EFFECT OF LECITHIN IN THE TREATMENT OF ETHANOL MEDIATED FREE RADICAL INDUCED HEPATOTOXICITY

Subir Kumar Das* and D.M.Vasudevan

Department of Biochemistry, Amrita Institute of Medical Sciences, Elamakkara, Cochin-682 026, Kerala

ABSTRACT

Alcoholic liver disease (ALD) develops as a consequence of priming and sensitizing mechanisms rendered by cross-interactions of primary mechanistic factors and secondary risk factors. Liver damage due to consumption of alcohol may be caused by oxygen radicals such as superoxide and hydroxyl radicals, generated during the metabolism of ethanol by the microsomal oxidizing system. Lecithin, an important class of phospholipids contains choline, which is considered as lipotropic factor. The effects of this lecithin as a hepatoprotective drug on body weight and antioxidant status of ethanol-exposed rats were studied. The results were compared with the effects of tocopheryl acetate. From the present study, it can be concluded that ethanol-induced stress can be partly prevented by tocopheryl acetate, and showed best result. Abstinence from alcohol also involved for little hepatic regeneration. Supplementation of lecithin showed better effect compared to abstinence from alcohol on reversing the effect of ethanol induced liver damage in the present study. Moreover, preventive measures were found to be better than curative treatment. Antioxidants are likely to provide beneficial effects on hepatocytes via desensitization against oxidant stress while inhibiting primary mechanism for expression of proinflammatory and cytotoxic mediators. However, abstinence from alcohol, proper nutrition, and supplementation of antioxidants, vitamins and hepatoprotective drugs are some of the therapeutic options.

KEY WORDS

S-Adenosyl methionine, Lecithin, Tocopherol, Oxidative stress, Glutathione

INTRODUCTION

Alcoholic liver disease (ALD) develops as a consequence of priming and sensitizing mechanisms rendered by cross-interactions of primary mechanistic factors and secondary risk factors. This concept, albeit not novel, is becoming widely accepted by the field, and more research is directed toward identifying and characterizing the interfaces of the cross interactions to help understand individual predisposition to the disease. Glutathione depletion is considered the most important sensitizing mechanism. One of the contributing factors is decreased methionine metabolism. But the key question is how altered methionine metabolism contributes to the pathogenesis of ALD? (1).

The liver plays a central role in methionine metabolism,

as half of the daily methionine is catabolized here (Fig.1). The first step in methionine metabolism is the formation of S-adenosylmethionine (SAM) catalyzed by methionine adenosyltransferase (MAT) (2, 3). Under normal conditions, most of the 6-8 gm of SAM generated per day is used in transmethylation reaction in which methyl groups are added to compounds and SAM is converted to S-adenosylhomocysteine (SAH) (3, 4). SAH is a potent competitive inhibitor of transmethylation reactions (3, 5). The reaction that converts SAH to homocysteine and adenosine is reversible and catalyzed by SAH hydrolase (2, 3). In vivo, the reaction proceeds in the direction of hydrolysis only if the products, adenosine and homocysteine, are rapidly removed (2, 3, 5).

There are three pathways that metabolize homocysteine. One is the trans-sulfuration pathway, which converts homocysteine to cysteine. This is a unique pathway present only in the liver and lens (6) that condenses homocysteine with serine to form cystathionine in a reaction catalyzed by cystathionine β -synthase (CBS) (4, 6). Cleavage of cystathionine, catalyzed by another enzyme, γ -cystathionase, then release free cysteine, the rate-limiting precursor for GSH synthesis (6). The other two pathways

Author for Correspondence :

Dr. Subir K. Das,
Department of Biochemistry
Amrita Institute of Medical Sciences,
Cochin 682 026, Kerala. India.
e-mail: subirkumardas @medical.amrita.edu

Fig. 1. methionine and GSH metabolism.

Methionine adenosyltransferase (MAT) catalyzes the synthesis of SAM for methionine and adenosine triphosphate; SAM is then converted to S-adenosylhomocysteine (SAH) as the methyl group is transferred. SAH hydrolase catalyzes the reversible hydrolysis of SAH to yield homocysteine and adenosine. Homocysteine in the liver can undergo three metabolic pathways, two of which regenerate methionine from homocysteine, the third is the transsulfuration pathway, which converts homocysteine to cysteine. In the transsulfuration pathway, cystathionine b-synthase catalyzes the formation of cystathionine. Both enzymes require normal levels of vitamin B6 as cofactor. Cysteine is the rate limiting precursor for GSH synthesis as glutamate cysteine ligase (GCL), the rate- limiting enzyme in GSH synthesis, catalyzes the formation of g-glutamylcysteine from cysteine and glutamate, and GSH synthetase catalyzes the formation of GSH from -glutamylcysteine and glycine. Methionine synthase regenerates methionine from homocysteine in a reaction that requires normal levels of folate and vitamin B12, and betaine homocysteine methyl transferase regenerates methionine from homocysteine that requires betaine.

resynthesize methionine from homocysteine (2, 4) (Fig. 1).

Ethanol feeding induced alterations in mitochondrial membrane phospholipid and fatty acid composition with resulting impairment in GSH uptake. Phospholipid methylation is required for maintenance of normal membrane fluidity and function (7). Reduced SAM level as a result of abnormalities in the methionine metabolism can affect mitochondrial GSH transport and sensitize the cell to oxidative stress-induced injury by this mechanism. Depletion of SAM may also lead to hypomethylation of phosphatidyl ethanolamine and a consequent reduction in the hepatic level of phosphatidyl choline (8). It is believed that the protective effect of SAM is via increased GSH level, changing in DNA-methylation status and inhibiting TNF release by macrophages. But, controversy has surrounded whether SAM can be taken up by hepatocytes (4). In the present study efficacy of lecithin to treat ethanol induced oxidative stress was compared with the effect of tocopheryl acetate (vitamin E) as well as capacity of hepatic regeneration during abstinence.

MATERIALS AND METHODS

Materials

Chemicals

Ethanol was purchased from Bengal Chemicals, Kolkata. Fine chemicals were purchased from Sisco Research Laboratory (SRL), India; and Sigma Chemical Co., St. Louis, USA; and analytical grade chemicals from E. Merck or SRL.

Animal Selection

16-18 week-old male albino rats of Wistar strain weighing 200- 220g were used. The animals were housed in plastic cages inside a well-ventilated room. The room temperature was maintained at $25 \pm 2^\circ\text{C}$ with a 12-h light/dark cycle. All rats had free access of standard diet (9); with modification containing 31% Bengal gram, 30% gingelly oil cake, 28% wheat, 10% polished rice, 0.5% salt mixture, 0.3% vitamin-mineral mixture, 0.2% yeast with fish or liver oil. Food and water were given *ad libitum*. The animals were weighed daily and their general condition and behavior were recorded including their daily intake of liquid.

The rats were divided into the following groups each containing 6 rats.

Group I: Control rats- which were fed normal diet and water.

Group II: Ethanol treated rats (1.6g ethanol/ kg body weight/ day for 4 weeks)

Group III: tocopheryl acetate +Ethanol treated rats

(1.6g ethanol and 80mg tocopheryl acetate / kg body weight/ day for 4 weeks)

Group IV: Ethanol followed by tocopheryl acetate treated rats (1.6g ethanol/kg body wt/ day for 4 wks, followed by 80mg tocopheryl acetate / kg body weight/ day for 4 wks)

Group V: Lecithin +Ethanol treated rats (1.6g ethanol and 500mg lecithin / kg body weight/ day for 4 weeks)

Group VI: Ethanol followed by lecithin treated rats (1.6g ethanol/kg body weight/ day for 4 weeks and followed by 500mg lecithin / kg body weight/ day for 4 wks)

Group VII: Ethanol treatment (1.6g ethanol/ kg body weight/ day) for 4 weeks and followed by 4 weeks abstinence.

Tocopheryl acetate was suspended in distilled water; and lecithin was freshly dissolved in distilled water during treatment. Ethanol was diluted with distilled water to get desired concentration and fed orally.

Methods

Experimental Procedure

At the end of the experimental period, the animals were sacrificed by cervical dislocation. The liver was dissected out and cleaned with ice-cold saline, blotted dry, and immediately transferred to the ice chamber. Various oxidative stress related parameters were estimated. The Animal Ethics Committee of the Institution approved the procedures.

Biochemical Methods

Liver was homogenized in 0.25 M sucrose solution, diluted with 0.9% saline, and these diluted samples were used for the estimation of tissue protein by the method of Lowry *et al.* (10). Ascorbic acid content was estimated using thiourea reagent and 2,4-dinitrophenylhydrazine (11). Lipid peroxidation was estimated using Trichloroacetic acid (TCA)-Thiobarbituric acid (TBA)- Hydrochloric acid (HCl) at 535 nm (12). The reduced glutathione (GSH) content of the tissue in 0.1M phosphate buffer (pH 7.4) was measured following the method of Ellman (13) using DTNB reagent [5,5'-dithiobis (2-nitrobenzoic acid)]. Catalase (EC 1.11.1.6) activity was determined based on the method as described by Beers and Sizer (14) with necessary modification. The tissues were homogenized in 0.05M phosphate buffered saline (pH 7.0). The rate of decomposition of H_2O_2 (2 ml, 30%) in 0.05 M-phosphate buffer (1 ml, pH 7.0) at 240nm after addition of homogenized tissue was noted. The specific activity was calculated assuming Enax $40 \text{ M}^{-1} \text{ cm}^{-1}$ for H_2O_2 at 240 nm (15). Glutathione reductase (GR, EC 1.6.4.2) activity was determined

at 340 nm by the method of Goldberg and Spooner (16). The tissue was homogenized using 0.05 M phosphate buffer of pH 6.5. Glutathione S-transferase (GST; EC 2.5.1.18) activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) (17). Glutathione peroxidase (GPx, EC 1.11.1.9) activity was measured as described by Paglia and Valentine (18). The superoxide dismutase (EC 1.15.1.1) activity was measured by the inhibition of auto oxidation of pyrogallol at 420 nm (19).

Results were expressed as mean $\pm$ SEM (standard error). Statistical significance was determined by Student's 't' test for unpaired data. The values of significance were evaluated with 'p' values. The differences were considered significant at $p < 0.05$.

RESULT

Preventive and curative effect effect of tocopheryl acetate and lecithin on percentage change in body weight of animals during the experiment in relation to its initial weight are presented in Figure 2. When rats were simultaneously exposed with ethanol and tocopheryl acetate or lecithin 39%, and 32.4% increase in body weight were found respectively. After ethanol treatment for 4 weeks, in the follow up treatment with tocopheryl acetate or lecithin for another 4 weeks, 34% and 29.5% increase in body weight were found, while abstinence showed 24.5% increase in body weight. The relative weights (g/ 100g body weight) of liver of different treatments are presented in Figure 3. No significant change was observed in liver weight.

Table 1 is showing the levels of ascorbic acid, thiobarbituric acid reactive substances (TBARS) and reduced glutathione (GSH) content in liver homogenate. No significant change was observed in ascorbic acid content. The level of lipid peroxidation in liver of all the tested groups was significantly higher than the control group. TBARS level was reduced by 20.6%, and 14.4% respectively during curative treatment with tocopheryl acetate and lecithin; while under preventive treatment by 21.9% and 15.5% respectively in comparison to ethanol fed groups. During abstinence reduction in TBARS level was 8.8%. The GSH content of liver homogenates of different groups were found to be significantly lower than the control group. GSH level was raised by 62.9% and 46.3% respectively during preventive treatment with tocopheryl acetate and lecithin when compared with ethanol fed rats. Same trend of response was observed in curative treatment; but preventive treatment showed better result than curative treatment.

The catalase activities of liver homogenates of lecithin and tocopheryl acetate treated groups were lower when compared with the control group (Table 2) and higher when compared with the ethanol treated group. On the other hand, the superoxide dismutase activities of liver of different groups were found to be higher than the activity observed in the control group. On the contrary, the liver superoxide dismutase activities of the treated groups were found to be lower than ethanol treated group.

Glutathione reductase and glutathione peroxidase activities of liver of all the tested groups were found

Fig. 2. Change in the body weight in different groups animals exposed to different treatment
Values are mean $\pm$ SE of 6 rats in each group

Fig. 3. Change in the liver weight in different groups animals exposed to different treatment
No significant change was observed in either group

Table 1. Effect of lecithin and tocopheryl acetate on ascorbic acid content, thiobarbituric acid reactive substances (TBARS) and glutathione levels in liver homogenate

Treatment	Ascorbic acid (mg/g tissue)		TBARS (mol MDA formed/min/100mg tissue)		GSH (g/mg tissue)	
Control	3.1 ± 0.18		0.665 ± 0.015		3.86±0.23	
Ethanol	2.53± 0.43		0.926 ± 0.023*		2.16±0.18*	
Abstinence	2.58± 0.26		0.844 ± 0.048*		2.74±0.24*	
	Curative	Preventive	Curative	Preventive	Curative	Preventive
Lecithin	2.66±0.17	2.62±0.19	0.792±0.016**	0.782±0.033**	3.06±0.14**	3.16±0.18**
Tocopheryl acetate	2.72±0.14	2.75±0.24	0.735±0.053**	0.723±0.046**	3.36±0.26#@	3.52±0.33#@

Values are mean±SEM of 6 rats in each group. *Indicates $p < 0.05$ when compared to normal healthy control. # Indicates $p < 0.05$ when compared to ethanol treated group. @ Indicates $p < 0.05$ when compared to abstinence group.

to be significantly lower in comparison to the enzyme activities of control group, and higher in comparison to ethanol treated group (Table 3). GR level was increased by 57.5% and 39.1% respectively during curative treatment with α -tocopherol and lecithin; in comparison to ethanol fed groups. During abstinence increase in glutathione reductase level was 27% in comparison to ethanol fed groups. GPx level was raised by 36.9% and 27.4% respectively during preventive treatment with α -tocopherol and lecithin when compared with ethanol fed rats. Liver glutathione

S-transferase activities of all the tested groups were found to be significantly higher than that of control group and lower than ethanol treated group. GST level was reduced by 37.9% and 30.9% respectively during curative treatment with α -tocopherol and lecithin; whereas under preventive treatment this level was reduced by 41.8% and 33.4% in comparison to ethanol fed groups. During abstinence reduction in GST level was 19%. However tocopheryl acetate and lecithin treated groups showed significantly lower value in comparison to the abstinence group.

Table 2. Effect of lecithin and tocopheryl acetate on catalase and superoxide dismutase activity in liver homogenate

Treatment	Catalase ^a		Superoxide dismutase ^b	
Control	38.76± 0.66		6.33±0.15	
Ethanol	32.63±0.83*		9.07±0.21*	
Abstinence	33.36±1.78*		8.62±0.0.73*	
	Curative	Preventive	Curative	Preventive
Lecithin	33.73±1.43*	34.63±1.12*	8.23±0.66*	8.12±0.0.58*
Tocopheryl acetate	36.22±1.54*#@	37.81±1.98#@	7.54±0.58*#	7.02±0.83*#@

a: mole H₂O₂ decomposed/ min/ mg protein; b: U/mg protein

Values are mean±SEM of 6 rats in each group. *Indicates p< 0.05 when compared to normal healthy control.

Indicates p< 0.05 when compared to ethanol treated group.

@ Indicates p< 0.05 when compared to abstinence group.

Table 3. Effect of lecithin and tocopheryl acetate on glutathione reductase, glutathione peroxidase, and glutathione s-transferase activity in liver homogenate

Treatment	Glutathione reductase ^a		Glutathione peroxidase ^b		Glutathione s-transferase ^c	
Control	56.38± 1.34		82.46±2.48		6.84±0.11	
Ethanol	26.77±0.84*		52.94±1.58*		13.26±0.31*	
Abstinence	34.23± 1.76*		59.63±4.02*		10.74±0.73*	
	Curative	Preventive	Curative	Preventive	Curative	Preventive
Lecithin	37.24±2.29*#	38.91±2.43*#@	63.26±2.53*#	67.43±3.26*#	9.16±0.63*#	8.83±0.83*#
Tocopheryl acetate	42.16±2.62*#@	44.36±2.83*#@	70.48±3.98*#@	72.46±2.13*#@	8.23±0.56*#@	7.71±0.46*#@

a: nmol NADPH breakdown/ min/ mg protein; b: nmol NADPH breakdown/ min/ mg protein;

c: mole CDNB conjugate formed/ min/ mg protein. Values are mean±SEM of 6 rats in each group.

*Indicates p< 0.05 when compared to normal healthy control.

Indicates p< 0.05 when compared to ethanol treated group.

@ Indicates p< 0.05 when compared to abstinence group.

DISCUSSION

The intragastric ethanol infusion technique allowed maximal ethanol consumption and absolute control over ethanol-induced liver injury. In the present study, a dose of 1.6 g ethanol/ kg body weight/ day for 4 weeks was used to induce maximum liver damage which can be reversed as observed by our previous study (20). Rats which were consuming high amount of alcohol showed a lower increase in body weight due essentially to fat mass reduction. Reduced adipose tissue may be the foremost cause of lower body weight (20). It is well known that animals with ALD exhibit impaired liver regeneration (21). In the present study, the regenerative capacity was observed by administering ethanol (1.6 g/ kg body weight/ day) for 4 weeks, followed by abstinence from alcohol for next

4 weeks. This group also acted as one of the control group. Tocopheryl acetate and lecithin exhibited an ability to counteract the alcohol-induced changes in the body weight and biochemical parameters in preventive and curative aspects in varying degree.

One of the proposed mechanisms of ethanol induced-toxicity is the membrane damage due to the direct effect of lipid peroxidation products (20), i.e., TBARS, which was found to be increased in the etanolic rats in the present study. Tocopheryl acetate and lecithin reversed this level significantly. These suggest that these treatments offered some protection against lipid peroxidation. The tissue ascorbic acid concentration reflects oxidative stress in hepatocytes.

GSH has been postulated to be an important pathogenic factor in alcoholic liver injury. The fall in

hepatic GSH sensitizes the liver to oxidative injury and sets up a vicious cycle. Chronic ingestion of ethanol resulted in a significant depletion of glutathione (GSH) content in liver (22). Raised levels of GSH have been reported to elicit a protective response against the toxic manifestations of chemicals, particularly those involving oxidative stress. Explanations of the possible mechanisms underlying the hepatoprotective properties of drugs include the prevention of GSH depletion (23) and destruction of free radicals (24).

The presence of superoxide dismutase (SOD) in various compartments of our body enables SOD to dismutate superoxide radicals immediately (20). In the present work, superoxide dismutase activities increased on ethanol exposure, and reduced significantly due to different treatment including abstinence from alcohol. Catalase activity was decreased in higher concentration of ethanol exposure might be due to loss of NADPH, or generation of superoxide, or increased activity of lipid peroxidation or combination of all (20). Different treatment increased its activity in the present study.

Glutathione reductase (GR) is concerned with the maintenance of cellular level of GSH by effecting fast reduction of oxidised glutathione to reduced form (20, 25). Chronic ingestion of ethanol resulted in a significant decrease in glutathione peroxidase (GPx) activity in liver may be due to either free radical dependent inactivation of enzyme or depletion of its co-substrates i.e. GSH and NADPH in ethanol treated rats (20, 26). Glutathione s-transferase (GST) plays an essential role in liver by eliminating toxic compounds by conjugating them with glutathione. Increased glutathione s-transferase activity and decreased glutathione reductase activity, followed by thiol depletion, are important factors sustaining a pathogenic role for oxidative stress (20, 27). Supplementation of tocopheryl acetate or lecithin either as therapeutic or prophylactic treatment in ethanol induced liver damage could partly reverse these enzyme levels in the present study.

From the present study it can be concluded that ethanol-induced stress can be partly prevented by tocopheryl acetate (vitamin E) supplementation. It is generally believed that α -tocopherol intercalates into the lipid bilayers of cell membranes and acts there directly to scavenge free radicals. Hydroxyl radical attack on tocopherol forms a stabilized phenolic radical that is reduced back to the phenol by ascorbate and NADH/NADPH reductase enzyme (28). Vitamin E may largely modulate the expression of the toxicity by GSH depleting agents (29).

Supplementation of lecithin also showed a little better effect than abstinence on reversing the effect of alcohol induced liver disease in the present study. In

one study, Lieber *et al.* (30) reported that choline supplementation failed to prevent alcohol-induced steatosis and fibrosis; while choline supplementation exerts moderate hepatotoxicity. However, in another study, Lieber *et al.* (31) concluded that some component of lecithin exerts a protective action against the fibrogenic effects of ethanol. In fact hepatic methionine level is dependent on the stage of liver injury in rats fed ethanol intragastrically. From the present study we conclude that the amount of lecithin (500 mg lecithin / kg body weight/ day for 4 weeks) used here was not sufficient, or some essential contributing factor was necessary, for reversing the oxidative stress mediated effect.

The hepatic regeneration as studied by change in body weight and biochemical parameters clearly indicated that different treatment showed different degree of activity. These activities were observed both in prophylactic and therapeutic treatment. However, preventive measures were better effective than curative treatment. From the present study, it can be concluded that tocopheryl acetate showed best result, followed by lecithin in the management of alcoholic liver disease. Abstinence from alcohol improved hepatic regeneration. Our future study will focus on the determination of optimum dose and other contributing factors are associated with lecithin activity.

REFERENCES

1. Tsukamoto, H. and Lu, S.C. (2001). Current concepts in the pathogenesis of alcoholic liver injury. *FASEB. J.* 15, 1335-1349.
2. Finkelstein, J.D. (1990). Methionine metabolism in mammals. *J. Nutr. Biochem.* 1, 228-237.
3. Mato, J.M., Alvarez, L., Corrales, F. and Pajares, M.A. (1994). S-adenosylmethionine and the liver. In: *The liver biology and Pathology*. Eds. Arias, I.M., Boyer, J.L., Fausto, N., Jakoby, W.B., Schachter, D.A. and Shafritz, D.A. Raven Press, New York; pp. 461-470.
4. Mato, J.M., Alvarez, L., Ortiz, P. and Pajares, M.A. (1997). S-adenosylmethionine synthesis: molecular mechanisms and clinical implications. *Pharmacol. Ther.* 73, 265-280.
5. Hoffman, D.R., Marion, D.W., Cornatzer, W.E. and Duerre, J.A. (1980). S-adenosylmethionine and S-adenosylhomocysteine metabolism in isolated rat liver. Effects of L-methionine, L-homocysteine, and adenosine. *J. Biol. Chem.* 255, 10822-10827.
6. Lu, S.C. (1998). Regulation of hepatic glutathione synthesis. *Semin. Liv. Dis.* 18, 331-343.
7. Lieber, C.S., Casini, A., DeCarli, L.M., Kim, C.I., Lowe, N., Sasaki, R. and Leo, M.A. (1990). S-

- adenosyl-L-methionine attenuates alcohol-induced liver injury in the baboon. *Hepatology*. 11, 165-172.
8. Lieber, C.S., Robins, S.J. and Leo, M.A. (1994). Hepatic phosphatidylethanolamine methyl transferase activity is decreased by ethanol and increased by phosphatidylcholine. *Alc. Clin. Exp. Res.* 18, 592-595.
9. Package of Practices Recommendation (2001). Veterinary & Animal Husbandary Department, Kerala Agriculture University, Mannuthy, Thrissur.
10. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with folin phenol reagent. *J. Biol. Chem.* 193, 265-275.
11. Roe, J.H. and Kuether, C.A. (1943). The determination of ascorbic acid in whole blood and urine through the 2,4-dinitrophenyl hydrazine derivative of dehydro ascorbic acid. *J. Biol. Chem.* 147, 399-401.
12. Sinnhuber, R.O., Yu, T.C. and Yu, T.C. (1958). Characterization of the red pigment formed in the thiobarbituric acid determination of oxidative rancidity. *Food Res.* 23, 626-630.
13. Ellman, G.L. (1959). The sulphhydryl groups. *Arch. Biochem. Biophys.* 32, 70-77.
14. Beers, R.F. and Sizer, I.W. (1952). A spectrophotometric method for measuring the breakdown of hydrogen peroxides by catalase. *J. Biol. Chem.* 195, 133-140.
15. Nelson, D. P. and Kiesow, L. A. (1972). Enthalpy of decomposition of hydrogen peroxide by catalase at 25°C (with molar extinction coefficients of H₂O₂ solutions in the UV. *Anal. Biochem.* 49, 474-478.
16. Goldberg, M.D. and Spooner, J.R. (1983). Glutathione reductase. In: *Methods Enzyme Analysis*. Eds. Bergmayer, H.U., Bergmayer J. and Grabi M. Academic Press, Inc., Florida,; Vol. III, 3rd edn. pp. 258-265.
17. Habig, W.H., Pabst, M.J. and Jakoby, W.B. (1974). Glutathione S-transferase, the first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* 249, 7130-7139.
18. Paglia, D.E. and Valentine, W.N. (1967). Studies on the quantitative and qualitative characterisation of erythrocyte glutathione peroxides. *J. Lab. Clin. Med.* 70, 158-159.
19. Marklund, S. and Marklund, G. (1974). Involvement of superoxide radical in the auto oxidation pyrogallol and a convenient assay for superoxide dismutase. *Eur. J. Biochem.* 47, 469-474.
20. Das, S.K. and Vasudevan, D.M. (2005). Effect of ethanol on liver antioxidant defense systems: a dose dependent study. *Ind. J. Clin. Biochem.* 20 (1), 79-83.
21. McClain, C., Hill, D., Schimdt, J. and Diehl, A.M. (2001). Cytokines and alcoholic liver disease. *Semin. Liv. Dis.* 13, 170-182.
22. Mari, M., Wu, D., Nieto, N. and Cederbaum, A.I. (2001). CYP2E1-dependent toxicity and up-regulation of antioxidant genes. *J. Biomed. Sci.* 8 (1), 52-55.
23. Campos, R., Garrido, A., Guerra, R. and Valenzuela, A. (1989). Silybin dihemisuccinate protects against glutathione depletion and lipid peroxidation induced by acetaminophen on rat liver. *Planta. Med.* 55, 417-419.
24. Valenzuela, A., Lagos, C., Schimdt, K. and Videla, K. (1985). Silymarin protection against hepatic lipid peroxidation induced by acute ethanol intoxication in the rat. *Biochem. Pharmacol.* 3, 2209-2212.
25. Dinu, V. and Zamfir, O. (1991). Oxidative stress in ethanol intoxicated rats. *Rev. Roum. Physiol.* 28 (1-2), 63-67.
26. Chandra, R., Aneja, R., Rewal, C., Konduri, R., Dass, S.K. and Agarwal, S. (2000). An opium alkaloid-*Papaverine* ameliorates ethanol-induced hepatotoxicity: diminution of oxidative stress. *Ind. J. Clin. Biochem.* 15 (2), 155-160.
27. Aniya, Y. and Daido, A. (1994). Activation of microsomal glutathione S-transferase in tert-butyl hydroperoxide-induced oxidative stress of isolated rat liver. *Jpn. J. Pharmacol.* 66 (1), 123-130.
28. Buettner, G.R. (1993). The pecking order of free radicals and antioxidants: lipid peroxidation, alpha tocopherol and ascorbate. *Arch. Biochem. Biophys.* 300, 535-543.
29. Maellaro, E., Casini, A.F., DelBello, B. and Comporti. (1990). Lipid peroxidation and antioxidant systems in the liver injury produced by glutathione depleting agents. *Biochem. Pharmacol.* 39 (10), 1513-1521.
30. Lieber, C.S., Leo, M.A., Mak, K.M., DeCarli, L.M. and Sato, S. (1985). Choline fails to prevent liver fibrosis in ethanol-fed baboons but causes toxicity. *Hepatology*. 5 (4), 561-572.
31. Lieber, C.S., DeCarli, L.M., Mak, K.M., Kim, C.I. and Leo, M.A. (1990). Attenuation of alcohol-induced hepatic fibrosis by polyunsaturated lecithin. *Hepatology*. 12 (6), 1390-1398.