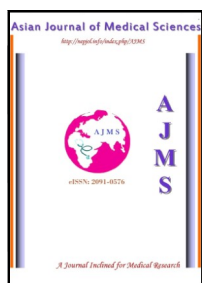


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Hepato-protective Potential of Hull Fraction from Indian Flaxseed Cultivar

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Abstract

Objective: Secoisolariciresinol diglucoside (SDG) isolated from hull fraction of Indian flaxseed cultivar was studied for its hepatoprotective potential by measuring the level of hepatic enzymes such as catalase, peroxidase and superoxide desmutase (SOD) upon feeding to albino rats.

Material & Methods: The animals were grouped into five groups (n=5): The first group served as normal and received normal diet without treatment of toxin and hull fraction of flaxseed. The second group was named the control and received a regular commercial diet. The third, fourth and fifth groups were fed with normal diet and supplemented with hull fraction of flaxseed (150 and 250 µg/kg) and standard SDG (150 µg/kg), that was mixed with olive oil for 14 days.

Results: Pretreatment of rats with 150 µg/kg b.w hull fraction of flaxseed followed by CCl₄ treatment caused restoration of catalase, SOD and peroxidase by 37.70%, 108.22% and 23.89% respectively as compared to control. The group treated with 250 µg/kg b.w hull fraction of flaxseed showed the restoration of 67.30%, 152.82% and 39.88% of catalase, SOD and peroxidase, respectively.

Conclusion: In conclusion, SDG fed in the form of flaxseed hull is responsible for its hepatoprotective properties. Further, it can be used as a potential source of nutraceutical agent and studies are required in this field to examine its use in large scale for food and feed industries.

Keywords: Flaxseed; hull fraction; SDG; antioxidant enzymes

1. Introduction

Carbon tetrachloride (CCl₄) is an industrial toxicant, known to cause not only the hepatic necrosis but also free radical generation in kidney, heart, lung, testis, brain and blood. Reactive oxygen species can destroy cellular membranes, cellular proteins, and nucleic acids. The toxic effects of CCl₄ are the consequences of production of free radicals, which initiate cell damage.¹ In recent years, flaxseed is gaining importance in diet supplements, as they possess nutraceutical properties. Flaxseed is one of the oldest domesticated crops and it is being increasingly used in the human diet because of its potential health benefits, particularly for cardiovascular

protection.² Flaxseed is the richest natural source of plant lignans, with secoisolariciresinol diglucoside (SDG) being the principal lignan compound, chemically 2, 3-bis [4-hydroxy-3-methoxyphenyl)methyl]-1,4-butane diglucoside. The concentrations of SDG in flaxseed vary with different cultivars. SDG concentrations in twenty-seven flaxseed species ranged from 1.19 to 2.59% for SDG and 0.22 to 0.5% (w/w) for its diastereoisomer.³ Flaxseed commonly contains 34-45.6% total fat, and α-linolenic acid alone represents 45-60% of the total fatty acid content in flax oil. Although α-linolenic acid is an ω-3 fatty acid, dietary α-linolenic acid did not show cholesterol-lowering effects in several clinical trials.⁴

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The plant lignans such as sesamin and sesamol in of sesame seed have been reported for their both in vivo and in vitro antioxidant activities.⁵ The lignan genistein has

been shown to be a potential antioxidant agent in vitro.⁶ In addition to SDG, the other lignans such as matairesinol, isolariciresinol and pinoresinol are also present in much lower amounts in flaxseed.⁷ There are reports on anticarcinogenic and antiestrogenic properties of flaxseed, which are attributed to the presence of lignans.⁸ Added to these, antioxidant activities of the flaxseed lignan, secoisolariciresinol diglucoside and its mammalian lignans enterodiol and enterolactone were also reported.⁹ Research evidence has also shown that SDG prevents/inhibits mammary carcinogenesis in rats.^{10,11} Other than antioxidant property, flaxseed lignans also cause changes in the menstrual cycle by prolonging the luteal phase and hormone progesterone.¹² Therefore, the incorporation of flaxseed in food and in animal diet has great advantages which helps prevention of diseases and the protection of health. As far as our literature survey could ascertain, no studies on the feeding of hull fraction of flaxseed alone has previously been published. The aim of this study was to determine the hepatoprotective potential of hull fraction of flaxseed supplementation on some of the hepatic biochemical markers using rats after treating with carbon tetrachloride as a toxin.

2. Materials and Methods

2.1. Seed material

The flaxseed (*L. usitatissimum*) was purchased from the local market. It was authenticated by the University of Agricultural Sciences, Hebbal, Bangalore, India, and the seed variety was identified as LVF-01. The sample (10 kg) was cleaned and subjected for dehulling process to obtain hull fraction of flaxseed using Kisan Krishi Yantra Udyog dehuller at Grain Science and Technology Department, CFTRI, Mysore, India

2.2 Chemicals

Analytical/HPLC grade solvents were obtained from Merck (Mumbai, India). Carbon tetrachloride (CCl₄), trichloroacetic acid (TCA), thiobarbituric acid (TBA), butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), nitroblue tetrazolium (NBT), potassium iodide (KI), potassium chloride (KCl), ethylene diamine tetra acetic acid (EDTA) and hydrogen peroxide (H₂O₂) were purchased from Loba Chemicals Ltd (Mumbai, India). Standard SDG (98% pure) was a generous gift sample from Dr. Lillian U. Thompson, Department of Nutritional Sciences, University of Toronto, Toronto, Canada.

2.3 High Performance Liquid Chromatography Analysis for SDG Content in Hull Fraction of Flaxseed

The SDG concentrate was prepared from hull fraction of flaxseed by the Klosterman method described by Rickard

et al.¹³ About 100 g of hull fraction obtained from flaxseeds was taken, defatted by extracting with petroleum ether (1:6 w/v) and CHCl₃ (1: 6 w/v). The defatted hull fraction of flaxseed was 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 was treated with 0.3 M aqueous Sodium methoxide in anhydrous CH₃OH and concentrated. The concentrate was acidified to pH 3.5 by adding H₂SO₄ and further eluted in silica column with CHCl₃:CH₃OH:H₂O 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).¹⁴ The SDG peaks of samples were identified by comparison with those of standard SDG.

2.4. Animals

All animal experiments were performed after due clearance from the Institutional Animal Ethics Committee. Male Wistar rats [Out B - Wistar, IND-Cft (2C)] weighing 180-220 ± 5 g were housed individually in steel cages at room temperature (28 ± 2 °C). A 12 h light/dark cycle was maintained and the rats received daily fresh pellet diet (Amrut feeds, Sangli, India) and had free access to tap water. The left-over diets were weighed and discarded.

2.5. Experimental Design

Albino rats of both sexes of the Wistar strain weighing 180-220 ± 5 g (25 in total) were used for the studies. The animals were grouped into five groups (n=5): a) the first group served as normal and received normal diet without treatment of toxin and hull fraction of flaxseed, b) the second group was named the control and received a regular commercial diet, c) the third, d) fourth and e) fifth groups were fed with normal diet and supplemented with hull fraction of flaxseed (150 and 250 µg/kg), standard SDG (150 µg/kg), which was mixed with olive oil for 14 days. 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. All the protocols were followed as per ethical committee guidelines after clearance for the experiment.

2.6. Catalase Assay

The catalase assay was carried out by the method of Aebi.¹⁵ One milliliter of liver homogenate from groups 1-5 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 protein.

2.7. Superoxide desmutase Assay

The assay of SOD was based on the reduction of NBT to water insoluble blue formazan, as described by Beuchamp and Fedovich.¹⁶ Liver homogenate (0.5 ml) was taken and 1 ml of 125mM sodium carbonate, 0.4 ml of 24 μM NBT, and 0.2ml of 0.1mM 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 protein.

2.8. Peroxidase Assay

The peroxidase assay was carried out as per the method of Alexander.¹⁷ Liver homogenate (0.5ml) was taken, and to this 1ml of 10mM KI solution and 1ml of 40mM sodium acetate solution were added. The absorbance of potassium periodide was read at 353nm, which indicates the amount of peroxidase. 20 μl of hydrogen peroxide (15mM) was added, and the change in the absorbance in 5min was recorded. Units of peroxidase activity were expressed as the amount of enzyme required to change 1unit OD per minute. The specific activity was expressed in terms of units per milligram protein.

2.9. Lipid peroxidation Assay

Thiobarbituric acid (TBA) reacts with malondialdehyde (MDA) to form a diadduct, a pink chromogen, which can be detected spectrophotometrically at 532 nm.¹⁸ Liver homogenate (0.5 ml) and 1 ml of 0.15M 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 2ml 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 $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$. One unit of lipid peroxidation activity was defined as the amount of TBA that converts to thiobarbituric acid reactive substances (TBARS). The specific activity was expressed in terms of units per milligram protein.

2.10. Determination of Protein

Protein content was determined by the method of Lowry's et. al.¹⁹

2.11. Statistical Analysis

Each biochemical experiments were done in triplicate. The results were expressed as mean \pm SD. The significance ($p < 0.05$) of the variables studied was assessed by one way analysis of variance (ANOVA).

3. Results

3.1. Isolation and Identification SDG Content in Hull Fraction of Flaxseed

The total SDG content was found to be 2.5% w/w in the seeds of *L. usitatissimum*. The HPLC chromatograms 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. The retention time for SDG was 30.91 min. The amount of SDG present in flaxseed was calculated.

3.2. Antioxidant enzymes

Among the animal groups, the groups that were treated with hull fraction of flaxseed at the concentrations of 150 and 250 $\mu\text{g}/\text{kg}$ body weight showed significant antioxidant activity, i.e., protection against oxidative stress when compared to control group. Treatment of toxin to hull fraction of flaxseed treated animals were assessed for protection in terms of hepatic enzymes, namely, catalase, peroxidase, superoxide desmutase and anti lipid peroxidation 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 hull fraction of flaxseed against radical mediated damage compared to control group. Lipid peroxidation increased by 1.6 fold compared to normal due to the CCl_4 treatment. However, pretreatment of rats with 150 $\mu\text{g}/\text{kg}$ of hull fraction of

flaxseed restored catalase, peroxidase and SOD activities; hence they were comparable with control values of respective enzymes.

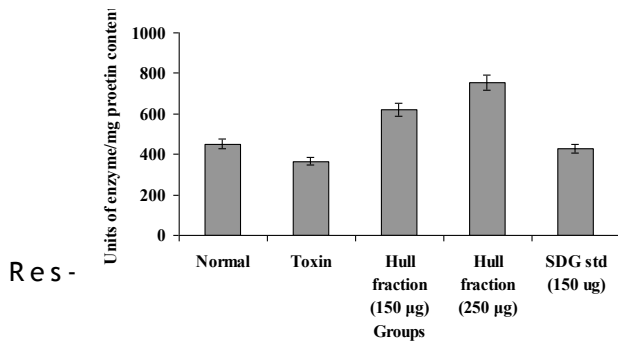


Fig. 1. Effects of hull fractions of flaxseed treatment on catalase levels in CCl_4 intoxicated rats. CCl_4 intoxicated rats were treated with vehicle (olive oil) and two doses of hull fractions of flaxseed at 150 and 250 $\mu\text{g}/\text{kg}$ body weight for 14 days. Results are expressed as means \pm SD (n =5).

toration of catalase was 37.70% and 67.30% when compared to the toxin treated group, respectively, in the case of 150 and 250 $\mu\text{g}/\text{kg}$ hull fraction of flaxseed (Figure 1). A similar trend was seen in the case of peroxidase and SOD enzymes (Figures 2 & 3).

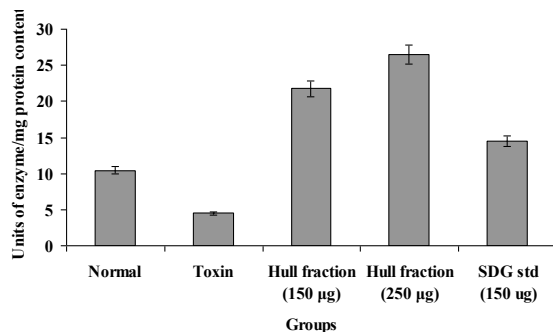


Fig. 2. Effects of hull fraction of flaxseed treatment on peroxidase levels in CCl_4 intoxicated rats. CCl_4 intoxicated rats were treated with vehicle (olive oil) and two doses of hull fractions of flaxseed at 150 and 250 $\mu\text{g}/\text{kg}$ body weight for 14 days. Results are expressed as means \pm SD (n =5).

Figure 4. shows that the lipid peroxidation was restored by 2.23 folds in case of 250 $\mu\text{g}/\text{kg}$ hull fraction of flaxseed treated group, and the same was restored by 1.14 folds in case of 150 $\mu\text{g}/\text{kg}$ hull fraction of flaxseed treated group. Thus, the experiment clearly shows the protection provided by feeding of hull fraction of flaxseed to these rats by way of maintaining the levels of radical scavenging enzymes even after treatment of toxin. Protection offered to the animals fed with hull fraction of flaxseed is evident from maintenance of the levels of the liver enzymes even after treatment of the toxin.

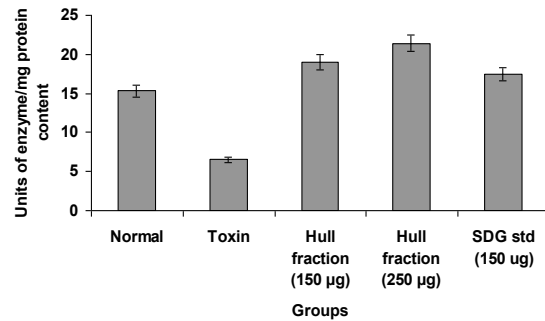


Fig. 3. Effects of hull fraction of flaxseed treatment on superoxide dismutase levels in CCl_4 intoxicated rats. CCl_4 intoxicated rats were treated with vehicle (olive oil) and two doses of hull fraction of flaxseed at 150 and 250 $\mu\text{g}/\text{kg}$ body weight for 14 days. Results are expressed as means \pm SD (n =5).

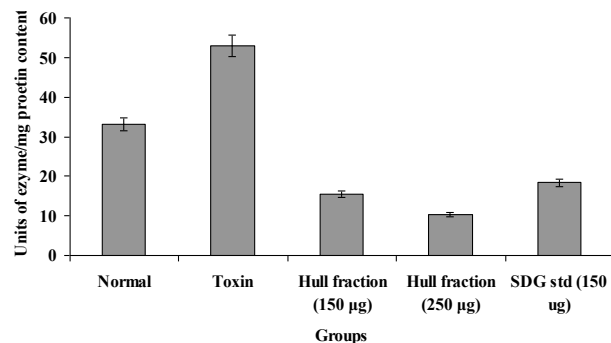


Fig. 4. Effects of hull fraction of flaxseed treatment on hepatic antilipid peroxidation levels in CCl_4 intoxicated rats. CCl_4 intoxicated rats were treated with vehicle (olive oil) and two doses of hull fraction of flaxseed at 150 and 250 $\mu\text{g}/\text{kg}$ body weight for 14 days. Results are expressed as means \pm SD (n =5).

4. Discussion

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 peroxy radical (CCl_3O_2) are involved in the pathogenesis of liver and kidney damage. The massive generation of free radicals in the CCl_4 induced liver damage provokes a sharp increase of lipid peroxidation in liver. When free radical generation is massive, the cytotoxicity effect is not localized but can be propagated intracellularly, increasing the interaction of these radicals with phospholipids structure and inducing a peroxidation process that destroys organ structure.¹⁴ SDG, the principal lignan of flaxseed is known for its valuable pharmacological activities such as antibacterial and antiplatelet activities.^{20,21} 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.^{14,20,21} There are number of reports on radical scavenging potentials of SDG as measured by in vitro models.^{6,22} However, no reports are available on their ability at in vivo conditions as number of factors decide the fate of these principal compounds in living systems. Flaxseed oil pre-treatment reduced radiation-induced sickness and mortality in mice by protecting against oxidative damage.²³ Protective role of flax lignans against lead acetate induced oxidative damage and hyperlipidemia in rat.²⁴ This can help enhance the biologically active lignans such as SDG through biotechnological route, which can help in making the flaxseed more popular as a nutraceuticals, functional 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. In conclusion, the present study indicated that the hull fraction of flaxseed is having potential hepatoprotective properties against carbon tetrachloride induced rats. The possible protection of hull fraction of flaxseed after feeding against chemically induced radical damage by in vivo and it showed clear potential for the utilization of hull fraction of flaxseed as a food additive and can also be used as a source of nutraceutical or nutritional supplements.

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