Protective role of *Ocimum canum* plant extract in alcohol-induced oxidative stress in albino rats

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ABSTRACT

Ethanol is the most frequently abused drug and causes a variety of pathological disturbances. It causes toxicity to tissues by generating free radicals during the course of its metabolism that can damage cellular structure and function, especially in hepatocytes. This study investigates the preventive and protective effects of *Ocimum canum* on alcohol-induced oxidative stress. Male Wistar rats were used in three separate experiments. First, two groups of six rats each (normal control and alcohol-treated) were used to establish hepatotoxicity. The alcohol-treated group showed a significant increase in TBARS and decreased activities of SOD, catalase, GSH, ∝-tocopherol and ascorbic acid. Second, the preventive effect of the *O. canum* extract was assessed. Four groups of rats (six in each group) were used and the experimental groups were treated with ethanol and graded doses of the extract for four weeks. Normal control and alcohol-treated groups were also assessed. Lipid peroxidation and antioxidant potential were quantified in plasma samples, which showed that the extract had a preventive effect. Third, the curative effect of the extract was assessed. The rats were divided into four groups comprising a normal control group on a normal diet and three other groups given alcohol for four weeks to establish alcohol toxicity. One of the alcohol groups was used as a control and the other two alcohol groups were given graded doses of the extract. After four weeks the rats were sacrificed in order to assess the lipid peroxidation and antioxidant potentials. The results indicated that the *O. canum* extract had hepatoprotective abilities against alcohol-induced oxidative stress.

KEY WORDS: Alanine transaminase.
Ascorbic acid.
Aspartate transaminase.
Catalase.
Glutathione.
Ocimum canum.
Superoxide dismutase.
Thiobarbituric acid.
Tocopherols.

Introduction

Alcohol abuse is a major problem worldwide. Alcoholic liver disease is responsible for 15–30% of admissions in many hospitals.[1] Ethanol consumption produces a wide variety of pathological disturbances in a number of organs. As it is a small molecule and is soluble in water and lipids, ethanol permeates all tissues and affects most vital functions in the liver, kidney, brain, heart and pancreas.[2]

There is increasing evidence to show that oxidative stress plays an important role in the toxic effects of ethanol, which has been found to cause accumulation of reactive oxygen species (ROS) including superoxide, hydroxyl radicals and hydrogen peroxide.[3] The ROS cause damage to the cellular membranes, proteins, carbohydrates and DNA, resulting in cellular injury.[4,5]

Increased oxidative stress occurs as a direct result of ethanol and its oxidation products ethyl and 1-hydroxyethyl radicals.[6] Furthermore, acetaldehyde, the product of ethanol oxidation, reacts with hepatic glutathione, a major liver cytosolic antioxidant, resulting it its depletion.[7] Oxidative stress is a key step in the pathogenesis of ethanol-associated liver injury.

Ethanol consumption induces an increase in lipid peroxidation, either by enhancing the production of ROS or by decreasing the level of endogenous antioxidants.[8] Reactive oxygen species include hydroxy ethyl radicals, superoxide radicals (O^-), hydroxy radicals (OH), peroxy radicals and hydrogen peroxide.[9] Moreover, ethanol metabolism increases the activity of CYP2E1, which catalyses the conversion of ethanol to acetaldehyde, which has a high rate of NADPH oxidase activity that leads to the production of various ROS.[10]

Plants are a valuable source of natural products for maintaining human health, and intensive studies have been devoted to natural therapies. The use of plant extracts and phytochemicals derived from medicinal plants are used extensively for pharmaceutical purposes

Recent studies confirm the presence of antioxidants in many medicinal plants. For example, *Withania somifera* affects the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in the rat brain,[11] *Scoparia dulcis*[12] is known for its free-radical scavenging activities, and *Smilex china*[13] is reported to have antioxidant properties.

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The cellular radical-scavenging systems include glutathione (GSH) and consist of an array of enzymatic and non-enzymatic reaction pathways involving the neutralisation of ROS.[14] Enzymes such as SOD (scavenges the superoxide ion by speeding up its dismutation) and CAT (a haem enzyme that removes hydrogen peroxide)[15] are involved. Other antioxidants that can counteract ROS include polyphenolic compounds such as flavonoids and phenolic acids commonly found in plants.

Developing antioxidant therapy is an important strategy

to improve the prevention and treatment of alcoholic liver injury. A number of plants have been shown to possess hepatoprotective properties by improving antioxidant status. *Ocimum* species are known for their medicinal properties and are used extensively in Ayurvedic preparations. *O. sanctum* (the holy basil) is reported to possess antioxidant properties.[6] The preventive and curative effect of *O. gratissimum* on alcohol-induced liver toxicity has also been reported.[17]

Ocimum canum possess antibacterial[18] and mosquito-repellent properties[19] and also lowers blood glucose level by facilitating the release of insulin from isolated pancreatic β-cells;[20] however, very little is known about its antioxidant properties. This study aims to determine the role of *O. canum* as a hepatoprotective and antioxidant agent in alcohol-induced oxidative stress in albino rats. A methanol extract of *O. canum* is evaluated for its abilities to scavenge free radicals, protect cell viability and inhibit the formation of lipid peroxides.

Materials and methods

Samples of *O. canum* were collected locally and identification was confirmed by Dr. M. P. Setshogo (University of Botswana Herbarium). The plant was cut into small pieces, dried in the shade, coarsely powered and soaked in 70% methanol for three days at room temperature. The extract was filtered and the solvent was removed using a Buchi-type rotary evaporator at 65°C. The yield was 7.8% (w/w). The methanol extract of *O. canum* (MEOC) was administered orally after dissolving it in a drug carrier (distilled water and Tween 80 [9:1]).

Male albino rats (Wistar strain, 200–250 g) were housed at an ambient temperature of 25(±2)°C, relative humidity of 50–55%, with a 12-h light/dark cycle. Water and food were available *ad libitum*. Experiments were conducted following internationally accepted principles for laboratory animal care.

All the chemicals used were of analytical grade and obtained from Sigma-Aldrich (St. Louis, USA).

Each experimental block included normal controls (six rats) and the experimental animals were divided into different groups according to the experimental design.

Effect of alcohol (Experiment 1)
The animals were divided into two groups of six animals. Group 1 (normal control) was administered 1.5 mL distilled water. Group 2 (alcohol control) was administered ethanol (5 g/kg body weight) daily for 30 days orally by gastric tube. Animals were bled from the tail (approximately 2 mL) every week for biochemical estimations.

Effects of extract and alcohol on normal rats (Experiment 2)
The animals were divided into four groups of six animals. Group 1 (normal control) received 1.5 mL of distilled water and 1.5 mL drug carrier. Group 2 (alcohol control) received ethanol (5 g/kg body weight) daily and 1.5 mL drug carrier. Group 3 (alcohol experiment 1) received ethanol (5 g/kg body weight) daily plus extract (80 mg/kg body weight daily) with 1.5 mL drug carrier. Group 4 (alcohol experiment 2) received ethanol (5 g/kg body weight) daily plus extract (120 mg/kg body weight) with 1.5 mL drug carrier. There was a 10 h gap between the two administrations. At the end of the experimental period (30 days) the rats were sacrificed and blood was collected for biochemical estimations.

Effect of extract on rats given alcohol for 30 days (Experiment 3)
This experiment was designed to establish the curative effect of the extract on alcohol-induced hepatotoxicity. The animals were divided into four groups. Group 1 (normal control) received 1.5 mL distilled water and 1.5 mL drug carrier. Group 2 (alcohol control) received ethanol (5 g/kg body weight) and 1.5 mL drug carrier. Group 3 (extract-treated 1) received 1.5 mL distilled water and extract (80 mg/kg body weight) with 1.5 mL drug carrier. Group 4 (extract-treated 2) received 1.5 mL distilled water and extract (120 mg/kg body weight) with 1.5 mL drug carrier. There was a 10 h gap between the two administrations. The experiment continued for 30 days, after which the animals were sacrificed and blood was collected for biochemical estimations.

Estimation of thiobarbituric acid reactive substances
Thiobarbituric acid reactive substances (TBARS) in plasma can be estimated by the method described by Niehaus,[21] with some modifications. Lipid peroxidation is measured by the formation of TBARS such as malondialdehyde (MDA), which is formed from the breakdown of fatty acids and serves as a convenient index for determining the extent of the peroxidation reaction. Briefly, 0.1mL plasma was treated with 2 mL of a TCA-TBA-HCl (1:1:1) mixture and incubated in boiling water for 10 min. The mixture was cooled and then 2 mL freshly prepared 1N NaOH was added. Absorbance (*A*) was measured at 535 nm.

Estimation of reduced glutathione
Reduced glutathione (GSH) was estimated by the method of Ellman.[22] Briefly, 25 mL plasma was mixed with 0.5 mL precipitating buffer (5% TCA in 1mmol/L EDTA), centrifuge and the supernatant collected and mixed with 2.5 mL 0.1 mol/L phosphate buffer (pH 8.0). Colour was developed by adding 100 µL DTNB (0.01%) and *A* measured at 412 nm.

Estimation of superoxide dismutase
Superoxide dismutase was assayed by the method of Kakkar *et al*.[23] Briefly, the reaction mixture contained 150 µL EDTA, 600 µl L-methionine, 300 µL nitro blue tetrazolium (NBT) and the volume was made up to 2.8 mL by adding SOD buffer. To the reaction mixture was added 200 µL lysate (except in the controls). To this was added 200 µL riboflavin to start the reaction. The test tube was kept under a fluorescent lamp and the reaction kinetics measured for 4 min. Absorbance was measured at 560 nm.

Estimation of catalase
Catalase was estimated by the method of Bisswagner.[24] Briefly, 0.2 mL plasma was added to 0.98 mL H_2O_2 solution (10 mmol/L). Absorbance was measured at 240 nm and the catalase activity was calculated using the extinction coefficient of H_2O_2 (0.071) and the activity was expressed as micromoles H_2O_2 oxidised per min/mg protein.

Estimation of ∝-tocopherol (vitamin E)
∝-tocopherol was estimated by the method of Martinek,[25] with some modification. Briefly, serum (0.25 mL mixed with

0.25 mL distilled water) was taken in a stoppered centrifuge tube, and 0.5 mL distilled water (blank) and 0.5 mL standard solution were taken in respective stoppered centrifuge tubes. To all three tubes was added 0.5 mL xylene. The tubes were stoppered, mixed and centrifuged. The xylene layer (containing the precipitated tocopherol) was pipetted carefully into a clean tube and mixed with 0.35 mL $\propto$-$\propto'$ dipyridyl reagent. The extinction of the test and standard against the blank was read at 460 nm. Dilution of serum was taken into consideration in the calculation.

Estimation of ascorbic acid (vitamin C)

The method of Roe and Kuether,[26] with little modification, was be used for the estimation of ascorbic acid (vitamin C) in plasma. Briefly, to 0.2 mL plasma and 0.2 mL distilled water was added 1.6 mL 10% TCA. The contents were mixed well and allowed to stand for 5 min, then centrifuged at 2000 rpm for 10 min. A blank (distilled water) and a standard were processed simultaneously. To 1.0 mL supernatant was added 0.4 mL DNPH reagent, and the tubes were incubated at 37°C for 3 h. The tubes were then kept in an ice bath and 1.6 mL cold 65% H_2SO_4 was added. The contents were mixed and the resultant colour was read at 520 nm after 30 min. Dilution of the serum was taken into consideration in the calculation.

Estimation of alanine transaminase and aspartate transaminase

Estimation of alanine transaminase (ALT) and aspartate transaminase (AST) was performed using kits obtained from Sigma. The manufacturer's guidelines were followed.

Statistical analysis

All the values are represented as mean (± SE). Data on biochemical investigations were analysed using analysis of variance (ANOVA) and the Statistical Analysis Software (SAS, SAS Institute 2002, 2003, Version 9.1) was used. $P<0.05$ was considered significant.

Results

Table 1 shows the results obtained in the first experiment. They clearly demonstrate evidence of lipid peroxidation. Table 2 shows the preventive effect of MEOC. The results show significant differences between the normal control and alcohol-treated groups, but non-significant differences between the normal control and the experimental groups. Table 3 shows the curative effect of MEOC. The results for the normal control are significantly different from those in the alcohol-treated groups, but not for all parameters in the experimental groups.

Discussion

Ethanol induces the production of free radicals due to oxidative stress, and these increase MDA levels and generally impair the antioxidant defence system.[27] Ethanol-induced tissue damage occurs in various organs but especially the liver, where ethanol is oxidised. However, tissues other than liver also show oxidative stress following acute or chronic alcohol ingestion.[3]

Lipid peroxidation, as reflected by TBARS values, was higher in all ethanol-treated groups in the present study. This clearly indicates the presence of oxidative stress in hepatic and extrahepatic tissues induced by ethanol and its oxidation products.[28] Ethanol is metabolised to acetaldehyde

Table 1. Effect of alcohol on albino rats after four weeks' administration.

S. No.	Parameters	Groups	Weeks			
			1	2	3	4
1	TBARS (nmol/L) Plasma	C	2.04±0.67	2.23±0.42	2.12±0.34	2.18±0.37
		E	2.67±0.67	3.67±0.89	5.23±0.54*	6.68±0.97*
2	GSH (mg/dL) Plasma	C	31.59±0.48	34.84±0.45	32.56±0.96	33.43±11
		E	29.67±0.45	26.45±0.82*	23.68±0.94*	19.49±0.97*
3	SOD (U/mg Hb) Haemolysate	C	2.64±0.17	2.52±0.48	2.87±0.22	2.68±0.56
		E	2.67±0.32	2.02±0.45	1.87±0.62	1.46±0.03*
4	CAT (U/mg Hb) Haemolysate	C	47.71±1.32	47.86±1.71	48.95±1.29	47.43±0.94
		E	47.57±1.04	42.84±1.45	36.87±1.54	28.93±1.05*
5	Vit C (mg/dL) Plasma	C	2.62±0.092	2.82±0.65	2.61±0.96	2.57±0.68
		E	2.82±0.86	1.87±0.69	1.05±0.64	0.98±0.56*
6	Vit E (mg/dL) Plasma	C	2.63±0.69	2.39±0.56	2.86±0.87	2.89±0.63
		E	2.74±0.23	1.89±0.76	1.05±0.12	0.79±0.09*
7	ALT (U/L) Plasma	C	48.32±0.57	48.14±1.06	49.85±0.63	47.85±0.54
		E	48.76±0.87	52.97±1.02	56.89±0.67	65.88±0.94*
8	AST (U/L) Plasma	C	38.78±2.5	37.67±0.89	38.34±1.23	38.92±0.78
		E	37.98±0.89	43.78±1.23*	56.12±0.89*	65.05±1.03*

C: Control rats received distilled water

E: Experimental rats received alcohol (5g/kg body wt) for four weeks

$n=6$ in each group, *$P<0.001$ compared with control.

Table 2. Preventive effects of MEOC on alcohol-induced oxidative stress.

S No.	Parameters	Groups			
		1	2	3	4
1	TBARS (nmol/L) Plasma	1.28±0.08	6.87±0.18*	1.34±0.95	1.37±0.88
2	GSH (mg/dL) Plasma	35.07±0.09	17.95±0.97*	30.01±0.94	32.28±1.68
3	SOD (U/mg Hb) Haemolysate	2.68±1.17	1.28±1.21*	2.48±0.98	2.52±0.91
4	CAT (U/mg Hb) Haemolysate	48.75±1.32	28.48±1.19*	42.86±1.10	46.28±0.96
5	Vit C (mg/dL.) Plasma	2.37±0.18	1.38±0.18*	2.36±0.19	2.35±1.03
6	Vit E (mg/dL) Plasma	2.28±0.14	0.94±1.23*	2.32±1.52	2.31±0.98
7	AST (U/L) Plasma	40.16±1.18	78.01±1.26*	38.15±0.98	37.19±0.91
8	ALT (U/L) Plasma	35.78±2.52	62.08±.056*	38.16±1.31	39.19±1.08

Group 1: Normal control (rats received distilled water).

Group 2: Ethanol control (rats received ethanol 5 g/kg body weight).

Group 3: Experimental rats administered ethanol (5 g/kg body weight) plus extract (80 mg/kg body weight).

Group 4: Experimental rats administered ethanol (5 g/kg body weight) plus extract (120 mg/kg body weight).

n=6 in each group, $^*P<0.001$.

by alcohol dehydrogenase in the liver and generates NADH and increased production of ROS by NADH in different organelles.[3,5] Later, this acetaldehyde is oxidised to acetate by aldehyde oxidase or xanthine oxidase, giving rise to ROS via P450 2EI, and excess ROS production plays an important role in the development of lipid peroxidation.[10,28]

Reduced glutathione determines the susceptibility of tissues to oxidative damage, and marked depletion of GSH occurs in a variety of tissues after acute and chronic ethanol intoxication.[29,30] Ethanol is believed to generate free radicals that inhibit GSH synthesis and deplete GSH levels in tissues, partly due to the binding of cysteine in GSH by acetaldehyde.[7] In the first experiment in the present study, a significant decrease in plasma GSH was observed in the ethanol-treated groups compared to the normal control groups in all the experiments ($P\leq0.0001$, F=834.94). In the second experiment, GSH levels were significantly different in the ethanol-treated groups, demonstrating that the *O. canum* extract had a preventive effect ($P\leq0.0001$, F=602.95). An increase in GSH levels in the third experiment shows that the extract also has restorative effect. ($P\leq0.0001$, F=767.11).

Superoxide dismutase scavenges the superoxide ions produced as cellular by-products of ethanol metabolism. The reduced SOD activity in the ethanol-treated group resulted in the accumulation of superoxide radicals and the production of oxidative stress. The MEOC was found to have good scavenging activity, mainly via superoxide anions, at all concentrations tested. The results show significant differences between the normal control and ethanol-treated groups ($P\leq0.0001$), but no significant different from the MEOC-treated groups

Catalase acts as a preventive antioxidant and plays an important role in protection against the effects of lipid

Table 3. Curative effects of MEOC on alcohol-induced oxidative stress.

S. No.	Parameters	Groups			
		1	2	3	4
1	TBARS (nmol/L) Plasma	1.31±0.18	6.97±0.38*	1.33±0.85	1.34±0.88
2	GSH (mg/dL)Plasma	35.97±0.59	18.25±0.17*	32.01±0.54	34.28±1.08
3	SOD (U/mg Hb) Haemolysate	2.78±1.37	1.08±1.31*	2.52±0.68	2.72±0.91
4	CAT (U/mg Hb) Haemolysate	46.65±1.42	30.48±1.18*	44.87±1.70	46.07±0.97
5	Vit C (mg/dL) Plasma	2.37±0.18	1.38±0.18*	2.36±0.19	2.35±1.03
6	Vit E (mg/dL) Plasma	2.35±0.15	1.04±1.24*	2.32±0.1.52	2.42±0.95
7	AST (U/L) Plasma	42.26±1.19	72.01±1.42*	39.15±1.08	38.9±0.91
8	ALT (U/L) Plasma	36.68±2.52	60.08±.057*	35.16±1.01	38.19±1.04

Group 1: Normal Control (rats received distilled water).

Group 2: Ethanol Control (rats received alcohol (5g/kg body weight).

Group 3: Experimental rats administered extract (80 mg/kg body weight).

Group 4: Experimental rats administered extract (120 mg/kg body weight).

n=6 in each group, $^*P<0.001$.

peroxidation. The inhibition of CAT activity suggests that there is increased synthesis of superoxide ions during ethanol ingestion because superoxide can be a powerful inhibitor of catalase.[31] In all three experiments conducted in the present study, significant differences were seen between the alcohol-treated groups and the normal controls ($P \leq 0.0001$), but no significant differences in the experimental groups.

Vitamin E is a major lipophilic antioxidant and plays an important role in the defence against oxidative stress. The significant decrease in vitamin E levels in the ethanol-treated groups ($P \leq 0.0001$, $F=73.4$) in this study shows the oxidative stress due to ethanol consumption might have resulted in the complete utilisation of vitamin E,[32] and this was prevented in the second experiment by MEOC ($P \leq 0.0001$, $F=507.83$). In the third experiment, vitamin E levels returned to normal in the ethanol-treated groups that received MEOC ($P \leq 0.0001$, $F=73.74$), and demonstrates that the extract has a curative effect.

Vitamin C and vitamin E work almost in the same way in the antioxidant system. There was a significant decrease in the levels of ascorbic acid in the ethanol-treated groups (Table 1; $P \leq 0.0001$, $F=285.55$) compared to the control groups, but did not show a significant difference in the MEOC-treated group that received ethanol ($P \leq 0.0001$, $F=71.23$).[33] In the third experiment, vitamin C did not show any significant difference between the normal control and experimental groups, but there was a significant difference from the ethanol-treated groups ($P \leq 0.0001$, $F=28.80$).

A significant increase in the levels of liver markers (ALT and AST) was observed in ethanol-treated groups, and this may be due to changes in membrane phospholipid composition caused by the peroxidation process, which in turn increases membrane permeability. Treatment with MEOC helped to prevent this in the second experiment and appeared to restore the normal condition to some extent in the third experiment.

In conclusion, the use of antioxidant is an important preventive method to minimise the pathological and toxic effects of oxidative stress. Thus, from the results presented here, *O. canum* would appear to have significant antioxidant properties and can partly prevent the consequences of ethanol-induced toxicity and to some extent reverse the consequences of ethanol toxicity. □

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