

PROTECTIVE EFFECT OF *Bacopa monnieri* LEAF EXTRACT AGAINST OXIDATIVE STRESS INDUCED HEPATOTOXICITY IN RATS

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ABSTRACT

Objective: The aim of the present study was to study the counteracting effect of *Bacopa monnieri* leaf extract against oxidative stress induced hepatotoxicity in rats.

Methods: Healthy male Wistar albino rats aged 6-8 weeks were randomly divided into control and experimental group. The experimental groups were treated with CCl₄ (2.0ml/kg body weight). Silymarin (a standard hepatoprotective antioxidant) was given at a dose of 25mg/kg body weight/day. Methanolic extract of *Bacopa monnieri* leaves (500mg/kg body weight) were administered to the CCl₄ treated rats for 21 days to study its protective effect and the extent of liver damage were evaluated by the activities of the liver marker enzymes and the lipid profile in the serum.

Results: On treatment with ethanol, a significant increase (P<0.05) in the activities of all the four serum marker enzymes (AST, ALT, ALP and γ -GT) were observed, which was further augmented on CCl₄ administration. The treatment with the plant extract was effective in restoring the activities close to the normal levels. A significant increase in the levels of serum cholesterol, triglycerides, free fatty acids and phospholipids in the alcohol treated group were observed and the levels were further increased in CCl₄ and alcohol treated group which was effectively counteracted by *Bacopa monnieri* leaf extract. The effect of the extract was comparable to the standard antioxidant silymarin.

Conclusion: The results of the present study indicate that the leaves of *Bacopa monnieri* possess hepatoprotective activity and can render protection against alcohol-CCl₄ induced toxicity.

Keywords: Ethanol, CCl₄, Oxidative stress, *Bacopa monnieri*, Hepatoprotection

INTRODUCTION

The role of free radicals and tissue damage in diseases, such as atherosclerosis, heart failure, neurodegenerative disorders, aging, cancer, diabetes mellitus, hypertension and several other diseases, is gaining a lot of recognition [1]. Both reactive oxygen species (ROS), as well as reactive nitrogen species (RNS), are products of normal cellular metabolism. They are well recognized as playing a dual role as both deleterious and beneficial species, in that they can be either harmful or beneficial to living systems [2].

The ability of ethanol to increase the production of reactive oxygen species and cause oxidative damage to lipids, proteins, and DNA has been demonstrated in a variety of systems, cells and species, including humans [3]. Chronic alcohol intake generates excess production of free radicals, where the antioxidant defenses are impaired, which results in sequential degradation of cell membranes by a process known as lipid peroxidation. This process may destroy the integrity of the membranes both within and surrounding the cells, seriously compromising cell function. Chronic and excess alcohol consumption may accelerate an oxidative mechanism directly or indirectly, which eventually produces cell death and tissue damage [4].

Carbon tetrachloride is one of the most commonly used hepatotoxins in the experimental study of liver damage. The hepatotoxic effects of this chemical is mostly based on membrane lipid peroxidation. Consequently, it leads to the induction of trichloromethyl radical that results in severe cell damage. The administration of CCl₄ in rats enhances hepatic protein oxidation and results in the accumulation of oxidized proteins in the liver [5]. CCl₄ treatment generates free radicals that trigger a cascade of events resulting in hepatic fibrosis [6]. The mechanism by which CCl₄ causes cell oxidative injury involves cytochrome P450 system that transforms CCl₄ into CCl₃ and then CCl₃ is transformed into a more reactive CCl₃O₂. CCl₃O₂ causes lipid peroxidation, disturbs Ca²⁺ homeostasis and eventually kills cells [7].

Currently, research interest has been focused on the role of antioxidants as well as antioxidant enzymes, in the treatment and prevention of the diseases mentioned above. The most commonly

used antioxidants at present are vitamins, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and *tert*-butylhydroquinone (TBHQ). However, they are suspected of being responsible for liver damage and acting as carcinogens in laboratory animals [8]. Therefore, the development and utilization of more effective antioxidants of natural origin is desirable.

Medicinal plants that historically have been useful, are obvious choices as potential sources of substances with significant pharmacological and biological activities. Phytomedicines exert their beneficial effects through the additive or synergistic action of several chemical compounds acting at single or multiple target sites associated with a physiological process in contrast to synthetic pharmaceuticals based upon a single chemical. This synergistic or additive pharmacological effect can be beneficial by eliminating the problematic side effects [9]. The therapeutic benefit of medicinal plants is often attributed to their antioxidant properties [10].

In recent years, considerable attention has been directed towards the identification of plants with antioxidant ability that may be used for human consumption. Therefore, research has focused on the use of antioxidants, with particular emphasis on naturally derived antioxidants, which may inhibit ROS production and may display protective effects [11].

Several medicinal plants have been analyzed for their biological activity and active constituents. One such medicinal plant is *Bacopa monnieri*, commonly known as Brahmi. Research has focused primarily on Bacopa's cognitive enhancing effects, specifically memory, learning and concentrations and the results support the traditional Ayurvedic claims. Not many studies have been done on the antioxidant and hepatoprotective effects of *Bacopa monnieri*. Hence, this study mainly focused on assessing the hepatoprotective effect of *Bacopa monnieri*.

MATERIALS AND METHODS

Chemicals

All reagents used in the experiment were of analytical grade and the kits used were procured from Span Diagnostics Limited, Sachin, India.

Collection of plant sample

The leaves of *Bacopa monnieri* were collected from the plants grown in pots in the University campus. They were washed thoroughly in running tap water in order to remove any dirt or soil particles adhered and blotted gently between folds of tissue paper to remove any water droplets.

Preparation of plant extract

Fresh leaves of *Bacopa monnieri* were collected and the methanolic extract of the leaves was prepared such that the final concentration was 500mg/ml. The methanol was evaporated and the residue was resuspended in water for gavage feeding.

Experimental design

Healthy male Wistar albino rats aged 6-8 weeks were procured from Small Animal Breeding Station, Trissur. The animals were randomly divided into seven groups of six animals each after an acclimatization period of two weeks. They were fed with standard pellet diet and 10% alcohol in drinking water *ad libitum*. The protocol was approved by the Institutional Animal Ethics Committee (623/02/b/CPCSEA).

Treatment groups

The oxidant used to induce stress *in vivo* was CCl₄, which is metabolized to the oxidative moiety by the cytochrome P450 2E1 (CYP2E1) isoenzyme. In the animals treated with oxidant, a 20 day pretreatment with alcohol (10%) in drinking water was given to induce CYP2E1 activity. CCl₄ was administered as a single subcutaneous injection (2.0ml/kg body weight) diluted 1:1 in paraffin oil on the 21st day. The methanolic extract of *Bacopa monnieri* was administered at a dose of 500mg/kg body weight. The experimental design was as follows:

Group I: Control

Group II: Alcohol

Group III: Alcohol + CCl₄

Group IV: Alcohol + methanolic extract of *Bacopa monnieri* leaves

Group V: Alcohol + methanolic extract of *Bacopa monnieri* leaves + CCl₄

Group VI: Alcohol + Silymarin

Group VII: Alcohol + Silymarin + CCl₄

Silymarin (a standard hepatoprotective antioxidant) was given at a dose of 25mg/kg body weight/day. Both the plant extracts and silymarin were administered by gastric intubation (gavage) for 21 days. After the treatment period of 21 days, the animals were sacrificed on the 22nd day by cervical dislocation.

The animals were quickly dissected and blood was collected by cardiac puncture. Serum was separated by centrifugation at 2000g at room temperature and plunged in cryovials at -85°C (Ilshin,

Korea). Serum samples were analyzed for liver marker enzymes and lipid profile.

Assay of serum marker enzymes

The marker enzymes for hepatic damage, namely aspartate transaminase (AST), alanine transaminase (ALT) [12], alkaline phosphatase (ALP) [13] and γ -glutamyl transpeptidase (γ -GT) [14], were estimated in the serum. All these enzymes were assayed using kits procured from Span Diagnostics Limited, Sachin, India.

Estimation of lipid profile

The levels of cholesterol [15], triglycerides [16], free fatty acids [17] and phospholipids [18] were estimated in the serum samples. The kits used for these assays were purchased from Span Diagnostics Ltd., Sachin, India.

Statistical analysis

All the parameters studied were analysed statistically using SigmaStat statistical package (Version 3.1). One way ANOVA with $P < 0.05$ was considered significant and, one way ANOVA followed by post-hoc Fischer analysis was done to test the levels of statistical significance.

RESULTS AND DISCUSSION

CCl₄ was used to induce oxidative stress in male Wistar rats. They were fed with alcohol to induce cytochrome P450 2E1 (CYP2E1), which enhances the oxidative damage caused by a subacute dose of CCl₄ injection. The animals were treated with the leaf extract and the effects in counteracting oxidative stress were studied, as explained in the methodology.

The extent of liver damage caused by alcohol-CCl₄ was assessed by estimating serum marker enzymes. The activities of AST, ALT (Table 1), ALP and γ -GT (Table 2) were estimated in the serum and the values are presented below.

On treatment with ethanol, a significant increase ($P < 0.05$) in the activities of all the four serum marker enzymes was observed, which was further augmented on CCl₄ administration. The treatment with the plant extract was effective in restoring the activities close to the normal levels. The effect of the extract was comparable to the standard antioxidant silymarin.

Cellular damage exhibits good correlation with enzyme leakage [19]. Serum AST, ALT, ALP and γ -GT are the most sensitive markers employed in the diagnosis of hepatic damage. The increase in the activities of these enzymes in serum and subsequent fall in the tissue might be due to the leakage of these cytosolic enzymes into the circulatory system, resulting from hepatocellular damage during ethanol administration. This is indicative of the onset of hepatocellular damage due to liver dysfunction and disturbance of the biosynthesis of these enzymes, with alteration in the permeability of liver membrane [20].

Table 1: Effects of *B. monnieri* leaf extract on the serum transaminase activities in oxidant challenged rats

Groups	AST activity (U/L)		ALT activity (U/L)	
	Without CCl ₄	With CCl ₄	Without CCl ₄	With CCl ₄
Alcohol	770.19 ± 10.04 ^a	948.36 ± 11.53 ^{ab}	47.41 ± 0.90 ^a	85.75 ± 1.42 ^{ab}
Leaf extract	471.31 ± 15.59 ^{ab}	528.29 ± 9.02 ^{bcd}	31.56 ± 2.08 ^b	38.53 ± 1.19 ^{abcd}
Silymarin	452.56 ± 13.20 ^{ab}	537.38 ± 9.17 ^{bcd}	31.31 ± 1.21 ^b	36.79 ± 1.23 ^{abcd}
LSD (5%)	18.960		2.347	

AST activity in untreated control: 519.0 ± 2.07 U/L

ALT activity in untreated control: 32.78 ± 1.0 U/L

Values are mean ± SD (n = 6)

Statistically significant ($P < 0.05$) compared to

a	-	Control
b	-	Alcohol treated group
c	-	Alcohol + CCl ₄ treated group
d	-	Plant extract / silymarin treated group

Table 2: Effects of *B. monnieri* leaf extract on the serum ALP and γ -GT activities in oxidant challenged rats

Groups	Serum ALP (U/L)		Serum γ -GT (U/L)	
	Without CCl ₄	With CCl ₄	Without CCl ₄	With CCl ₄
Alcohol	159.85 ± 2.21 ^a	235.69 ± 1.33 ^{ab}	33.66 ± 1.14 ^a	53.40 ± 2.84 ^{ab}
Leaf extract	110.62 ± 3.69 ^{ab}	131.42 ± 2.78 ^{bcde}	14.25 ± 1.84 ^b	21.56 ± 0.61 ^{abcde}
Silymarin	106.41 ± 1.66 ^{ab}	126.34 ± 4.07 ^{bcd}	13.33 ± 0.94 ^b	15.48 ± 0.83 ^{bc}
LSD (5%)	4.260		2.588	

ALP activity in untreated control: 127.66 ± 0.76 U/L

γ -GT activity in untreated control: 15.43 ± 0.78 U/L

Values are mean ± SD (n = 6)

Statistically significant (P < 0.05) compared to

a	-	Control
b	-	Alcohol treated group
c	-	Alcohol + CCl ₄ treated group
d	-	Plant extract / silymarin treated group
e	-	Silymarin + CCl ₄ treated group

The administration of *Rhoicissus tridentata* extracts after CCl₄ intoxication in rats resulted in significantly reduced concentrations of ALT and ASP [21]. CCl₄-induced hepatotoxicity in rats, as judged by the raised serum enzymes, was prevented by pretreatment with the aqueous and methanolic extracts of *Phyllanthus niruri*, demonstrating their hepatoprotective action [22]. The elevated serum enzymatic levels of AST, ALT, ACP and ALP in rats induced with liver toxicity using paracetamol were significantly restored towards normalization by pre-treatment with Chrysophanol and methanol fraction of *Cassia occidentalis* [23].

The partially purified petroleum ether extractable fraction of the whole plant *Aerva lanata* restored the elevated activities of liver marker enzymes against liver damage induced by carbon

tetrachloride (CCl₄) in Sprague Dawley rats [24]. Oral pretreatment with the chloroform extracts of *Terminalia catappa* significantly reduced the increased serum AST and ALT activities in CCl₄ treated rats [25]. The hydroalcoholic extract of the aerial part of *Cajanus cajan* showed a significant reduction in serum enzyme aspartate aminotransferase (AST) and alanine aminotransferase (ALT) against carbon tetrachloride induced liver damage in wistar rats [26].

SERUM LIPID PROFILE

The serum of the treated rats was analyzed to estimate the lipid profile. The parameters analyzed were total cholesterol, triglycerides, free fatty acids and phospholipids. The results are presented in Table 3 and 4.

Table 3: Effects of *B. monnieri* leaf extract on serum cholesterol and triglyceride levels in oxidant challenged rats

Groups	Serum cholesterol (mg/dl)		Serum triglycerides (mg/dl)	
	Without CCl ₄	With CCl ₄	Without CCl ₄	With CCl ₄
Alcohol	107.87 ± 0.85 ^a	156.35 ± 2.07 ^{ab}	174.18 ± 1.95 ^a	212.87 ± 4.50 ^{ab}
Leaf extract	65.51 ± 0.88 ^{ab}	76.44 ± 0.87 ^{abcd}	112.60 ± 5.17 ^{ab}	124.20 ± 2.59 ^{bcd}
Silymarin	62.55 ± 1.84 ^{ab}	76.42 ± 0.99 ^{abcd}	110.33 ± 7.55 ^{ab}	123.39 ± 5.83 ^{bcd}
LSD (5%)	2.264		8.080	

Levels of serum cholesterol in untreated control: 68.22 ± 0.88 mg/dl

Levels of serum triglycerides in untreated control: 130.35 ± 0.85 mg/dl

Values are mean ± SD (n = 6)

Statistically significant (P < 0.05) compared to

a	-	Control
b	-	Alcohol treated group
c	-	Alcohol + CCl ₄ treated group
d	-	Plant extract / silymarin treated group

Table 4: Effects of *B. monnieri* leaf extract on the serum free fatty acid and phospholipid levels in oxidant challenged rats

Groups	Serum free fatty acids (mg/dl)		Serum phospholipids (mg/dl)	
	Without CCl ₄	With CCl ₄	Without CCl ₄	With CCl ₄
Alcohol	204.17 ± 3.30 ^a	249.53 ± 4.04 ^a	181.73 ± 3.54 ^a	212.33 ± 3.24 ^{ab}
Leaf extract	121.31 ± 4.01 ^b	147.47 ± 2.42 ^{abcde}	97.11 ± 0.84 ^{ab}	119.57 ± 2.56 ^{abcde}
Silymarin	111.10 ± 3.06 ^{ab}	129.66 ± 2.85 ^{bcd}	95.26 ± 1.95 ^{ab}	111.85 ± 3.85 ^{abcd}
LSD (5%)	5.427		4.660	

Levels of serum free fatty acid in untreated control: 124.48 ± 0.79 mg/dl

Levels of serum phospholipid in untreated control: 106.50 ± 0.79 mg/dl

Values are mean ± SD (n = 6)

Statistically significant (P < 0.05) compared to

a	-	Control
b	-	Alcohol treated group
c	-	Alcohol + CCl ₄ treated group
d	-	Plant extract / silymarin treated group
e	-	Silymarin + CCl ₄ treated group

Ethanol treatment caused a significant increase in the levels of serum lipids. This was further augmented on exposure to a single assault of CCl₄. Treatment with the extract brought down the lipid levels even below the control. The standard antioxidant silymarin was also very effective in reducing the levels of the lipids to the control levels.

There was a significant increase in the levels of serum cholesterol, triglycerides, free fatty acids and phospholipids in the alcohol treated group and the levels were further increased in CCl₄ and alcohol treated group. This shows that these lipids are secreted from the liver at an increased rate. The administration of *B. monnieri* leaf extract was effective in counteracting the oxidative stress induced damage by decreasing the serum lipid levels of rats.

Our results are supported by a number of research findings, where plant extracts have exhibited hypolipidemic effects. Aqueous extract of propolis, a resinous wax-like beehive product, significantly reduced the levels of serum and tissue triglycerides, serum cholesterol, total and esterified cholesterol in rat tissue [27]. An aqueous extract of *Ajuva iva* lowered plasma cholesterol and triglyceride levels in streptozotocin induced diabetic rats [28]. An ethanolic extract of *Beta vulgaris* roots exhibited significant dose-dependent hepatoprotective activity against CCl₄-induced hepatotoxicity in rats, by showing a decline in the levels of serum markers, namely cholesterol and triglyceride [29]. The significant increase in the levels of cholesterol, triglycerides, phospholipids and free fatty acids in the liver and kidney, caused by the administration of streptozotocin in rats were brought down to normalcy on treatment with Aloe vera leaf gel [30].

CONCLUSION

In conclusion, it can be said that the levels of serum marker enzymes were elevated on treatment with alcohol and CCl₄. The administration of *B. monnieri* leaf extract caused a significant decrease in the activities of serum marker enzymes, indicating its protective effect, wherein no toxic effects were observed. Similar observations were made in the lipid levels also. The results of the present study prove the hepatoprotective effect of *B. monnieri* leaf extract and thus can render protection against alcohol-CCl₄ induced toxicity.

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