

Prophylactic Action of Flaxseed Oil against Cyclophosphamide Induced Oxidative Stress in Mice Liver

Dr. Brij Mohan Singh

Associate Professor, P.G. Department of Zoology, S. K. Govt. Girls P.G. College, Sikar (Rajasthan) 332001, India

Abstract: The study has been investigated that liver responsible for the detoxification of damaging electrophiles generated during oxidative stress in mammals. Although liver is enriched with endogenous antioxidants, but exogenous antioxidants are more helpful to detoxify toxicity generated by prooxidants. Author found decreased LDL activity in cyclophosphamide (CP) administered mice (90.36%) and further increase in flaxseed supplementation group (131.51%) indicating the protective mechanism of lignan component of the flaxseed oil. Similarly, significant increase were found in HDL, Cholesterol, triglycerides activities after CP administration were increased by 104.07%, 108.84% and 185.78% in comparison to control group (32.90 ± 0.34 , 14.70 ± 0.41 and 12.80 ± 0.80 $\mu\text{g/ml}$) and further increased in flaxseed treated mice by 140.18%, 155.78% and 400.46% respectively in comparison to control group of mice. There was also a remarkable elevation in the serum protein markers TNF- α , ALP, ALT, and AST. They were increased by 202.10%, 254.95%, 604.08% and 172.75% in CP administered group in comparison to control group and further increased by 241.68%, 492.48%, 366.78% and 142.29% in flaxseed supplemented group of mice. These pathological and biochemical changes correlated well with the altered enzyme activities. The results indicate the anti-oxidative properties of flaxseed oil which showed its prophylactic property against the cyclophosphamide-induced biochemical alterations in mice liver.

Keywords: Cyclophosphamide, prooxidants, oxidative stress, flaxseed oil.

1. Introduction

Recently, an interest has been generated to develop potential drug of plant origin for the modification of drug toxicity. Plant products appear to have an advantage over the synthetic compounds in terms of low/ no toxicity at the effective dose with no side effects. Keeping in view the fact, this study was carried out to evaluate the role of flaxseed oil against different pro-oxidants like cyclophosphamide (CP) induced oxidative stress. Flaxseed is a major source of lignan; they are widely distributed in plant derived food items and are believed to protect against cancer. The richest source of lignan is flaxseed or linseed which has been reported to contain glycosides of secoisolariciresinol as a major lignan together with small amount of matairesinol, isolariciresinol and pinorensinol (Eliasson *et al.*, 2003). Prasad (2005) found that secoisolariciresinol isolated from flaxseed reduces hypercholesterolemic atherosclerosis, decrease serum cholesterol, LDL-C, and lipid peroxidation products. This increases HDL-C and antioxidant reserve. Flaxseed were found to inhibit human breast cancer and metastasis and reduce expression of insulin like growth factor and epidermal growth factor receptors (Chen *et al.*, 2002). The role of lignan rich flaxseed oil against cyclophosphamide induced oxidative stress has also been evaluated (Manda and Bhatia, 2003).

Oxidative stress refers to the cytotoxic consequence of reactive oxygen byproducts; superoxide anions and hydroxyl radicals which are generated as metabolites of normal and aberrant metabolic processes that utilize molecular oxygen. Oxidative stress leads to lipid peroxidation, protein and carbohydrate oxidation and metabolic disorders. Lipid peroxidation within the membrane has a devastating effect on the functional state of

the membrane because it alters membrane fluidity, typically decreasing it and thereby allowing ions such as Ca^{++} to leak in to the cell. The preservation of the cellular membrane integrity through protection or repair mechanism capable of neutralizing oxidative reaction warrants an attention. *Origanum vulgare* is used to cure respiratory disease, hypoglycemic diseases and leukemia (Sheibani *et al.*, 2010). The major constituent of this plant extract is rosmarinic acid and origanol A and B have antioxidative activities (Kulisic *et al.*, 2007); Matsuura *et al.*, 2003). Some other reports showed that components of the aqueous of origanum plant extract such as ursolic acid, rosmarinic acid, exert potent antioxidant activities by scavenging free radicals (Di Sotto *et al.*, 2010; Lambert *et al.*, 2001). Liver is the primary organ of drug metabolism and is mainly responsible for the detoxification of damaging electrophiles generated during oxidative stress. Although mammalian liver is enriched with endogenous antioxidants and related enzymes but on acute drug toxicity supplementation of exogenous antioxidants became essential. Liver of mammals has been reported as highly susceptible to pro-oxidants. Hepatic injury can lead to life threatening complications when the entire or most of the liver exposed to toxic substances. Therefore the present study is aimed to reveal the mechanism of hepatoprotection by flaxseed oil in albino mice.

2. Materials and Methods

2.1 Animal Selection

40 Adult (6-8 week old) Swiss albino mice (*Mus musculus*) from an inbred colony, of body weight 150–200 gm have been selected from Animal Care Centre, Department of Zoology, University of Rajasthan, Jaipur. The animals were kept in a 12-hour light/dark cycle, at a temperature of 22°C

$\pm 2^{\circ}\text{C}$ with relative humidity $50\% \pm 20\%$, and ventilated with filtered non-recycled air. They were fed on standard chow pellets which were purchased from animal food Shoppe, Raja Park, Jaipur and tap water for the entire test period. Experimental procedures were carried out according to the Guidelines of National Institute of Health for Animal Care. The procedure was approved by the Local Ethics Committee, of university of Rajasthan, Jaipur.

Flaxseed oil has been given orally to mice. Different concentrations of flaxseed oil were given to the mice for the selection of optimum dose in terms of ml/kg body weight. After supplementation of 15 days, sublethal concentration (0.005 mg/L) of pro-oxidant cyclophosphamide (CP) has been injected to the animal. The survivability and body weight were checked up to 30 days. The dose of pro-oxidant (CP) which produced 50% mortality within 30 days was estimated. The group which showed maximum survivability and healthier condition were selected for the study.

2.2 Chemicals and reagents

Flaxseed was purchased from General store, central park, Jaipur. cyclophosphamide and other chemicals used in this study were of high analytical grade. Kits for cholesterol, triglyceride and HDL-cholesterol, Kits for high sensitivity TNF- α kits, Quantikine Immunoassay kits, and PON-1 kit was purchased from Sharma chemical suppliers and Co. Agra.

2.3 Experiment

Swiss albino mice have been divided in to 3 groups.

- (i) Control group, which were not received any other treatment.
 - (ii) Second group were administered with cyclophosphamide prooxidant (CP).
 - (iii) Third group of mice were already cyclophosphamide injected and given single dose of flaxseed oil (200 ml/kg body weight) orally (CP+FSO).
- Mice were sacrificed at various intervals ranging between 1-30 days.

2.4 Observation

Mice of all three groups after treatment were observed for survivability, body weight changes, sickness induced due to prooxidant and other abnormalities. All groups of mice were sacrificed at various intervals and liver were studied time to time for qualitative and quantitative parameters color, weight of liver, histopathological and biochemical analysis by standard methods in Rajiv pathological laboratory and Rathi hospital and diagnostic center, Sikar.

2.5 Histopathological analysis

The livers were fixed in 10% neutral buffered formalin, sliced transversely, paraffin embedded, and prepared as 5 μm thick sections that were stained with hematoxylin and eosin (H&E) for compound microscopic evaluation (Figure-2). Serum levels of hepatic markers, including ALT, AST, and ALP, were evaluated based on standard methods of

Ahmadi *et al.*, 2011. Three factors, hepato-cellular necrosis, level of inflammation in the portal area and lymphocytic inflammatory infiltrations, were evaluated using a semi-quantitative method. To estimate the ALT and AST serum activities, commercially available enzymatic kits (based on the reaction of 2,4-dinitrophenylhydrazine with pyruvate and/or oxaloacetate to yield a brown-colored complex in alkaline medium) were used. Serum alkaline phosphate (ALP) activity was measured using a spectrophotometric method. The results are expressed in the unit of $\mu\text{g}/\text{mg}$. At least three random visual fields from each animal were scored in a blinded manner by two expert pathologists.

2.6 Statistical Analysis

Various statistical analyses like t-test, variance (ANOVA) and DMRT etc. were performed with help of a computer in order to ascertain the level of significance. Descriptive statistics including frequency, mean, and standard deviation were used to describe different characteristics of data. The results were at the 5% level of significance.

3. Results and Discussion

For the sake of convenience, this section was divided in to following two steps:

(a) Assessment of lipid profile

Lipid profile improving effect of flaxseed has long been studied by researchers and many literatures have related its effect to high fiber, ALA and lignans content of flaxseed (Thakur *et al.*, 2009). The low density lipoprotein (LDL) level of mice has significantly decreased (90.36%) in comparison to control group $34.56 \pm 2.23 \mu\text{g}/\text{ml}$ in the cyclophosphamide administered group-II and further increase by 131.51% in group-III which is cyclophosphamide administered with flaxseed oil (Data of table-1 and figure-1). This could be due to the protective effect of lignan complex of flaxseed oil in reducing the extent of hypercholesterolemic atherosclerosis (Newairy and Abdou, 2009). On the otherhand, in high density lipoproteins (HDLs) a significant increase to control group ($32.9 \pm 0.34 \mu\text{g}/\text{ml}$) which was 104.07% in the cyclophosphamide administered group-II and further increase by 140.18% in group-III which is flaxseed supplemented group combined with cyclophosphamide prooxidant (Table-1 and Figure-1).

The significant increase in HDL due to flaxseed supplementation may be explained by the stimulation of lipid peroxidation. The most previous studies reported no change in HDL activities in response to dietary flaxseed (Dupasquer *et al.*, 2006). Activation of lipoprotein lipase enzyme activity also speeds up the conversion of very low density lipoprotein (VLDL) to HDL (Press *et al.*, 2003; Lee and Prasad, 2003). A remarkable elevation in cholesterol activity 108.84% ($16.0 \pm 5.30 \mu\text{g}/\text{ml}$) in CP administered group-II and 155.78% ($22.90 \pm 2.25 \mu\text{g}/\text{ml}$) in CP + flaxseed supplementation group-III is observed in comparison to control level $14.7 \pm 0.41 \mu\text{g}/\text{ml}$ (Table-1 and Figure-1). This elevation might be attributed to protective effects of lignan complex. The difference of all figures and data were significantly ($p < 0.001$) determined.

The comparison of triglyceride level of control and experimental group-II and flaxseed supplemented group-III has revealed that significant elevation in plasma triglyceride level was $23.78 \pm 2.37 \mu\text{g/ml}$ (185.78%) and $51.26 \pm 1.38 \mu\text{g/ml}$ (400.46%) respectively relative to control group-I i.e. $12.80 \pm 0.80 \mu\text{g/ml}$ (Table-1 and Figure-1). Elevated triglyceride level could be due to decreased activity of lipoprotein lipase enzyme in liver in acute myocardial ischemia (Sarifkan and Zaker, 2012). Alterations in the lipid profiles of isoproterenol-treated rats, attributed to enhanced lipid biosynthesis by cardiac cyclic adenine monophosphate (cAMP) may be responsible for the triglyceride elevation in group II (Santhil *et al.*, 2007).

(b) Assessment of serum profile

The extents of hepatic damage were assessed by measuring the levels of alkaline phosphate (ALP), alanine transaminase (ALT), and aspartate transaminase (AST) in the circulation (Bhattacharjee & Sil, 2007). The effect of flaxseed oil pretreatment on the serum TNF- α , ALP, ALT, AST activities after CP administration were increased by 202.10%, 254.95%, 604.08% and 172.75% in comparison to control group (13.29 ± 0.03 , 33.30 ± 0.04 , 11.26 ± 1.37 and $43.01 \pm 3.12 \mu\text{g/ml}$) and further increased by 241.68%, 492.48%, 366.78% and 142.29% in group-III in comparison to control group as shown in Table-1 and figure-1. TNF- α is a prominent element in the cytokine hypothesis of heart disease and has been the focus of research in myocardial ischemia (Kupai *et al.*, 2009). However, the majority of clinical dietary intervention trials were investigated the effects flaxseed on markers of hepatic inflammation. They have reported no effect on the serum levels of IL-6, TNF- α , soluble intercellular cell adhesion molecule-1, sVCAM-1, monocyte chemo-attractant protein-1, CRP, or serum amyloid A (SAA) protein (Dodin *et al.*, 2005 and Kaul *et al.*, 2008). Recent researches reported that moderate endurance activity influenced circulating cytokine levels specially the TNF- α levels which could be due to production of IL-6 produced by exercising muscle as an anti-inflammatory effect (Wood *et al.*, 2009). In the present study, a single CP dose resulted in a significant increase in the activities of serum ALT, AST, and ALP in the liver of mice. However, pretreatment with flaxseed oil significantly lowered the serum levels of hepatic markers, and the levels were comparable with the control animal values (Figure-2). The restoration of the levels of these marker in animals pretreated with flaxseed oil indicates the protective activity of flaxseed oil in the liver. This protective effect might be from flaxseed oil scavenging activity for the toxic metabolites produced during CP activation by liver microsomal enzymes. These data suggest a hepatoprotective role of flaxseed oil.

In the current study, histopathological and biochemical examinations proved that CP causes liver damage as evidenced by the observation of necrotic hepatocytes with small crushed nuclei, a portal space with severe inflammation, and hepatocytes surrounded by lymphocytic infiltration. These observations might be caused by the sequential membrane damaging potential of the CP's metabolites as shown in Figure-3. These pathological changes correlated well with the altered enzyme activities, and these findings are supported by another previous study (Senthilkumar *et al.*, 2006). Flaxseed oil pretreatment effectively alleviated the CP-induced hepatic histopathological changes; abnormal pathological findings, such as tissue injury and necrosis; and protected tissues from oxidative damage (Figure-3 a,b,c). These histopathological and biochemical observations suggest that flaxseed oil could protect tissues from damage and thus decrease the leakage of enzymes (AST, ALT, and ALP) into the circulation.

4. Conclusion

In this study, flaxseed oil showed dose-dependent protective effects and reduced cyclophosphamide-induced hepatotoxicity in mice. Administration of flaxseed oil to mice for limited days prior to CP injection attenuated the serum levels of hepatic markers. Histopathological examinations confirmed the cyto-protective effects flaxseed oil and for improving defense mechanisms in physiological systems against oxidative stress and inflammation caused by CP. This oil is more useful against CP-induced liver damages because CP-induced tissue damage might be alleviated by antioxidant activity, free radical scavenging, increased activity of the antioxidant defense systems, and membrane stabilizing properties of flaxseed oil. Because flaxseed oil has an additive therapy and herbal medicine for tumors, it could be a potential agent for a safe supplemental agent against the side effects of chemotherapy.

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Table 1: Levels of cholesterol, triglycerides, LDL, HDL in liver and TNF- α , ALP, ALT and AST serum level plasma of control and experimental groups of mice [Data are represented as the mean \pm SD of five mice]

Parameters↓	Group-I (Control Group)	Group-II (Only CP given)	Difference in (gp-I) and gp-II (%)	Group-III (CP+FSO)	Difference in (gp-I) and gp-III (%)
LDL ($\mu\text{g/ml}$)	34.56 ± 2.23	31.23 ± 3.24	90.36%	45.45 ± 1.31	131.51%
HDL ($\mu\text{g/ml}$)	32.9 ± 0.34	34.24 ± 5.22	104.07%	46.12 ± 4.50	140.18%
Cholesterol ($\mu\text{g/ml}$)	14.7 ± 0.41	16.0 ± 5.30	108.84%	22.90 ± 2.25	155.78%
Triglycerides ($\mu\text{g/ml}$)	12.80 ± 0.80	23.78 ± 2.37	185.78%	51.26 ± 1.38	400.46%
TNF- α ($\mu\text{g/ml}$)	13.29 ± 0.03	26.86 ± 1.32	202.10%	35.12 ± 1.20	241.68%
ALP ($\mu\text{g/ml}$)	33.30 ± 0.04	84.90 ± 2.10	254.95%	65.50 ± 1.20	492.48%
Serum ALT ($\mu\text{g/ml}$)	11.26 ± 1.37	68.02 ± 2.33	604.08%	41.3 ± 2.21	366.78%

Serum AST ($\mu\text{g/ml}$)	43.01 \pm 3.12	74.3 \pm 1.23	172.75%	61.2 \pm 1.34	142.29%
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Where CP: Cyclophosphamide; FSO: Flaxseed oil; LDL: Low density lipids; HDL: High density lipids; TNF: Tumour necrosis factor; ALP: Alkaline phosphatase; ALT: Alanine transaminase; AST: Aspartate transaminase.

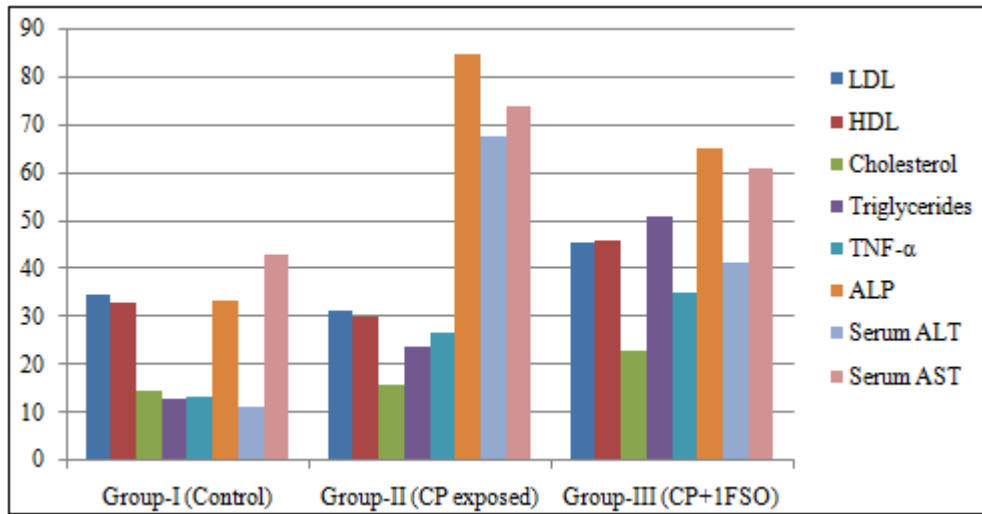


Figure 1: Showing Comparison of the parameters cholesterol, triglycerides, LDL, HDL in liver and TNF- α , ALP, ALT, AST and other serum level plasma of control and experimental groups.

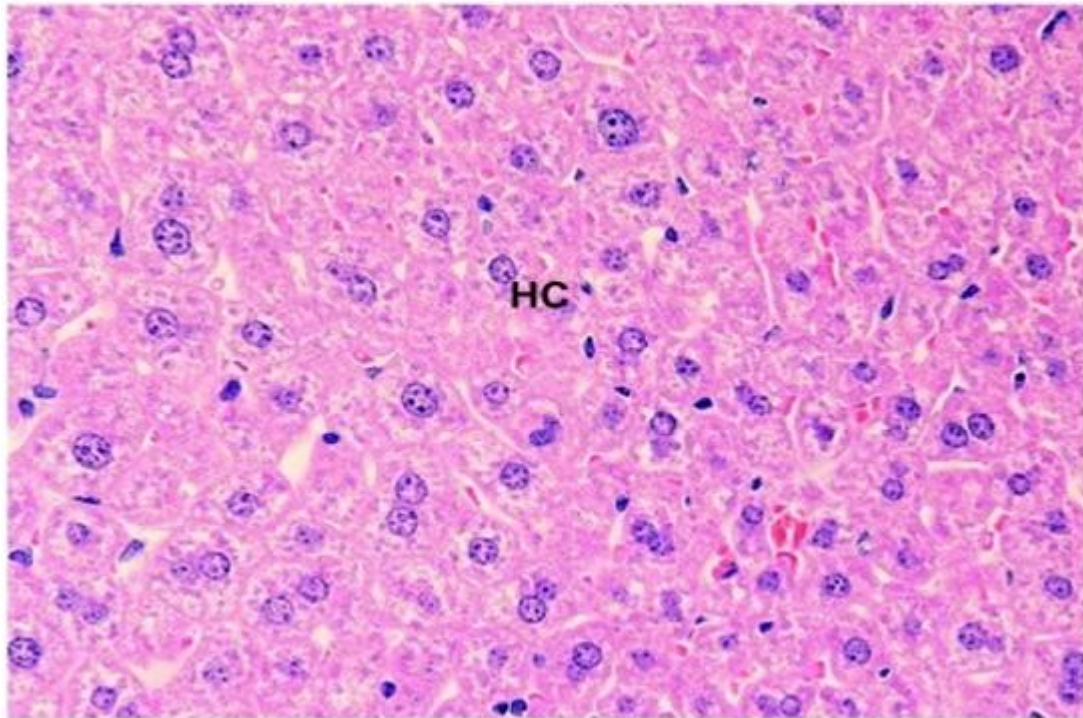


Figure2: Section of liver showing normal hepatocytes in the liver of control mice

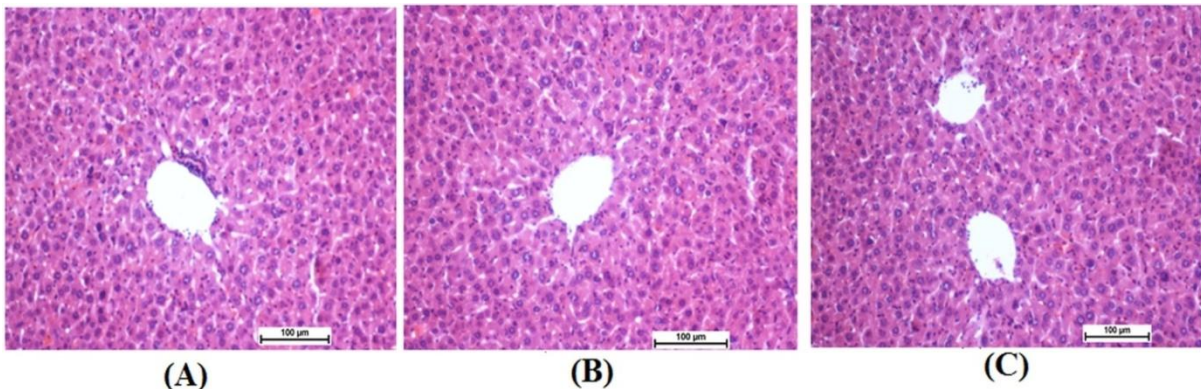


Figure 3: A, B and C Showing sequential cytototoxic effects of CP in mice liver

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