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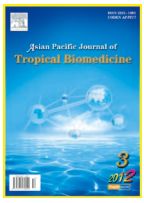

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Study of Aging and Hepatoprotective Activity of *Vitis vinifera* L. Seeds in Albino Rats

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ABSTRACT

Objective: Present study was conducted to investigate in liver of rats from 8–12 weeks old to 20 weeks old, the age dependent changes, carbon tetrachloride mediated changes, and the hepatoprotective effect shown by the seeds of *Vitis vinifera* L. **Method:** The hepatoprotective activity was studied by observing the effect of 100 mg/kg dose of ethanolic extract of grape seeds on carbon tetrachloride induced hepatotoxicity in albino rats and results were compared with those of the aged group results. **Results:** 100 mg/kg b.w. of ethanolic extract of *Vitis vinifera* seeds produced highly significant decrease in AST, ALT, ALP, bilirubin, albumin levels and significant decrease in the TSP levels compared to the toxic group levels. The levels of AST, ALT, ALP, bilirubin and albumin in aged control rats were found to be significantly higher than the levels in young control animals. MDA levels were slightly higher while GSH levels were lower in aged control rats as compared to young control rats. MDA levels in the toxic group showed highly significant increase compared to the young control levels. Ethanolic extract of seeds of *Vitis vinifera* significantly lowered the MDA levels. Histopathology results reveal that 100mg/kg/day dose of ethanolic extract of seeds of *Vitis vinifera* L. cured the hepatic damage to a great extent which was induced by CCl_4. **Conclusions:** Aging leads to the changes in the hepatic structure which are comparable to the changes induced by low doses of a hepatotoxin and the ethanolic extract of seeds of *Vitis vinifera* L. was effective in bringing about functional improvement of hepatocytes exposed to free radical attack, which was confirmed by biochemical and histological observations.

1. Introduction

The aging is a very complex biological process. In addition to individual genetic factors, the external influences such as nutrition, smoking, alcohol, environmental conditions etc. can strongly contribute to its anticipated appearance[1]. A particular attention in this respect has been paid to the biological action of free radicals, especially to oxygen species (OH, peroxyl, ozone and other oxidizing species), which are causing 'oxidative stess'[2]. The liver regulates many important metabolic functions. Hepatic injury is associated with distortion of these metabolic functions[3]. Additionally it is the key organ of metabolism and excretion. Morphological changes in the hepatic sinusoid with old age are increasingly recognized. These include thickening and defenestration of the liver sinusoidal endothelial cell, sporadic deposition of collagen and basal lamina in the extracellular space of Disse, and increased numbers of fat engorged, non-activated stellate cells[4]. Antioxidants of natural origin have attracted special interest because they can protect human body from free radicals without producing toxic effects[5]. It is already reported that natural antioxidants, especially phenolics and flavonoids, found in plants are the most bioactive. Plants available worldwide already reported for their antioxidant activity are well known, famous for their uses and readily available[6]. Grapes (*Vitis vinifera*) have been heralded for their medicinal and nutritional value for thousands of years. Grape seeds are a particularly rich source of complex polymers of flavonoids such as gallic acid, the monomeric flavan-3-ols catechin, epicatechin, gallocatechin, epigallocatechin, epicatechin-3-ogallate, dimeric, trimeric and even more polymeric

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proanthocyanidins. Grape seed extract of *Vitis vinifera* L. has in vivo antioxidant property and could be as important as vitamin E in preventing oxidative damage in tissues by reducing the lipid oxidation and/or inhibiting the production of free radicals[7].

The aim of the present study was to investigate in liver of rats from 8-12 weeks old to 20 weeks old, the age dependent changes, carbon tetrachloride mediated changes, and the hepatoprotective effect shown by the seeds of *Vitis vinifera* L. by examining the systems involved in the glutathione redox cycle and the concerted action of antioxidant enzyme defenses.

2. Materials and methods

2.1. Plant material and preparation of extract

Grape seeds were bought from a local supermarket (Khari bavli) in Delhi. They were dried for 1hour at 60 °C in an oven. Seeds were identified as *Vitis vinifera* L. by M.P. Sharma, Prof. Department of Botany Jamia Hamdard University, New Delhi.

After collection and authentication the plant materials were shade dried and powdered separately. All plant material was passed through sieve no. 40 and used for extraction. Ethanolic extract of the powdered plant material was prepared by the method given by Alkofahi *et al*[8].

2.2. Animals and treatments

The study was carried out for 21 days. Five groups of male albino rats of Wistar strain, four young (8-12 weeks old) and one old age group (16-20 weeks old) weighing 200-250 g and 300-350 g respectively were procured from central animal house facility Jamia Hamdard. The animals were kept in polypropylene cages (6 in each cage) under standard laboratory conditions (12 hour light and 12 hour dark: day and night cycle) and had a free access to commercial pelleted diet and tap water ad libitum. All studies were performed in accordance with the guidelines of Institutional Animal Ethics Committee (IAEC)

Group first served as young control and received a single daily dose of normal saline (0.3 mL). Group two served as aged control and received a daily single dose of normal saline as in group I rats. Group three animals received carbon tetrachloride (0.7 ml/kg body weight, *i.p.* 1:1 v/v mixture of CCl_4 and liquid paraffin) alone on first day and served as toxic young control[9]. Fourth group served as young standard control and the animals received a single dose of CCl_4 (as in group 2 animals) on first day along with a single dose of standard Silymarin (Sivylar-140, Ranbaxy) (25mg/kg p.o.) daily for 20 days[10]. Group five animals received a single dose of CCl_4 (0.7 ml/kg *i.p.* in liquid paraffin) and a daily single dose (100mg/kg b.w.) of ethanolic extract of *Vitis vinifera*. Animals were sacrificed 48 h after the last dose of the drug. The blood was collected and liver samples were dissected.

2.3. Estimations in serum

The collected blood was allowed to clot and serum was separated at 2 500 rpm for 15 min and the biochemical parameters like serum enzymes: aspartate aminotransferase (AST, U/L)[11], serum glutamate pyruvate transaminase (ALT, U/L)[11], serum alkaline phosphatase (ALP, IU/L)[12] and total bilirubin (mg/dL)[13] were assayed using assay kits.

2.4. Estimations in liver

2.4.1. Assessment of lipid peroxidation (LPO)

The dissected out liver samples were washed immediately with ice cold saline to remove as much blood as possible. 10% w/v tissue homogenate was prepared in ice cold 0.15 M KCl for TBARS. 1 ml of suspension medium was taken from the 10% tissue homogenate. A total of 5 mL of 30% TCA was added to it, followed by 0.5 mL of 0.8% TBA reagent. The tubes were then covered with aluminium foil and kept in shaking water bath for 30 min at 80 °C. After 30 min tubes were taken out and kept in ice cold water for 30 min. These were then centrifuged at 3 000 rpm for 15 min. The absorbance of the supernatant was read at 540 nm at room temperature against blank. Blank consisted of 1 mL distilled water, 0.5 mL of 30% TCA and 0.5 mL of 0.8% TBA[14].

2.4.2. Assessment of reduced glutathione (GSH) activity

This spectrophotometric procedure is based on the method of Ellman *i.e.* 5,5′-dithiobis-(2-nitrobenzoic acid) or DTNB, is reduced by SH groups to form 1 mole of 2-nitro-5-mercaptobenzoic acid per mole of SH. The reaction mixture contained equal volumes of 4% sulfosalicylic acid and tissue samples homogenized in 4 vol. of ice cold 0.1 mL phosphate buffer (pH 7.4). The method used for estimating GSH in this study also measures non-protein sulfhydryl concentration inclusive of GSH. However, 80-90% of the non-protein sulfhydryl content of the cell represents free endogenous GSH. Enzyme activity was expressed as milligram per hundred grams[15].

2.4.3. Protein estimation

Protein reacts with Folin's ciocalteau phenol reagent to give a coloured complex. The colour so formed is due to the reaction of alkaline copper with protein as in Biuret test and the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein[16]. 500 mg of liver tissue was homogenized in 5 mL 0.15 M KCl and centrifuged at 10 000 rpm for 10 min. 1 ml of supernatant was mixed with 5 mL of alkaline copper solution and allowed to stand at room temperature for 10 min. 0.5 mL of Folin's reagent (1:2) was added and tubes were shaken to mix the solution. After 30

min the absorbance was read at 750 nm against appropriate blank. The protein content was expressed in mg.

2.5. Histopathological studies

For histological studies, the liver tissues were fixed with 10% phosphate buffered neutral formalin, dehydrated in graded (50-100%) alcohol and embedded in paraffin. Thin sections (5 μm) were cut and stained with routine hematoxylin and eosin (H & E) stain for photo microscopic assessment. The initial examination was qualitative, with the purpose of determining histopathological lesions in liver tissue[17].

2.6. Statistical analysis

Results are presented as Mean±SEM of six animals used in each group. Data were subjected to statistical analysis through one way analysis of variance (ANOVA) taking significant at 5% level of probability followed by Student's *t*-test taking significant at $P \leqslant 0.05$[18].

3. Results

3.1. Serum enzymes

Levels of the serum marker enzymes of hepatic damage, AST, ALT, ALP, bilirubin and albumin increased significantly (while level of total serum proteins (TSP) decreased significantly) in group 3 rats which were treated with only CCl_4 compared to both young control (group 1) and aged control (group 2) rats. The levels of AST, ALT, ALP, bilirubin and albumin in aged control rats were found to be significantly higher than the levels in young control animals. 100 mg/kg b.w. of ethanolic extract of *Vitis vinifera* seeds produced highly significant decrease in AST, ALT, ALP, bilirubin, albumin levels and significant decrease in the TSP levels compared to the toxic group levels. (Table 1)

3.2. Tissue estimations

The levels of MDA were slightly higher in aged control rats (group 2) as compared to young control rats (group 1). MDA levels in the toxic group showed highly significant increase compared to the young control levels. Ethanolic extract of seeds of *Vitis vinifera* significantly lowered the MDA levels in the group 5 rats. GSH levels were found slightly lower in the aged control rats compared to the young control rats. CCl_4 produced a highly significant fall in the GSH levels. Grape seed extract produced significant fall in the GSH levels in the group 5 rats. There was seen a significant different in the tissue protein levels of young control and aged control rats. CCl_4 produced a highly significant increase in the protein levels (group 3), while 100 mg/kg p.o. dose of *Vitis vinifera* seed extract reduced the levels significantly towards the normal level (Table 2).

3.3. Histopathological results

The results are shown in Figure 1. Histopathological examination of the liver slides of rats of young (group 1) and aged (group 2) normal control showed normal parenchyma and normal portal tract. Livers of the rats administered only

Table 1

Analysis of different serum parameters.

Treatments	AST (IU/mL)	ALT (IU/mL)	ALP (KA units)	TSP (g/dL)	Bilirubin (g/dL)	Albumin (g/dL)
Group 1	28.3±3.38c	34.8±1.72c	29.1±0.98c	15.2±0.9b	0.75±0.03c	4.9±0.29c
Group 2	61.6±2.66cz	84.4±7.9cy	30.4±0.5cy	12.9±1.1	2.3±0.1cz	5.36±0.19cx
Group 3	136.1±5.6z	104.5±3.76z	48.8±0.45z	9.5±0.54y	8.5±0.33z	10.5±0.28z
Group 4	79.8±2.83cz	39.5±5.03c	30.7±0.69c	14.3±0.66b	1.0±0.04c	6.4±0.04cz
Group 5	96.9±3.55cz	47.7±6.1c	27.2±1.2c	14.9±1.03b	2.5±0.07c	3.9±0.22c

a $P<0.05$, b $P<0.01$, c $P<0.001$ compared with the toxic group

x $P<0.05$, y $P<0.01$, z $P<0.001$ compared with the young control group

Each observation is expressed as Mean±SEM and $n=6$

Table 2

Analysis of different liver tissue parameters.

Treatments	MDA (nmol/g)	GSH (μg/mg)	Tissue protein (mg/mL)
Group 1	0.9±0.19c	17.5±2.17c	1.5±0.08c
Group 2	1.3±0.02c	15.8±0.98c	1.8±0.11cx
Group 3	4.6±0.24z	3.4±0.4z	2.8±0.19z
Group 4	3.0±0.18cz	8.3±0.25y	2.6±0.06z
Group 5	1.2±0.27c	13.5±2.79c	1.9±0.1c

a $P<0.05$, b $P<0.01$, c $P<0.001$ compared with the toxic group

x $P<0.05$, y $P<0.01$, z $P<0.001$ compared with the young control group

Each observation is expressed as Mean±SEM and $n=6$

CCl_4 (toxic control, group 3) showed moderate inflammation of the portal triad; fatty change and necrosis of the periportal zone and they also showed severe necrosis, sinusoidal dilatation, inflammation, hemorrhage and vascular congestion of the centrizonal area. Rat livers treated with CCl_4 along with Silymarin (100 mg/kg/day) showed almost normal appearance of liver parenchyma. However, a necrotic focus was seen in the periportal area. Animals that had received CCl_4 along with extract of *Vitis vinifera* (100 mg/kg/day) showed a little fatty change in periportal zone. Centrizonal area showed mild sinusoidal dilatation, moderate inflammation and mild haemorrhage.

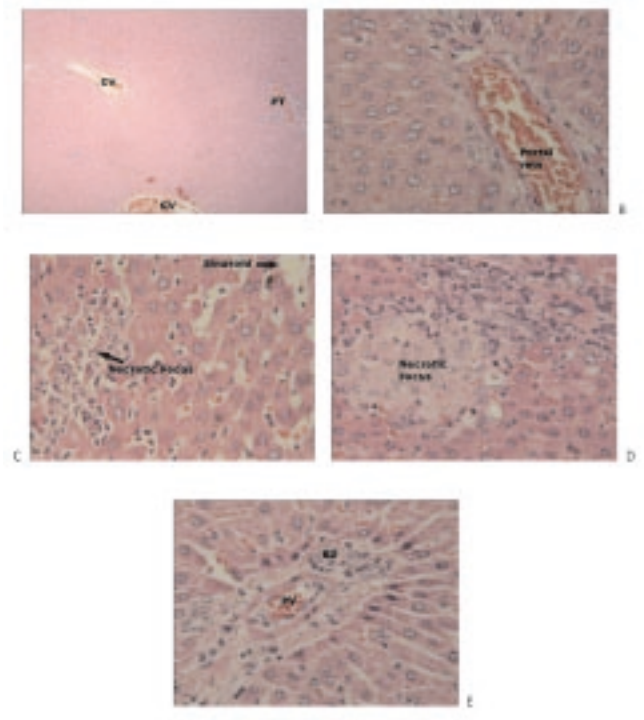

Figure 1.

1(A): Liver from young control group animal showing normal liver parenchyma.

1(B): Liver from aged control group animal showing normal liver parenchyma.

1(C): Liver from animal treated with CCl_4 only showing sinusoidal dilatation and a focus of necrosis with inflammatory cell infiltration and haemorrhage.

1(D): Liver from animal treated with CCl_4 and Silymarin showing the necrotic focus in the periportal area. 1(E): Photomicrograph of liver from animal treated with CCl_4 and 100 mg/kg b.w. of alcoholic extract of Vitis vinifers L. seed extract showing a normal hepatic parenchyma and a normal portal triad.

4. Discussion

The aim of the present study was to investigate in liver of rats from 8-12 weeks old to 20 weeks old, the age dependent changes, carbon tetrachloride mediated changes, and the hepatoprotective effect shown by the seeds of *Vitis vinifera* L. by examining the systems involved in the glutathione redox cycle and the concerted action of antioxidant enzyme defenses. The aged control group was included in this study to ascertain that there are age associated changes prevalent in the structure and functional capacity of the hepatocytes. These age associated changes can be correlated to the toxic control group which was given a onetime low dose of CCl_4. It has been reported that CCl_4 produces lipid peroxidation which may cause peroxidative tissue damage in inflammation, cancer, aging, ulcers, cirrhosis and atherosclerosis. Therefore inhibition of cytochrome P450-dependent oxygenase activity could cause a reduction in the level of toxic reactive metabolites and a decrease in tissue injury. On the other hand, an elevation of plasma AST, ALT, ALP activities could be regarded as a sign of damage to the liver membrane.

In this study AST, ALT and liver MDA levels decreased significantly ($P<0.001$) by 28.82%, 54.3% and 74.5% respectively in rats treated with ethanolic extract of *Vitis vinifera* L. seed extract (100 mg/kg b.w.) in comparison with the toxic group. GSH level was significantly increased ($P<0.001$) by 74.83% with grape seed extract. Levels of other enzymes in which significant changes ($P<0.001$) were observed are ALP (-44%), bilirubin (-84%), albumin (-59.7), total serum protein (+35.84, $P<0.01$) as compared to the young toxic group. The reversal of the damage in CCl_4 induced hepatic damage by ethanolic extract by *Vitis vinifera* could be explained by the prevention of leakage of intracellular enzymes through its membrane stabilizing effects[19]. Glutathione (GSH), extensively found in cells, protects them against electrophillic attacks provided by xenobiotics such as free radicals and peroxides. The elevation of MDA levels, which is one of the end products of lipid peroxidation in the liver tissue, and the reduction of hepatic GSH levels are important indicators of liver damage in CCl_4 intoxicated rats[20]. In this study it was ascertained that MDA levels have been suppressed and CCl_4 induced depletion of GSH was prevented significantly ($p<0.001$) by 74.83% when compared with toxic group by treatment with *Vitis vinifera* L.

Histopathology results reveal that 100mg/kg/day dose of ethanolic extract of seeds of *Vitis vinifera* L. cured the hepatic damage to a great extent which was induced by CCl_4.

On the other hand, the liver enzyme levels in the aged control group were increased as AST -54% ($P<0.001$), ALT-58.73% ($P<0.01$), ALP-4.3% , bilirubin-67% ($P<0.001$), albumin-8% ($P<0.05$), MDA-31.39% ($P<0.001$) and tissue protein-16.6% ($P<0.05$) when compared with the young control group. This implies that there were age associated changes in antioxidant defense of hepatocytes. From histological observations no significant changes were seen in the aged group.

It can be concluded that aging leads to the changes in the hepatic structure which are comparable to the changes induced by low doses of a hepatotoxin and the ethanolic

extract of seeds of *Vitis vinifera* L. was effective in bringing about functional improvement of hepatocytes exposed to free radical attack, which was also confirmed by histological observations.

Conflict of interest statement

We declare that we have no conflict of interest.

6. Acknowledgement

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