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Ursolic Acid Isolated from *Eucalyptus tereticornis* Protects Against Ethanol Toxicity in Isolated Rat Hepatocytes†

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Ursolic acid is the active material isolated from the leaves of the Eucalyptus hybrid *E. tereticornis*. In the present study, it has shown a significant preventive effect *in vitro* against ethanol-induced toxicity in isolated rat hepatocytes. Compared with the incubation of isolated hepatocytes with ethanol only, the simultaneous presence of ursolic acid in the cell suspension preserved the viability of hepatocytes and reversed the ethanol-induced loss in the level of all the marker enzymes (AST, ALT and AP) studied. Ethanol alone resulted in a 48%–54% decrease in the viability and a 42%–54% reduction in the biochemical parameters of the hepatocytes. Ursolic acid showed a concentration dependent (1–100 μg/mL) preventive effect (12%–76%) on alcohol-induced hepatocyte toxicity by restoring the altered parameters. The results thus suggest the effective use of an *in vitro* test system as an alternative for *in vivo* assessment of hepatoprotective activity of purified material. **Copyright © 2000 John Wiley & Sons, Ltd.**

Keywords: In vitro prevention; ursolic acid; ethanol toxicity; hepatocytes; marker enzymes.

INTRODUCTION

Exposure to many drugs and industrial chemicals as well as excessive alcohol use leads to liver injury and other end-organ damage. In the past years, a number of herbal compounds and formulations with diverse chemical compositions from plants used in the Indian traditional medicinal system have been evaluated for their putative hepatoprotective/antihepatotoxic activity. Isolated chemical constituents from some of these plants have been evaluated for their protective activity against chemically induced toxicity in experimental models under *in vitro, ex vivo*, and *in vivo* conditions. Conventionally, chemical toxicants such as paracetamol, galactosamine and carbon tetrachloride are used in animal models to provoke hepatotoxicity followed by assessment of the hepatoprotective properties of a given natural or synthetic agent (Dwivedi *et al.*, 1990; Visen *et al.*, 1991 a, b; Visen *et al.*, 1996; Rastogi *et al.*, 1996). However, alcohol-induced hepatotoxicity has not been used often for bioevaluation of the natural hepatoprotective compounds.

Alcohol abuse-related metabolic derangements affect almost all body organs and their functions (Lieber, 1989; Agarwal and Goedde, 1990). The liver is one of the prime target organs of alcohol-related diseases. Alcohol in large amounts is directly toxic to the liver, although nutritional deficiencies may play a secondary and accelerating role. Chronic alcohol abuse provokes successive hepatic changes consisting of hepatic steatosis (fatty liver), fibrosis, alcoholic hepatitis and cirrhosis (Lieber, 1989). Individual susceptibility to the development of alcoholic liver disease may be due to a number of genetic and environmental factors including gender, immunological disturbances, nutrition, and polymorphism of alcohol and acetaldehyde metabolizing enzymes (Agarwal and Goedde, 1990). The direct toxic effects of alcohol and alcohol-related physical alterations have been attributed to the generation of reducing equivalents as NADH and the formation of acetaldehyde rather than to ethanol itself. Acetaldehyde is produced from ethanol by alcohol dehydrogenase (ADH). Acetaldehyde is far more toxic than the parent compound. Acetaldehyde damages mitochondria, reducing the level of aldehyde dehydrogenase (ALDH) and thus further impairing the metabolism of acetaldehyde. An enhanced lipid peroxidation as a mechanism of alcoholic liver injury may function by the formation of oxygen free-radicals, direct impact of ethanol-derived free-radicals and via acetaldehyde (Lieber, 1989). Acetaldehyde can covalently bind with a number of proteins, phospholipids and nucleic acids to form protein adducts, resulting in antibody production, enzyme inactivation and decreased DNA repair. Moreover, acetaldehyde promotes glutathione depletion, free-radical mediated toxicity, and lipid peroxidation. Alcohol abuse-related hepatotoxicity may also arise via the microsomal ethanol oxidizing system (MEOS) which is highly induced in chronic alcohol abuse.

Ursolic acid is a pentacyclic triterpenoid (saponin) that exists widely in food, medicinal herbs and other plants. Ursolic acid, isolated from the leaves of the plant Eucalyptus hybrid *E. tereticornis*, has been found to possess a wide variety of pharmacological activities such as antiinflammatory, antiulcer, and cytotoxic activities.

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Table 1. *In vitro* preventive effect of ursolic acid on the viability of rat hepatocytes against alcohol-induced cellular toxicity

Parameter	Control (Group I)	Ethanol (Group II)	Ursolic acid + Ethanol[a] (Group III) 1(μg/mL)	10(μg/mL)	100(μg/mL)
% Viable cells (Trypan blue exclusion test)	98.1 ± 2.1	45.2 ± 3.1[b]	51.5 ± 2.1	67.2 ± 1.5[c]	84.6 ± 2.3[c]
Oxygen uptake (μL/h/mg protein)	4.12 ± 0.9	2.12 ± 0.7[b]	2.45 ± 0.1	2.69 ± 0.1[c]	3.54 ± 0.04[c]

[a] Ethanol concentration: 40 μL/mL of 40% v/v.
[b] ($p < 0.001$).
[c] ($p < 0.001$).
Values are mean ± SD of six samples.
Group II compared with Group I.
Group III compared with Group II.

We have recently shown that ursolic acid acts as a hepatoprotective against chemical toxicity (Saraswat *et al.*, 1996). In the present study we have examined the effect of ursolic acid on ethanol-induced toxicity in isolated rat hepatocytes as an *in vitro* hepatic model. Changes in selected marker enzymes were recorded after incubation of isolated rat hepatocytes with ethanol, with and without treatment with ursolic acid, under *in vitro* conditions.

MATERIALS AND METHODS

Animals. Adult albino Druckery rats of either sex weighing 250–270 g were obtained from the Division of Laboratory Animals of the Central Drug Research Institute (CDRI), Lucknow. The animals were maintained under control conditions and diet.

Chemicals and reagents. Ursolic acid was kindly supplied by Dr Rameshwar Dayal, Forest Research Institute, Dehradun. Other reagents were obtained commercially from standard companies.

Isolation of hepatocytes. The hepatocytes were isolated as described earlier (Visen *et al.*, 1991a).

Assessment of the effect of ursolic acid on ethanol toxicity. Hepatocytes were incubated with 40 μL/mL 40% ethanol with and without ursolic acid (1, 10 and 100 μg/mL) for 24 h at 37 °C. The viability of the hepatocytes was measured by the trypan blue exclusion test and oxygen uptake test. Cellular damage was assessed by estimating the levels of the marker. Three different dilutions (20, 40 and 80 μL/mL) of a 40% ethanol solution (v/v) were used to standardize the dose of ethanol which would impart about 50% toxicity to hepatocytes as judged by the viability test. After incubation, the cells were subjected to the trypan blue exclusion test and oxygen uptake test. About 50% reduction in viable hepatocytes was recorded at an ethanol (40% v/v) concentration of 40 μL/mL. This dose was, therefore, used in all experiments reported here.

Trypan blue test. One drop of hepatocyte suspension (containing approximately 10 000 to 12 000 cells) was mixed with three drops of trypan blue solution (0.2%). The unstained viable cells were counted under a microscope and the percentage calculated accordingly.

Oxygen consumption. The oxygen uptake by hepatocytes, as a measure of viability, was determined by using a Gilson Oxygraph (Estabrook, 1967). The reaction mixture contained 1.7 mL of HEPES buffer and 0.1 mL of hepatocyte suspension. Oxygen consumption was evaluated as μL of oxygen utilized/h/mg protein.

Assay of marker enzymes. Alcohol-related cellular damage was assessed by estimating the levels of marker enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP), alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). Assay of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were carried out according to the method of Reitman and Frankel (1957). Alkaline phosphatase was estimated as described by Kind and King (1954). Alcohol dehydrogenase (ADH) and ALDH activities were assayed according to Agarwal and Goedde (1990).

Table 2. *In vitro* effect of ursolic acid on alcohol-induced cellular toxicity in isolated rat hepatocytes as judged by changes in marker enzymes

Parameter (U/mg protein)	Control (Group I)	Ethanol (Group II)	Ursolic acid + Ethanol[a] (Group III) 1(μg/mL)	10(μg/mL)	100(μg/mL)
AST	26.1 ± 1.3	12.6 ± 0.7[b]	14.2 ± 1.9	18.2 ± 1.8[c]	19.8 ± 2.1[c]
ALT	24.2 ± 2.1	11.4 ± 0.7[b]	13.5 ± 2.2	16.7 ± 2.7[c]	19.6 ± 2.0[c]
AP	18.6 ± 0.5	10.3 ± 0.3[b]	11.6 ± 1.9	13.8 ± 2.6[c]	15.7 ± 2.0[c]

[a] Ethanol concentration: 40 μL/mL of 40% v/v.
[b] ($p < 0.001$).
[c] ($p < 0.001$).
Values are mean ± SD of six samples.
Group II compared with Group I.
Group III compared with Group II.

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Table 3. *In vitro* effect of ursolic acid on ADH and ALDH activity in isolated rat hepatocytes against ethanol induced toxicity

Parameter (U/mg protein)	Control (Group I)	Ethanol (Group II)	Ursolic acid + Ethanol[a] (Group III) 1(μg/mL)	10(μg/mL)	100(μg/mL)
ADH	0.81 ± 0.1	0.47 ± 0.03[b]	0.53 ± 0.06	0.60 ± 0.07c	0.71 ± 0.09c
ALDH	24.2 ± 2.1	11.4 ± 0.7[b]	13.5 ± 2.2	16.7 ± 2.7c	19.6 ± 2.0c

[a] Ethanol concentration: 40 μL/mL of 40% v/v.
[b] ($p < 0.001$).
[c] ($p < 0.001$).
Values are mean ± SD of six samples.
Group II compared with Group I.
Group III compared with Group II.

Statistical analysis. Application of variance analysis (ANOVA) followed by individual comparison by Student's *t*-test was used to determine the significance of difference of mean (±SD) values.

RESULTS

Effect on viability of hepatocytes

As shown in Table 1, ursolic acid showed a significant protection against ethanol-induced damage in isolated hepatocytes from rats under *in vitro* conditions. The simultaneous presence of ursolic acid in the ethanol-treated cell suspension preserved the viability of the hepatocytes. The protective effect of ursolic acid was concentration dependent, almost 71%–74% protection was observed at a concentration of 100 μg/ml ursolic acid.

Effect on marker enzymes in hepatocytes

The level of marker enzymes, ALT, AST and AP was reduced significantly following incubation of the hepatocytes with 40 μl/mL of 40% ethanol at 37 °C for 24 h. This loss in marker enzyme activities was prevented (up to 65% in the presence of 100 μg/mL ursolic acid (Table 2).

Effect on alcohol metabolizing enzymes in hepatocytes

ADH and ALDH activities were also found to be reduced (42%–47%) as a result of ethanol treatment. When hepatocytes were incubated with ethanol in the presence of three different concentrations of ursolic acid (1, 10 and 100 μg/mL there was a marked prevention in the loss of enzyme activities. In the presence of 10 μg/mL ursolic acid only about 40% protection of ADH and ALDH activities was noted as compared with the control values, while a 100 μg/mL of ursolic acid concentration imparted about 70%–76% protection. The results are given in Table 3.

DISCUSSION

Various biochemical state markers are currently in use for the routine detection of alcohol abuse and alcohol–related liver damage. Elevated AST in serum is a general indicator of tissue and organ damage caused by either alcohol, viral infections, drugs or toxins. During unspecific hepatic damage, serum levels of AST increase significantly, while ALT level tends to increase to a greater extent in alcoholic liver disease. Acute alcohol drinking does not effect the serum AP concentrations but elevated levels of this enzyme have been reported in alcoholics and heavy drinkers. In the present *in vitro* study, following incubation of hepatocytes with alcohol, a marked decrease in the viability of hepatic cells as well as enzyme levels in the hepatocytes was noticed after 24 h of the period. Leakage of cytoplasmic enzymes and increased uptake of trypan blue dye are good markers of damage to the plasma membrane (Zimmerman and Mao, 1965; Zahn and Braunbeck, 1995). In our present findings the change in the permeability of plasma membrane might be due to peroxidation of lipid by the generation of oxygen as free-radical (Salaspero, 1989). Alcohol also decreased the alcohol metabolizing enzymes (ADH and ALDH) in isolated hepatocytes. The major impact of alcohol and its metabolites seems to fall on the hepatic mitochondria which subsequently cause a reduction in ADH and ALDH, thus further impairing the metabolism of acetaldehyde. These results are in correlation with earlier findings in alcohol induced hepatotoxicity *in vivo* and *in vitro* (Rastogi *et al.*, 1996; Visen *et al.*, 1996). Our earlier work on an *ex vivo* test system also showed the loss of enzyme levels in the isolated rat hepatocytes followed by an increase in serum (Visen *et al.*, 1991 a, b). One of the long-term goals of developing a natural hepatoprotective agent is to use it for prevention and treatment of alcoholic liver disease. No study has been reported yet on the effect of ursolic acid in alcohol-related hepatotoxicity. In this preliminary study, using isolated hepatocytes as a hepatic model to study alcohol-related injury under *in vitro* conditions, a significant degree of protection by ursolic acid was recorded. Probably the hepatoprotectives interfere in the handling of acetaldehyde generated from ethanol. This is evident from the reversal of the loss in marker enzyme levels in hepatocytes simultaneously incubated with ursolic acid and also by observing an increase in the viability of hepatocytes as assessed by trypan blue exclusion and the rate of oxygen uptake tests. The effect was found to be concentration dependent. Antagonism of the toxic effects of galactosamine, thioacetamide and carbon tetrachloride, as shown in earlier studies (Saraswat *et al*, 1996) indicate that ursolic acid might protect the liver by increasing protein synthesis, reducing free-radical generation and exerting anticholestatic activity. Thus, ursolic acid merits further investigation as a

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potential hepatoprotective agent against alcohol-related liver damage.

Though the exact mechanism of protection by ursolic acid has yet to be fully elucidated, hepatotoxicant and hepatoprotective molecules seem to interact with constituents of cellular and intracellular membranes and alter their functions. Apparently, ursolic acid modulates the handling of the acetaldehyde generated from ethanol. Thus, alcohol-induced *in vitro* cellular toxicity can serve as a physiological model to test the efficacy of known and unknown hepatoprotective compounds. Further, this also indicates the usefulness of *in vitro* models in the case of active materials because of the ready interaction in the hepatocytes.

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