Pharmacological Reports
2006, 58, 41–47
ISSN 1734-1140

Impact of ursolic acid on chronic ethanol-induced oxidative stress in the rat heart

Rajendrasozhan Saravanan, Viswanathan Pugalendi

Department of Biochemistry, Faculty of Science, Annamalai University, Annamalainagar – 608 002, Tamil Nadu, India

Correspondence: Viswanathan Pugalend, e-mail: drkvp@sify.com

Abstract:
Oxidative stress plays an important role as a mediator of myocardial damage produced by ethanol. This work was designed to investigate the effect of ursolic acid (UA), a reported radical scavenger and antioxidant, on oxidative stress in the heart of chronically ethanol-administered rats. Chronic ethanol administration (7.9 g/kg/day for 60 days) caused tissue damage that was manifested by the elevation of serum lactate dehydrogenase (LDH) and creatine phosphokinase (CPK). It also induced oxidative stress in the heart by increasing the lipid peroxidation process and by decreasing the antioxidant capacity of the heart. After the induction of toxicity (i.e. initial 30 days), treatment groups received UA (10, 20 and 40 mg/kg/day) along with ethanol for another 30 days. Coadministration of UA effectively (20 mg/kg/day) restored the activities of marker enzymes. It also controlled the oxidative stress by decreasing lipid peroxidation products (manifested by decreased lipid peroxidation products such as thiobarbituric acid reactive substances – TBARS, lipid hydroperoxides – LOOH and conjugated dienes – CD), increasing the activities of free radical scavenging enzymes (superoxide dismutase – SOD, catalase – CAT, glutathione peroxidase – GPx and glutathione S-transferase – GSH) and increasing the levels of non-enzymic antioxidants such as reduced glutathione, ascorbic acid and α-tocopherol. These findings demonstrate that UA acts as a protective agent against ethanol-induced abnormalities in the heart by reducing the lipid peroxidation process and by enhancing the antioxidant capacity.

Key words:
ursolic acid, triterpenoid, ethanol, heart, oxidative stress, antioxidants

Abbreviations: CAT – catalase, CD – conjugated dienes, CPK – creatine phosphokinase, GPx – glutathione peroxidase, GSH – reduced glutathione, GST – glutathione S-transferase, LDH – lactate dehydrogenase, LOOH – lipid hydroperoxides, SOD – superoxide dismutase, TBARS – thiobarbituric acid reactive substances, UA – ursolic acid

Introduction

Alcohol abuse continues to remain as a prevalent social problem contributing to severe heart problem in alcoholics [1]. Moderate drinkers exhibit lower rate of coronary heart disease-related mortality than both heavy drinkers and abstainers [21]. Ethanol is reported to induce cellular protection in mouse cardiac myocytes against sustained stimulated ischemia *in vitro* [30]. But the chronic abuse of alcohol has been known to adversely affect the health of many individuals, causing life-threatening conditions in numerous organs including heart, brain, pancreas, and liver. With respect to the heart, alcohol abuse has proven to be a major cause of non-ischemic cardiomyopathy in Western society [2, 38]. Alcoholic cardiomyopathy is a common diagnosis in long-term alcoholics [8]. Re-

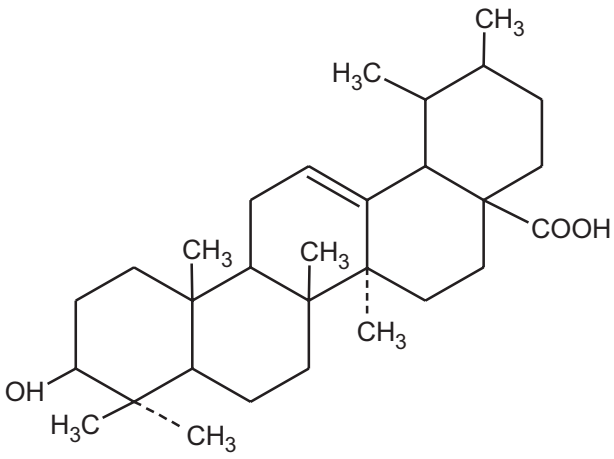

Fig. 1. Structure of ursolic acid

active oxygen species, generated from ethanol metabolism, have been indicated to play a role in the onset of heart disturbances in ethanol-intoxicated animals [27, 36]. Oxidant balance in the heart has a very important role in protecting the heart and in allowing normal cardiac contractile performance. In general, the amount of antioxidants in the heart is sufficient to protect it from any oxidant production that might occur under normal circumstances [29]. Reduction of heart anti-oxidative capacity and morphological changes has been observed in animals chronically intoxicated with ethanol [9, 28]. Antioxidants may have protective role in these conditions. Edés et al. [9] have reported the protective effect of antioxidants against myocardial lipid peroxidation in rats after chronic alcohol ingestion.

Herbal and natural products with antioxidant capacity have been used for centuries in every culture throughout the world. Scientists and medical professionals have shown increased interest in this field as they recognize the true health benefits of these remedies. There is a growing interest in the elucidation of the biological roles of triterpenoid compounds, the major components of some traditional medicinal plants, in terms of hepatoprotective, analgesic, antitumor, antiinflammatory and cardiotonic effects [22]. Among these, ursolic acid (UA; 3-hydroxy-urs-12-en-28-oic acid), a steroid-like triterpene compound (Fig. 1), is present in many kinds of medicinal plants, such as *Eriobotrya japonica, Rosmarinus officinalis*, *Melaleuca leucadendron, Ocimum sanctum, Glechoma hederaceae* [22] and *Piper betle* [35] in the form of free acid or as aglycone of triterpenoid saponins. It is reported to have strong hepatoprotective activity against ethanol [41]. Balanehru et al. [5] have reported that protective effect of UA against free radical damage was apparently stronger in the heart than in the liver under *in vitro* condition. Since it is not known whether UA protects the heart from oxidative stress *in vivo,* the present study was planned to unravel the effect of UA on ethanol-induced oxidative stress in the heart of albino Wistar rats. In particular, we focused on its effects upon tissue lipid peroxidation and antioxidant levels in the heart of ethanol-administered rats.

Materials and Methods

Healthy male albino Wistar rats (140–160 g) purchased from Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical Collage, Annamalai University were housed in polypropylene rat cages in a room with controlled temperature (24 ± 2°C) and light (lights on 0600 to 1800). They were fed with standard pellet diet (Agro Corporation Private Limited, Bangalore, India) and water *ad libitum*. The experimental and animal-handling procedures were approved by the Institutional Animal Ethics Committee and were performed in accordance with the guidelines of U.S. National Institutes of Health as specified in the "Guide to the Care and Use of Experimental Animals".

UA (95% purity) was received from Sami Labs Ltd. (The Research and Manufacturing Arm of Sabinsa Corporation), Bangalore, India. UA was isolated from the leaves of *Melissa officinalis* by solvent extraction, concentration, pH adjustments, salt formation, chromatography (preparative HPLC) and crystallization. All the chemicals used in this study were of analytical grade.

The animals were randomly divided into six groups of eight rats in each. Alcoholic rats (toxicity control) received ethanol (7.9 g/kg, i.e. 5 ml of 20% ethanol/100 g) as an aqueous solution for 60 days by intragastric intubation (*po*). Normal control rats received glucose solution equivalent to the calorific value of ethanol (15 g of glucose/kg, i.e. 5 ml of 30% glucose solution/100 g). After the induction of toxicity (i.e. initial 30 days), treatment groups received ursolic acid for 30 days at three different doses (10, 20, 40 mg/kg/day)

Tab. 1. Experimental design; UA – ursolic acid

Group	Treatment and duration	
	1–30 days	31–60 days
1	Glucose (7.5 g/kg, twice a day (09.00 a.m. and 5.00 p.m.), i.e. 15 g/kg/day)	
2	Glucose (15 g/kg/day)	Glucose (15 g/kg) + UA (10 mg/kg, twice a day, i.e., 20 mg/kg/day)
3	Ethanol (3.95 g/kg, twice a day, i.e. 7.9 g/kg/day)	
4	Ethanol (7.9 g/kg/day)	Ethanol + UA (5 mg/kg/twice a day, i.e. 10 mg/kg/day)
5	Ethanol (7.9 g/kg/day)	Ethanol + UA (10 mg/kg/twice a day, i.e. 20 mg/kg/day)
6	Ethanol (7.9 g/kg/day)	Ethanol + UA (20 mg/kg/twice a day, i.e. 40 mg/kg/day)

along with ethanol. This experimental design is clearly demonstrated in Table 1. The total experimental duration was 60 days. Rats in all the groups were sacrificed by decapitation 24 h after the last treatment. Blood was collected and allowed to coagulate at ambient temperature for 30 min. Serum was separated by centrifugation at 2000 rpm. The heart tissue was sliced into pieces and homogenized in cold 50 mM phosphate buffer (pH 7.4) to give 10% homogenate (w/v). The homogenate was centrifuged at 1000 rpm for 10 min at 0°C in cold centrifuge. The supernatant was separated and used for various estimations.

The activities of lactate dehydrogenase (LDH, E.C. 1.1.1.27) and creatine phosphokinase (CPK, E.C. 2.7.3.2) were assayed by the methods described by King [20] and Okinaka et al. [32], respectively. Thiobarbituric acid reactive substances (TBARS) were estimated [31] as an index of oxidative stress. Since this method quantitates malondialdehyde-like material and does not specifically measure malondialdehyde or lipid peroxidation, other indexes that reflect lipid peroxidation, including lipid hydroperoxides (LOOH) [18] and conjugated dienes (CD) [34], were also estimated in the heart. Protein was estimated by the method of Lowry et al. [23], using bovine serum albumin as standard. The antioxidant potential of heart was determined by assaying the activities of superoxide dismutase (SOD, E.C. 1.15.1.1), catalase (CAT, E.C. 1.11.1.6) glutathione peroxidase (GPx, E.C. 1.11.1.9) and glutathione S-transferase (GST, E.C. 2.5.1.18) according to the methods of Kakkar et al. [19], Sinha [42], Rotruck et al. [40] and Habig et al. [14], respectively. Reduced glutathione [10], ascorbic acid [39] and α-tocopherol [4] were estimated according to the procedures described earlier.

Values are expressed as means ± SD of eight rats in each group. Statistical evaluation was done using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). A probability of $p < 0.05$ was considered as significant.

Results

Figures 2 and 3 show the activities of marker enzymes such as LDH and CPK in the serum of control and ex-

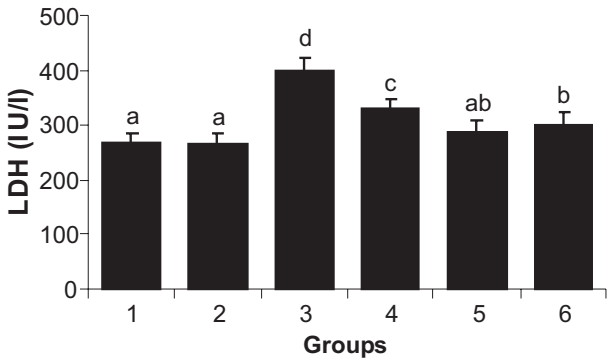

Fig. 2. Activities of lactate dehydrogenase (LDH) in the serum of rats treated with ethanol and/or ursolic acid. Values are given as means ± SD for eight rats in each group. Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT)

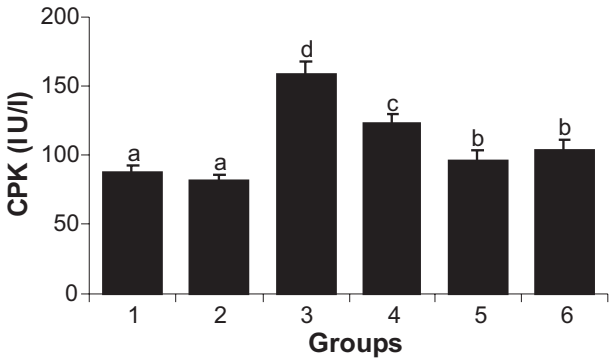

Fig. 3. Activities of creatine (CPK) phosphokinase in the serum of rats treated with ethanol and/or ursolic acid. Values are given as means ± SD for eight rats in each group. Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT)

Tab. 2. Levels of lipid peroxidation markers in the heart of rats treated with ethanol and/or ursolic acid (UA)

Group	TBARS (mmol/100 g of tissue)	LOOH (mmol/100 g of tissue)	CD (mmol/100 g of tissue)
Control	0.59 ± 0.03^{b}	82.77 ± 7.30^{ab}	43.24 ± 3.02^{b}
Control + UA (20 mg/kg)	0.47 ± 0.04^{a}	79.13 ± 6.07^{a}	38.92 ± 1.64^{a}
Ethanol	1.06 ± 0.09^{d}	134.16 ± 9.83^{c}	72.18 ± 4.64^{c}
Ethanol + UA (20 mg/kg)	0.63 ± 0.06^{c}	95.04 ± 9.51^{b}	46.77 ± 3.72^{b}

Values are given as means ± SD for eight rats in each group. Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT); TBARS – thiobarbituric acid reactive substances, LOOH – lipid hydroperoxides, CD – conjugated dienes

Tab. 3. Activities of enzymic antioxidants in the heart of rats treated with ethanol and/or ursolic acid (UA)

Group	SOD (U^a/mg of protein)	CAT (U^b/mg of protein)	GPx (U^c/mg of protein)	GST (U^d/mg of protein)
Control	7.21 ± 0.58^{ab}	46.94 ± 2.79^{ab}	6.16 ± 0.43^{a}	3.75 ± 0.34^{ab}
Control + UA (20 mg/kg)	7.71 ± 0.72^{a}	49.17 ± 3.43^{a}	6.28 ± 0.57^{a}	3.89 ± 0.54^{a}
Ethanol	4.37 ± 0.39^{c}	33.78 ± 3.14^{c}	3.46 ± 0.38^{b}	2.32 ± 0.18^{c}
Ethanol + UA (20 mg/kg)	6.65 ± 0.45^{b}	42.96 ± 2.94^{b}	5.89 ± 0.56^{a}	3.44 ± 0.24^{b}

Values are given as means ± SD for eight rats in each group. Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT). U^a – Enzyme concentration which gives 50% inhibition of NBT reduction/min; U^b – μmoles of hydrogen peroxide consumed/min; U^c – μmol of glutathione consumed/min; U^d – μmoles of CDNB-GSH conjugate formed/min; SOD – superoxide dismutase, CAT – catalase, GPx – glutathione peroxidase, GST – glutathione-S-transferase

perimental groups of rats. Marked elevation in the activities of these enzymes were observed in ethanol-intoxicated rats when compared with normal control, whereas ursolic acid coadministration significantly ($p < 0.05$) prevented these alterations when compared to untreated alcoholic rats. Coadministration of UA at a dose of 20 mg/kg/day showed the highest activity as compared to other doses.

Chronic ethanol administration significantly enhanced the myocardial lipid peroxidation as compared to normal control (Tab. 2). The levels of lipid peroxidation markers such as TBARS, LOOH and CD were maintained at near normal levels in rats coadministered with UA. Ursolic acid exhibited its potent antioxidant activity by decreasing the levels of TBARS and CD in control rats administered with UA as compared to normal control rats.

As depicted in Table 3, the activities of SOD, CAT, GPx and GST were substantially decreased in the heart of ethanol-administered group. In response to UA treatment, the activities of these enzymic antioxidants were significantly ($p < 0.05$) increased as compared to untreated alcoholic rats.

Table 4 shows the levels of non-enzymic antioxidants in control and experimental animals. The levels of reduced glutathione, ascorbic acid and α-to-

Tab. 4. Levels of non-enzymic antioxidants in the heart of rats treated with ethanol and/or ursolic acid (UA)

Group	GSH (μg/mg of protein)	Vitamin C (μg/mg of protein)	Vitamin E (μg/mg of protein)
Control	9.43 ± 0.83^{ab}	0.42 ± 0.04^{ab}	2.98 ± 0.27^{a}
Control + UA (20 mg/kg)	10.14 ± 0.96^{a}	0.45 ± 0.03^{a}	3.12 ± 0.19^{a}
Ethanol	6.28 ± 0.48^{c}	0.29 ± 0.03^{c}	1.71 ± 0.23^{c}
Ethanol + UA (20 mg/kg)	9.08 ± 0.79^{b}	0.40 ± 0.04^{b}	2.62 ± 0.25^{b}

Values are given as means ± SD for eight rats in each group. Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT); GSH – reduced glutathione

copherol were found to be reduced in the heart of ethanol-administered rats as compared to those of control rats. UA-treated alcoholic rats showed elevated levels of these antioxidants as compared to ethanol-administered rats.

Discussion

Alcohol is easily absorbed and distributed into tissues high in water content and blood flow, including heart [12]. The acute and chronic administration of alcohol in several human and animal models has demonstrated the cardiotoxic effect of ethanol [6]. Increased activity of serum LDH and CPK is a well-known diagnostic marker of myocardial injury and was reported to increase during alcoholic cardiomyopathy [7]. These cytosolic enzymes are released from the heart into blood stream during myocardial membrane damage [44]. However, these enzymes are not restricted to cardiac muscle tissue and their activity increases in the serum in non-cardiac tissue injuries also [11]. LDH and CPK are reported to increase in alcoholic liver injury [24] and alcoholic myopathy [25], respectively. Thus, the increased activities of these enzymes observed in ethanol-administered rats not only reflect the alcoholic myocardial injury but may also reflect the alcoholic liver and muscle injury. Leakage of these cytosolic enzymes increased due to the change in the permeability of plasma membrane resulted as a consequence of peroxidation of membrane by oxygen- and ethanol-derived free radicals. Coadministration of UA gave protection against ethanol by reversing the changes produced by ethanol. The protective ability of UA may be due to its cell membrane stabilizing effect [15] and radical scavenging potency [5]. In this concern, Saraswat et al. [41] also reported the membrane stabilizing effect of UA *in vitro* against ethanol. Since 20 mg/kg/day showed the highest activity when compared to other doses, this dosage was used for the studies on oxidative stress.

Earlier studies have shown that reactive oxygen species (ROS) are involved in ethanol-induced oxidative tissue injury [33]. The levels of lipid peroxidation markers were found to be increased in the heart of ethanol-administered rats as compared to control. Enhanced cardiac lipid peroxidation resulting from ethanol administration could play an important role in the ethanol-induced impairment of myocardial function [3]. UA-coadministered rats showed decreased levels of lipid peroxidation as compared to that of untreated alcoholic rats. It has been already reported that UA is a protector against lipid peroxidation and useful in the management of myocardial injury [5]. Thus, UA prevents the oxidative damage to rat heart membrane primarily through a free radical scavenging mechanism. This establishes that the antioxidant effects of UA [45] may be responsible for its antiperoxidative and cardioprotective effect against ethanol.

Antioxidants constitute the foremost defense system that limit the toxicity associated with free radicals. The myocardium has enzymic and non-enzymic systems to neutralize free radicals. The enzymes SOD, CAT, GPx and GST, as well as the non-enzymic antioxidants reduced glutathione, α-tocopherol and ascorbic acid are the main antioxidants [29]. But heart tissue has less antioxidant enzyme activity compared to liver and some other tissues. Therefore, it may be more vulnerable to peroxidative damage due to oxidative stress [16, 43]. Our results show decreased myocardial SOD, CAT, GPx and GST activities in chronically ethanol-administered rats. Thus, the inability of the myocardial cells to scavenge superoxide radical and hydrogen peroxide (which are usually scavenged by the enzymic antioxidants) leads to oxidative damage. We have observed the restoration of the activities of enzymic antioxidants in UA-coadministered rats. UA had a strong inhibitory activity against superoxide formation and showed superoxide scavenging activity [26]. It was also reported to reduce both intracellular ROS levels and the rate of H_2O_2-induced apoptotic cell death [17]. Because of these activities, it was expected that UA may decrease the workload of enzymic antioxidants and the radical-induced damage of cellular proteins (including enzymic antioxidants) and thereby enhance the activities of enzymic antioxidants. The decrease in the tissue lipid peroxidation on UA treatment can be correlated with the elevated activities of enzymic antioxidants.

Chronic ethanol intake induces changes in components of myocardial antioxidant defense system [9]. We have observed a lower level of cardiac GSH in chronically ethanol-administered rats, which could contribute to the increase in free radical formation in tissues. Other researchers have also reported the decreased level of GSH in the heart of alcoholic rats [13]. Apart from GSH, α-tocopherol and ascorbic acid are important free-radical scavengers which pro-

tect cell membrane against toxic agents such as alcohol. They are active in different media: while tocopherol is located in the lipid membrane environment, ascorbate is hydrophilic [46]. The levels of these vitamins were decreased in ethanol-treated rats. Since GSH is involved in the recycling of vitamin C and E by mediating the reduction of dehydroascorbate and the oxidized form of α-tocopherol [37], GSH deficiency would be expected to produce deficiency of vitamin C and vitamin E in the heart of alcohol-fed rats. Coadministration of UA with ethanol increased the levels of GSH, ascorbic acid and α-tocopherol as compared to alcoholic rats and thereby improved the antioxidant/prooxidant balance of the tissue. The enhanced antioxidant capacity along with the decreased levels of lipid peroxidation in ursolic acid coadministered rats reflects the decreased oxidative injury in the heart tissue.

Multiple mechanisms may interplay in the cardioprotective effect of UA. The present observation suggests that ursolic acid coadministration with ethanol would protect the heart from ethanol-induced oxidative damage probably by suppressing cellular oxidative stress. Thus UA could be of use in the treatment of alcoholic cardiomyopathy, and warrants further detailed evaluation.

Acknowledgments:
The authors thank Indian Council of Medical Research, New Delhi, India for their financial support in the form of senior research fellowship and Sami Labs Ltd., Bangalore, India for the kind supply of authentic ursolic acid.

References:

1. Aberle NS II, Ren J: Experimental assessment of the role of acetaldehyde in alcoholic cardiomyopathy. Biol Proced Online, 2003, 5, 1–12.
2. Andersson B, Waagstein F: Spectrum and outcome of congestive heart failure in a hospitalized population. Am Heart J, 1993, 126, 632–640.
3. Antonenkov VD, Pirozhkov SV, Popova SV, Panchenko LF: Effect of chronic ethanol, catalase inhibitor 3-amino-1,2,4-triazole and clofibrate treatment on lipid peroxidation in rat myocardium. Int J Biochem, 1989, 21, 1313–1318.
4. Baker H, Frankel O, Angelis B, Feingold S: Plasma tocopherol in man at various times after ingesting free or acetylated tocopherol. Nutr Res, 1980, 21, 531–536.
5. Balanehru S, Nagarajan B: Intervention of adriamycin-induced free radical damage. Biochem Int, 1992, 28, 735–744.
6. Beckemeier ME, Bora PS: Fatty acid ethyl esters: potentially toxic products of myocardial ethanol metabolism. J Mol Cell Cardiol, 1998, 30, 2487–2494.
7. Chrostec L, Szmitkowski M: Enzymatic diagnosis of alcoholism-induced damage of internal organs. Psychiatr Pol, 1989, 23, 353–360.
8. Doll R, Peto R, Hall E, Wheatley K, Gray R: Mortality in relation to consumption of alcohol: 13 years' observations on male British doctors. Br Med J, 1994, 309, 911–918.
9. Edés I, Toszegi A, Csanady M, Bozoky B: Myocardial lipid peroxidation in rats after chronic alcohol ingestion and the effects of different antioxidants. Cardiovasc Res, 1986, 20, 542–548.
10. Ellman GL: Tissue sulfhydryl groups. Arch Biochem Biophys, 1959, 82, 70–77.
11. Fontes JP, Goncalves M, Ribeiro VG: Serum markers for ischemic myocardial damage. Rev Port Cardiol, 1999, 18, 1129–1136.
12. Goldstein DB: The Pharmacology of Alcohol, Oxford University Press, New York, 1983.
13. Guerri C, Grisolia S: Changes in glutathione in acute and chronic alcohol intoxication. Pharmacol Biochem Behav, 1980, 13, 53–61.
14. Habig WH, Pabst MJ, Jakoby WB: Glutathione S-transferase. The first enzymatic step in mercapturic acid formation. J Biol Chem, 1974, 249, 7130–7139.
15. Han SK, Ko YI, Park SJ, Jin IJ, Kim JM: Oleanolic acid and ursolic acid stabilize liposomal membranes. Lipids, 1997, 32, 769–773.
16. Husain K, Somani SM: Response of cardiac antioxidant system to alcohol and exercise training in the rat. Alcohol, 1997, 14, 301–307.
17. Jeon BW, Kim KT, Chang SI, Chang SI, Kim HY: Phosphoinositide 3-OH kinase/protein kinase B inhibits apoptotic cell death induced by reactive oxygen species in *Saccharomyces cereviseae*. J Biochem, 131, 696–699.
18. Jiang ZY, Hunt JY, Wolff SP: Ferrous ion oxidation in the presence of xylenol orange for detection of hydroperoxides in low density lipoprotein. Anal Biochem, 1992, 202, 384–389.
19. Kakkar P, Das B, Viswanathan PN: A modified spectrophotometric assay of superoxide dismutase. Ind J Biochem Biophys, 1984, 21, 130–132.
20. King J: The dehydrogenases or oxidoreductase-lactate dehydrogenase. In: Practical Clinical Enzymology. Ed. Van D, Nostrand, London, 1965, 83–93.
21. Klatsky AL: Epidemiology of coronary heart disease – influence of alcohol. Alcohol Clin Exp Res, 1994, 18, 88–96.
22. Liu J: Pharmacology of oleanolic acid and ursolic acid. J Ethnopharmacol, 1995, 49, 57–68.
23. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with Folin-phenol reagent. J Biol Chem, 1951, 193, 265–275.
24. Magarian GJ, Lucas LM, Kumar KL: Clinical significance in alcoholic patients of commonly encountered laboratory test results. West J Med, 1992, 156, 287–294.
25. Martin F, Ward K, Slavin G, Levi J, Peters TJ: Alcoholic skeletal myopathy, a clinical and pathological study. Q J Med, 1985, 55, 233–251.

26. Martin-Aragon S, De Las Heras B, Sanchez-Reus MI, Benedi J: Pharmacological modification of endogenous antioxidant enzymes by ursolic acid on tetrachloride-induced liver damage in rats and primary cultures of rat hepatocytes. Exp Toxic Pathol, 2001, 53, 199–206.
27. McCord JM: Free radicals and heart disease. In: Nutritional Impact of Food Processing. Ed. Somogyi JC, Miller HR, Karger, Basel (Switzerland), 1989, 327–337.
28. McDonough KH: Antioxidant nutrients and alcohol. Toxicology, 2003, 189, 89–97.
29. McDonough KH: The role of alcohol in the oxidant-antioxidant balance in heart. Front Biosci, 1999, 4, D601–D606.
30. Nayeem MA: Ethanol induced delayed cellular protection in mouse cardiac myocytes: role of inducible nitric oxide synthase. Pol J Pharmacol, 2003, 55, 595–602.
31. Nichans WG Jr, Samuelson B: Formation of malondialdehyde from phospholipid arachidonate during microsomal lipid peroxidation. Eur J Biochem, 1968, 6, 126–130.
32. Okinaka S, Kumagai H, Ebashi S, Sugita H, Momoi H, Toyokura Y, Fujie Y: Serum creatine phosphokinase. Activity in progressive muscular dystrophy and neuromuscular diseases. Arch Neurol, 1961, 4, 520–525.
33. Preedy VR, Richardson PJ: Ethanol-induced cardiovascular disease. Br Med Bull, 1994, 50, 152–163.
34. Rao KS, Recknagel RO: Early onset of lipoperoxidation in rat liver after carbon tetrachloride administration. Exp Mol Pathol, 1968, 9, 271–278.
35. Rastogi RP, Mehrotra BN: Compendium of Indian Medicinal Plants, vol. 5, Drug Research Properties: A Central Drug Research Institute Series, Publication and Information Directorate, New Delhi, India, 1998, 649.
36. Reinke LA, Lai EK, DuBose CM, MacCay PB: Reactive free radical generation *in vivo* in heart and liver of ethanol-fed rats: Correlation with radical formation *in vitro*. Proc Natl Acad Sci USA, 1987, 84, 9223–9227.
37. Rivers JM: Safety of high-level vitamin C ingestion. Ann NY Acad Sci, 1987, 498, 445–454.
38. Roberts WC, Siegel RJ, McManus BM: Idiopathic dilated cardiomyopathy: analysis of 152 necropsy patients. Am J Cardiol, 1987, 60, 1340–1355.
39. Roe JH, Kuether CA: The determination of ascorbic acid in whole blood and urine through the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid. J Biol Chem, 1943, 147, 399–407.
40. Rotruck JT, Pope AL, Ganther HE, Swanson AB: Selenium: Biochemical roles as a component of glutathione peroxidase. Science, 1973, 179, 588–590.
41. Saraswat B, Visen PKS, Agarwal DP: Ursolic acid isolated from *Eucalyptus tereticornis* protects against ethanol toxicity in isolated rat hepatocytes. Phytother Res, 2000, 14, 163–166.
42. Sinha AK: Colorimetric assay of catalase. Anal Biochem, 1972, 47, 389–394.
43. Somani SM: Exercise training and tissue specific antioxidant enzymes. In: Pharmacology in Exercise and Sports. Ed. Somani SM, CRC Press, Boca Raton, FL, 1996, 7–96.
44. Wexler BC, Kittinger GW: Myocardial necrosis in rats: Serum enzymes-adrenal steroids and histopathological alterations. Circ Res, 1963, 13, 159–171.
45. Wu JW, Lee MH, Ho CT, Chang SS: Elucidation of the chemical structures of natural antioxidants isolated from rosemary. J Am Oil Chem Soc, 1982, 59, 339–345.
46. Yu BP: Cellular defenses against damage from reactive oxygen species. Physiol Rev, 1994, 74, 139–162.

Received:
February 10, 2005; in revised form: October 5, 2005.