatic enzymes, namely, catalase, peroxidase, superoxide desmutase, and antilipid peroxidase, which served as biochemical markers. Statistical analysis revealed that all the treatments had significant influence on the level of these enzymes, indicating the protective ability of the flaxseed against radical mediated damage compared to control group (**Table 1**). Treatments of rats with toxin (carbon tetrachloride) at 2.0 g kg^{-1} body weight significantly reduced the levels of catalase, peroxidase, and SOD by 40%, 181.42%, and 123.7%, respectively (**Figures 2**−**4**). On the other hand, lipid peroxidation increased by 20.0% compared to normal due to the CCl_4 treatment (**Figure 5**). However, pretreatment of rats with 0.75 g kg^{-1} of flaxseed (5.0% feed) restored catalase, peroxidase, and SOD activities, and hence they were comparable with control values of respective enzyme. Restoration of catalase was 40% and 95.31% when compared to the toxin treated group, respectively, in the case of 0.75 g kg^{-1} and 1.5 g kg^{-1} of flaxseed (**Figure 2**). A similar trend was seen in the case of peroxidase and SOD enzymes (**Figures 4** and **5**).

Thus the experiment clearly shows the protection provided by feeding flaxseed to these rats by way of maintaining the levels of radical scavenging enzymes even after treatment of toxin. The lipid peroxidation was restored by 1.2−fold in the case of the 1.5 g kg^{-1} flaxseed treated group, and it was restored by 1.06-fold more in the case of the 0.75 g kg^{-1} flaxseed treated

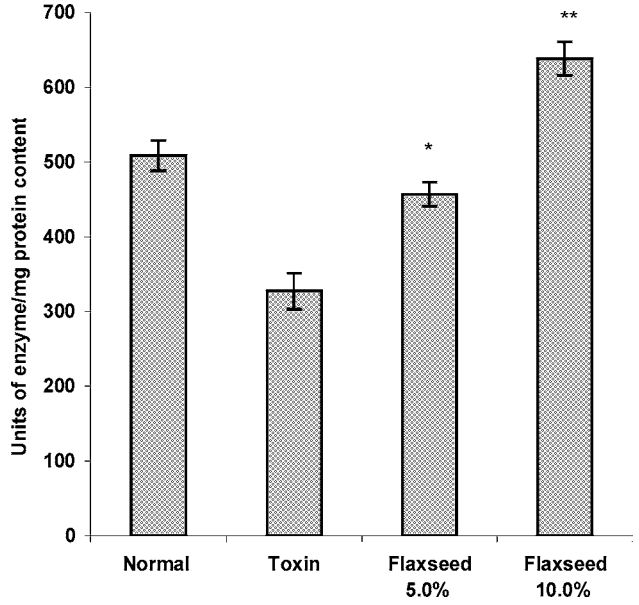

Figure 2. Effect of flaxseed treatment on hepatic catalse levels in CCl_4 intoxicated rats. CCl_4 intoxicated rats were treated with vehicle (olive oil) and two doses of flaxseed at 5.0% and 10.0% for 14 days. Animals of the normal group were treated with equal amounts of olive oil. Liver homogenate was obtained after CCl_4 treatment. Catalase content was determined as reported in the Materials and Methods section. Results are expressed as means ± SEM ($n = 6$). *$P < 0.05$ when compared to normal and **$P < 0.001$ as compared with the toxin treated group.

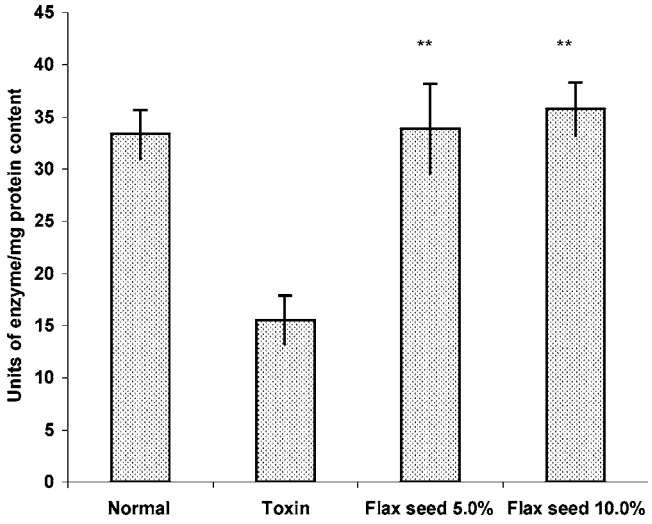

Figure 3. Effect of flaxseed treatment on hepatic peroxidase levels in CCl_4 intoxicated rats. CCl_4 intoxicated rats were treated with vehicle (olive oil) and two doses of flaxseed at 5.0% and 10.0% for 14 days. Animals of the normal group were treated with equal amounts of olive oil. Liver homogenate was obtained after CCl_4 treatment. Peroxidase content was determined as reported in the Materials and Methods section. Results are expressed as means ± SEM ($n = 6$). **$P < 0.001$ as compared with the toxin treated group.

group. The effect of free radicals on the mean liver detoxification enzymes (catalase, SOD, and peroxidase) reduced the enzyme activity, mainly due to enzyme inactivation during the catalytic cycle.

Carbon tetrachloride has been extensively studied as a liver toxicant, and its metabolites such as trichloromethyl radical (CCl_3^{∞}) and trichloromethyl peroxyl radical ($CCl_3O_2^{\infty}$) are involved in the pathogenesis of liver (*8*) and kidney damage. The massive generation of free radicals in the CCl_4 induced

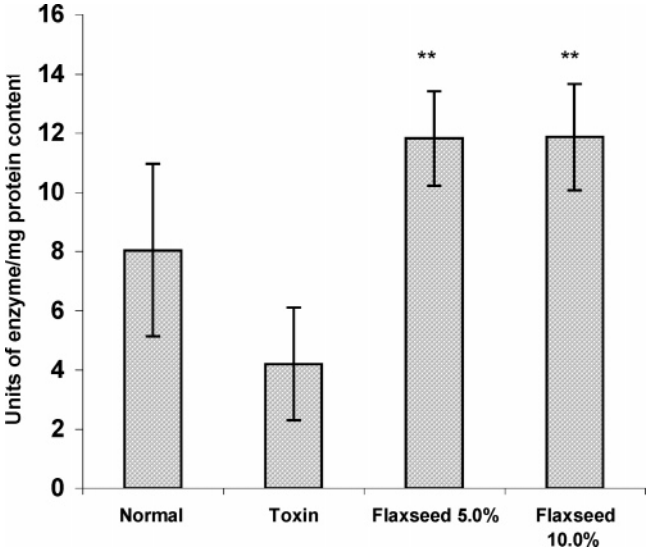

Figure 4. Effect of flaxseed treatment on hepatic superoxide desmutase levels in CCl_4 intoxicated rats. CCl_4 intoxicated rats were treated with vehicle (olive oil) and two doses of flaxseed at 5.0% and 10.0% for 14 days. Animals of the normal group were treated with equal amounts of olive oil. Liver homogenate was obtained after CCl_4 treatment. Superoxide desmutase content was determined as reported in the Materials and Methods section. Results are expressed as means ± SEM ($n = 6$). **$P < 0.001$ as compared with the toxin treated group.

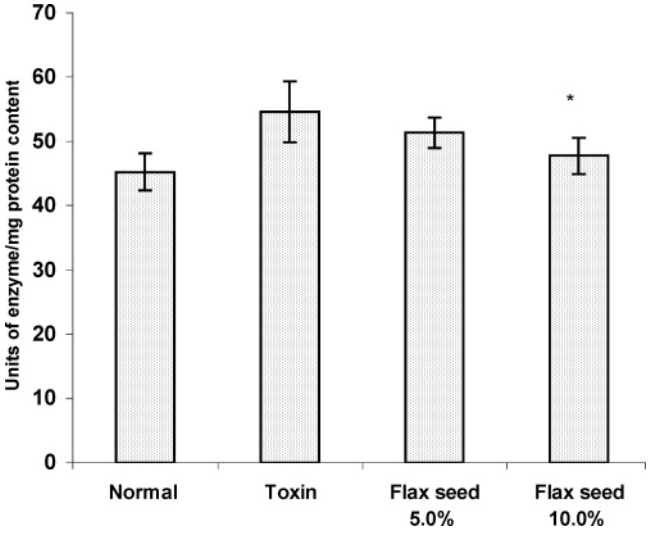

Figure 5. Effect of flaxseed treatment on hepatic antilipid peroxidation content in CCl_4 intoxicated rats. CCl_4 intoxicated rats were treated with vehicle (olive oil) and two doses of flaxseed at 5.0% and 10.0% for 14 days. Animals of the normal group were treated with equal amounts of olive oil. Liver homogenate was obtained after CCl_4 treatment. Antilipid peroxidation content was determined as reported in the Materials and methods section. Results are expressed as means ± SEM ($n = 6$). *$P < 0.05$ when compared to the toxin treated group.

liver damage provokes a sharp increase of lipid peroxidation in liver. When free radical generation is massive, the cytotoxic effect is not localized but can be propagated intracellularly, increasing the interaction of these radicals with phospholipid structure and inducing a peroxidation process that destroys organ structure (*40*).

Secoisolariciresinol diglucoside is principal lignan, chemically 2,3-bis[(4-hydroxy-3-methoxyphenyl)methyl]-1,4-butane diglucoside. It is known for its valuable pharmacological activities such as antibacterial and antiplatelet activity (*41*). Along with

SDG, other lignans are known for their chemopreventive effects on various tumors such as colon, skin, and mammary gland (*42*, *43*). Researches have shown that these lignans are beneficial in some of the major health hazards including cardiovascular diseases, menopausal symptoms, and osteoporosis (*44*, *45*).

There are a number of reports on radical scavenging potentials of SDG as measured by in vitro models (*46*, *47*); however, no reports are available on their ability at in vivo conditions as a number of factors decide the fate of these principal compounds in living systems. Hence the present study was aimed at finding the possible protection of flaxseed upon oral feeding against chemically induced radical damage by in vivo, and the results of the present study indicate that flaxseed can be a promising nutritional source of highly potential antioxidants.

In conclusion, flaxseed is known for its high content of protein (~26%), dietary fiber (~40%), fat (~ 32%), and essential minerals (4%) as major nutritional components (*48*). Oil constitutes about 32−35% of the whole seed; this includes some of the biologically active essential fatty acids such as ω-3 fatty acids and ω-6 fatty acids. Apart from SDG, other lignans like matairesinol and pinorecinol, which are diphenolic, may also contribute to various biological activities.

This can help enhance the biologically active lignans such as SDG through biotechnological or other route, which can help in making the flaxseed more popular as a nutraceutical and a prophylactic, as it is already known for its utility in conventional food. It can also help in different diseases in humans related to stress or free radicals and can be examined in individual diseases for the best suitability as therapeutic or prophylactic. The study can also give insight in understanding the possible biological active lignans from other natural sources for their utilization as a source of antioxidant.

ABBREVIATIONS USED

LDL, low density lipoproteins; CCl_4, carbon tetrachloride; SOD, superoxide dismutase; SDG, secoisolariciresinol diglucoside.

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