HEPATOPROTECTIVE EFFECT OF B-SITOSTEROL ON LIPID PEROXIDATION
AND ANTIOXIDANT STATUS IN ETHANOL-INDUCED HEPATOTOXIC RATS

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ABSTRACT
Liver is the key organ regulating homeostasis in the body. It plays a key role in food processing and in the process of detoxification. The present study was designed to investigate the effect of β-sitosterol on lipid peroxidative products such as thiobarbituric acid reactive substance (TBARS) and lipid hydroperoxide and antioxidant status in ethanol-induced hepatotoxic rats. Ethanol induction (3 g/kg) caused toxicity to liver that lead to various physiological and biochemical alterations. Albino Wistar rats were divided in to 5 groups, β-sitosterol was suspended in carboxymethyl cellulose and administered to rats orally at the dosage of 20 and 40 mg/kg/day for 35 days. Hepatotoxic rats had elevated levels of TBARS and hydroperoxide and decreased antioxidant activities (both enzymatic and non-enzymatic) when compared with normal control rats. Treatment with β-sitosterol shows the decreased level of lipid peroxides and increased levels of antioxidants in ethanol-induced hepatotoxic rats. These results demonstrated that β-sitosterol protected liver damage and it may have beneficial properties in the prevention of liver toxicity.

KEYWORDS: Hepatotoxic, lipid peroxidation, antioxidant, ethanol, β-sitosterol

INTRODUCTION
Liver is one of the largest organs in human body and the chief site for intense metabolism and excretion which play an important role in the maintenance, performance and regulating homeostasis of the body. It is involved with almost all the biochemical pathways to growth, fight against disease, nutrient supply, energy provision and reproduction (Nasir, et al., 2013). The major functions of the liver are carbohydrate, protein and fat metabolism, detoxification, secretion of bile and storage of vitamin. Thus, to maintain a healthy liver is a crucial factor for overall health and well being. Liver is continuously and variably exposed to environmental toxins and abused by poor drug habits and alcohol which can eventually lead to various liver ailments like hepatitis, cirrhosis and alcoholic liver disease (Noorani, et al., 2010).

Alcohol-induced liver injury is one of the world’s major health problems and excessive alcohol consumption caused several fetal diseases, which are evidenced by considerable experimental and clinical studies (Castilla, et al., 2004). Alcohol was absorbed through the mucous membrane of the oesophagus and stomach, and it metabolized entirely in the liver (Zhang, et al., 2008). When alcohol was metabolized in liver, it gives rise to oxidative stress by the generation of excess amounts of reactive oxygen species (ROS), and further affected antioxidant defence system (Ozaras, et al., 2003). 80-90% of alcohol breakdown in liver metabolism in the cells leads to ROS production (Pronko, et al., 2002).

Phytochemicals are a large group of plant-derived compounds hypothesized to be responsible for much of the disease protection conferred from diets high in fruits, vegetables, beans, cereals and plant-based beverages such as tea and wine. Phytochemicals are classified in to alkaloids, glycosides, flavonoids, phenolics, saponins, tannins, terpenes, anthraquinones and steroids (Arts, and Hollman, 2005). Plant steroids (or steroid glycosides) also referred to as ‘cardiac glycosides’ are one of the most naturally occurring plant phytoconstituents that have found therapeutic applications as cardiac drugs (Firm,
2010). Ergosterol, stigmasterol and β-sitosterol are three important sterols in fungal and plant samples. β-sitosterol (24-ethyl-5-cholestan-3-ol), a well-known plant sterol (Fig 1), has been reported to reduce serum cholesterol levels and to prevent cardiovascular events mainly by inhibition of cholesterol absorption in the intestines (Pouteau, et al., 2003). β-sitosterol is also known to regulate key molecules involved in inflammation, the immune response, anti-cancer defenses and apoptosis. In addition, the plasma β-sitosterol concentration was found to be significantly reduced in type 2 diabetes patients, suggesting a possible role of β-sitosterol in lowering the blood glucose level (Awad, and Fink, 2000). The aim of the present study is to investigate the hepatoprotective effect of β-sitosterol on lipid peroxidation and antioxidant status in ethanol-induced hepatotoxic rats.

![Figure 1: Structure of β-Sitosterol](image)

**MATERIALS AND METHODS**

**Experimental Animals**

All the experiments were done with male albino Wistar rats weighing 150-200 g, obtained from the Venkateswara Enterprises, Bangalore. They were housed in polypropylene cages (47x34x20 cm) lined with husk, renewed every 24 hours under 12:12 hour light/dark cycle at around 22˚ ± 2˚C and had free access to water and food. The rats were fed on a standard pellet diet (Pranav Agro Industries Ltd., Maharashtra, India). The pellet diet consisted of 22.02% crude protein, 4.25% crude oil, 3.02% crude fibre, 7.5% ash, 1.38% sand silica, 0.8% calcium, 0.6% phosphorus, 2.46% glucose, 1.8% vitamins and 56.17% nitrogen free extract (carbohydrates). The diet provides metabolisable energy of 3,600 kcal. The experiment was approved by Institutional Animal Ethics Committee (IAEC) of Muthayammal College of Arts & Science, Rasipuram, Tamilnadu, India, (Clearance No: 1416/Po/a/11CPSCEA & 7 March 2011) and carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

**Drugs and Chemicals**

β-Sitosterol was purchased from Sigma Chemical Company, St. Louis, MO, USA. Ethanol was purchased from Changshu Yangyuan Chemicals Pvt. Ltd., China. Thiobarbituric acid (TBA), 1, 1',3',3'-tetramethoxy propane, butylated hydroxy toluene (BHT), xylenol orange, dithionitro bis benzoic acid (DTNB), ascorbic acid, 2, 2' dipyrilid, p-phenylene diamine and sodium azide were obtained from Himedia laboratory, Mumbai, India. All other chemicals were obtained under analytical grade.

**Experimental Induction of hepatotoxicity**

Ethanol (3 gm/kg) was dissolved in water and injected intragastrically, for a period of 35 days (Rajat, and Gill, 1999). β-sitosterol (20 and 40 mg/kg) was suspended in carboxy methyl cellulose (CMC) and administered to rats orally using an intragastric tube daily for a period of 35 days.

**Experimental Design**

In this experiment, a total of 30 rats (18 toxicity surviving rats, 12 control rats) were used. The rats were divided into 5 groups of 6 rats in each.

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
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<tr>
<td>1</td>
<td>Normal control rats</td>
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<td>2</td>
<td>Normal rats treated with β-</td>
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<td>sitosterol (40 mg/kg)</td>
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<tr>
<td>3</td>
<td>Ethanol control rats</td>
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<tr>
<td>4</td>
<td>β-sitosterol (20 mg/kg) + Ethanol</td>
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<tr>
<td>5</td>
<td>β-sitosterol (40 mg/kg) + Ethanol</td>
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After the last treatment, all the rats were sacrificed by cervical decapitation. Plasma was separated from blood after centrifugation. The tissues (liver and kidney) were excised immediately from the rats, washed off blood with ice-cold physiological saline. A known weight of the liver and kidney were homogenized in appropriate buffer solution. The homogenate was centrifuged and the supernatant was used for the estimation of various biochemical parameters.

**Biochemical estimations**

The level of plasma TBARS was estimated by the method Yagi (1987), and the tissue TBARS was estimated by the method of Fraga et al. (1988). The levels of lipid hydroperoxides (HP) were estimated by the method of Jiang et al. (1992). The activity of SOD, catalase and GPx was assayed according to the procedures of Kakkar et al. (1984), Sinha (1972) and Rotruck et al. (1973) respectively. The level of ceruloplasmin was estimated by the method of van der Heide (1961). The level of GSH was estimated by the method of Ellman (1959). The level of vitamin C
and E was estimated by the methods of Omaye et al. (1979) and Baker et al. (1980) respectively.

Statistical analysis
Statistical analysis was performed by one way analysis of variance (ANOVA) followed by Duncan’s Multiple Range Test (DMRT) using Statistical Package for the Social Sciences (SPSS) software package version 16.00. P values <0.05 were considered significant.

RESULTS
Effect of β-sitosterol on lipid peroxidation in plasma and tissues
The levels of lipid peroxidative products (TBARS and hydroperoxides) in plasma and tissue of normal and ethanol-induced rats were presented in Figs 2, 3, 4 & 5. Ethanol-induced rats show the elevated levels of TBARS and hydroperoxides in plasma and tissue. Treatment with β-sitosterol in ethanol-induced rats showed reversal of these parameters to near normal levels.

Figure 2: Effect of β-sitosterol on the levels of plasma thiobarbituric acid reactive substances (TBARS) in normal and ethanol-induced hepatotoxicity in rats.

![Graph 2]

Each value is mean ± S.D. for six rats in each group.
Values not sharing a common superscript (a-d) differ significantly with each other (P<0.05, DMRT).

Figure 3: Effect of β-sitosterol on the levels of thiobarbituric acid reactive substances (TBARS) in tissues (liver and kidney) in normal and ethanol-induced hepatotoxicity in rats.

![Graph 3]

Each value is mean ± S.D. for six rats in each group.
Values not sharing a common superscript (a-d) differ significantly with each other (P<0.05, DMRT).
Effect of β-sitosterol on antioxidants in serum and tissues

The activities of enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in the liver and kidney of normal and ethanol-induced rats are shown in Figs 6, 7 & 8. Ethanol-induced rats showed significant reduction in the activity of SOD, CAT and GPx. Oral administration of β-sitosterol exerted a significant effect on the antioxidants in ethanol-induced rats.
Figure 6: Effect of β-sitosterol on the activities of superoxide dismutase (SOD) in tissues (liver and kidney) in normal and ethanol-induced hepatotoxicity in rats.

U – Enzyme concentration required to inhibit the chromogen produced by 50% in one minute.
Each value is mean ± S.D. for six rats in each group.
Values not sharing a common superscript (a-d) differ significantly with each other (P<0.05, DMRT).

Figure 7: Effect of β-sitosterol on the activities of catalase in tissues (liver and kidney) in normal and ethanol-induced hepatotoxicity in rats.

U – μmol of hydrogen peroxide consumed per minute.
Each value is mean ± S.D. for six rats in each group.
Values not sharing a common superscript (a-d) differ significantly with each other (P<0.05, DMRT).

Figure 8: Effect of β-sitosterol on the activities of glutathione peroxidase (GPs) in tissues (liver and kidney) in normal and ethanol-induced hepatotoxicity in rats.

U – μg glutathione consumed.
Each value is mean ± S.D. for six rats in each group.
Values not sharing a common superscript (a-d) differ significantly with each other (P<0.05, DMRT).
The levels of non-enzymatic antioxidants such as reduced glutathione (GSH), ceruloplasmin, vitamin C and vitamin E in the plasma, liver and kidney of normal and ethanol-induced rats are shown in Figs 9, 10, 11, 12, 13 & 14. The level of ceruloplasmin, GSH, vitamin C and E was found to be significantly reduced in serum, liver and kidney of hepatotoxic rats. Oral administration of β-sitosterol significantly increased the levels of ceruloplasmin, GSH, vitamin C and E in ethanol-induced rats.

For all the parameters, β-sitosterol treatment at 40 mg/kg to ethanol-induced hepatotoxic rats showed better effect than β-sitosterol 20 mg/kg. β-sitosterol treatment (40 mg/kg) to normal rats didn’t show any significant effect.

**Figure 9: Effect of β-sitosterol on the levels of plasma ceruloplasmin in normal and ethanol-induced hepatotoxicity in rats.**

![Graph showing the effect of β-sitosterol on plasma ceruloplasmin levels.]

Each value is mean ± S.D. for six rats in each group.

Values not sharing a common superscript (a-d) differ significantly with each other (P<0.05, DMRT).
Figure 10: Effect of β-sitosterol on the levels of reduced glutathione (GSH) in plasma in normal and ethanol-induced hepatotoxicity in rats.

Each value is mean ± S.D. for six rats in each group. Values not sharing a common superscript (a-d) differ significantly with each other (P<0.05, DMRT).

Figure 11: Effect of β-sitosterol on the levels of reduced glutathione (GSH) in tissues (liver and kidney) in normal and ethanol-induced hepatotoxicity in rats.

Each value is mean ± S.D. for six rats in each group. Values not sharing a common superscript (a-d) differ significantly with each other (P<0.05, DMRT).
Figure 12: Effect of β-sitosterol on the levels of vitamin C and vitamin E in plasma in normal and ethanol-induced hepatotoxicity in rats.

Each value is mean ± S.D. for six rats in each group.
Values not sharing a common superscript (a-d) differ significantly with each other (P<0.05, DMRT).

Figure 13: Effect of β-sitosterol on the levels of vitamin C in tissues (liver and kidney) in normal and ethanol-induced hepatotoxicity in rats.

Each value is mean ± S.D. for six rats in each group.
Values not sharing a common superscript (a-d) differ significantly with each other (P<0.05, DMRT).

Figure 14: Effect of β-sitosterol on the levels of vitamin E in tissues (liver and kidney) in normal and ethanol-induced hepatotoxicity in rats.

Each value is mean ± S.D. for six rats in each group.
Values not sharing a common superscript (a-d) differ significantly with each other (P<0.05, DMRT).
DISCUSSION
Liver is an important organ actively involved in several metabolic functions mainly in detoxification of toxicants (Meyer, and Kulkarni, 2001). Chronic alcohol provokes successive hepatic changes, consisting of alcoholic fibrosis, alcoholic hepatitis and cirrhosis (Kai, 1995). Increasing evidence support the hypothesis that ethanol-induced tissue damage may be a consequence of oxidative stress and nutritional deficiencies (Anbu, and Anuradha, 2012).

In liver, ethanol is metabolized to highly toxic metabolite acetaldehyde that interacts with the cellular macromolecules (lipids and proteins) and leads to the damage of membrane lipids besides altering the enzyme activities (Negre, et al., 1999). Alcohol dehydrogenase (ADH) leads to the formation of acetaldehyde from ethanol with the simultaneous reduction of NADP to NADH which in turn elevates xanthine oxidase activity and leads to the enhanced production of superoxide (Lieber, 2005).

Increased lipid peroxidation associated with chronic ethanol administration has often been used as an indicator of oxidative stress in both animal models and human clinical trials (Nordmann, 1994). Ethanol-induced rats showed increased plasma and tissue levels of lipid peroxidation markers such as TBARS and lipid hydroperoxides. The increased peroxidation can result in changes in cellular metabolism of the hepatic and extrahepatic tissues. Increased accumulation of lipid peroxidation products in cells can result in cellular dehydration, whole cell deformity and cell death (Winrow, et al., 1993).

β-sitosterol (20 and 40 mg/kg) co-administered rats for a period of 35 days showed significantly lowered levels of these lipid peroxidative markers compared to ethanol-induced rats. Decreased lipid peroxidation with β-sitosterol administration suggests a decreased impact of reactive oxygen species (ROS) on lipid membranes, and therefore increased protection against ethanol-induced liver injury. Thus the inhibition of lipid peroxidation by β-sitosterol may be one of the mechanisms by which β-sitosterol exerts its protection against ethanol-mediated tissue injury.

Oxidative stress results from a disturbance in the balance between generated oxidants and antioxidants in favour of the oxidants. This is often caused by an increase in the generation of ROS and a decrease in the activity of antioxidant system (Postmal, et al., 1996). According to Wu and Cederbaum (2003), chronic alcohol consumption not only activates free radical generation, but also alters the levels of both enzymatic and non-enzymatic endogenous antioxidant systems. This results in oxidative stress with cascade of effects, thus, affecting both functional and structural integrity of cell and organelle membranes (De leve, et al., 1996).

Free radical scavenging enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are the first line of defense against oxidative injury. The second line of defense consists of the non-enzymatic scavengers, such as GSH, ascorbic acid (vitamin C) and α-tocopherol (vitamin E), which scavenge residual free radicals escaping decomposition by the antioxidant enzymes. Moreover, enzymatic antioxidants are inactivated by the excessive levels of free radicals and hence the presence of non-enzymatic antioxidants is presumably essential for the removal of these radicals (Allen, 1991).

Decrease in enzyme activity of SOD is a sensitive index in hepatocellular damage and is the most sensitive enzymatic index in live injury. It scavenges the superoxide anion to form hydrogen peroxide and thus diminishing the toxic effect caused by radicals. Decrease in SOD production can be attributed to an enhanced superoxide generation and utilization of this enzyme during reactive metabolites detoxification. High amounts of superoxide inhibit catalase, which is another important antioxidant enzyme (Flora, 2002). CAT decomposes hydrogen peroxide and protects the tissues from highly reactive hydroxyl radicals (Chance, and Greenstein, 1992). Therefore reduction in the activity of CAT may result in a number of deleterious effects due to the assimilation of superoxide radical and hydrogen peroxide. GPx works in tandem with CAT to scavenge excess H₂O₂ as well as other free radicals in response to oxidative stress. The equilibrium between these enzymes is important for the effective removal of oxidative stress in intracellular organelles. The sulphydryl group is subjected to modification, which is invariably affecting the activity of the enzyme. This antioxidant defense system is significantly altered by ethanol administration (Veerappan, et al., 2004).

Ethanol-induced rats showed the decreased activities of these enzymes; result in the accumulation of highly reactive free radicals, leading to deleterious effects such as loss of cell membrane integrity and membrane function (Krishnakantha, and Lokesh, 1993). Treatment with β-sitosterol significantly increases the hepatic and renal SOD, catalase and GPx activity and thus reduces reactive free radical induced oxidative damage to liver.
Glutathione is one of the most abundant tripeptide, non-enzymatic biological antioxidant present in the liver. It removes free radical species such as hydrogen peroxide, superoxide radicals and maintains membrane protein thiols. Also it is a substrate for glutathione peroxidase (Prakash, et al., 2001), decreased level of GSH is associated with an enhanced lipid peroxidation in ethanol-induced rats.

Vitamin C and E both are naturally occurring free radical scavengers (Yu, 1994). Both vitamin C and E are known to be decreased in liver diseases, particularly in alcoholics (Bjorneboe, et al., 1987). Under these conditions, thiol compounds, such as GSH, might be involved in regenerating α-tocopherol from its radical form (Wefers, and Sies, 1988). The observed decrease in the levels of vitamin C and E may be due to their increased utilization for scavenging ethanol- and/or oxygen-derived radicals.

Administration of β-sitosterol in ethanol-induced rats significantly increased the levels of these non-enzymatic antioxidants. The ability of β-sitosterol might be potentially useful in countering free radical-mediated injuries involved in the development of liver damage caused by alcohol abuse.

β-sitosterol acts as a competitive inhibitor of cholesterol absorption. Hence administration of β-sitosterol decreases the levels of cholesterol and other lipids in ethanol-induced rats which is one of the indirect effects of β-sitosterol on the lipid peroxidative products as well as antioxidants. Additionally β-sitosterol itself having strong antioxidant action (Yoshida, and Niki, 2003) as well as free radical scavenging activity (Backhouse, et al., 2008). Thus the free radical scavenging antioxidant and cholesterol lowering property are collectively responsible for the hepatoprotective action in ethanol-induced rats. However further clinical evaluation is required for β-sitosterol to become a drug for alcoholism.

REFERENCES


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