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Original Article

Anti-hepatotoxic effect of *Casuarina stricta* and *Casuarina suberosa* extracts on alcohol-induced liver toxicity in rats

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ABSTRACT: In recent years, there has been a global trend toward the use of natural phytochemicals present in natural resources, such as fruits, vegetables, oilseeds, and herbs, as antioxidants and functional foods. The aim of the present study was to investigate the protective and antioxidant effects of methanolic extract of *Casuarina stricta* and *Casuarina suberosa* leaves on ethanol induced hepatotoxicity in rats. The ethanol intoxication (1 ml of 40 % ethanol for 100 gm body weight for 6 weeks) to rats resulted in a significant increase in serum ALT, AST, γ glutamyl transferase (γ GT), hydroxyproline, MDA level and a significant decrease in serum albumin, total protein, A/G ratio, total antioxidant capacity, SOD, catalase activities and GSH level, $P < 0.05$. The treatment with *Casuarina stricta* and *Casuarina suberosa* extract at a dose of 250 mg/Kg body weight together with ethanol for 6 weeks successfully prevented the alterations of studied parameters in the treated groups. Both extracts significantly increased the total antioxidant capacity. The experimental results indicate that, both *Casuarina* extracts have excellent hepatoprotective effect.

KEYWORDS: *Casuarina stricta*, *Casuarina suberosa*, liver, antioxidants, ethanol

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INTRODUCTION

The Alcoholic liver disease (ALD) remains a serious health problem. ALD is a common consequence of prolonged and heavy alcohol intake. Changes fatal to the liver include fatty liver, hepatitis and hepatic cirrhosis (Matsuda *et al.*, 1991). Multiple mechanisms are likely to be involved in the pathogenesis of these problems; especially those associated with the toxic substances generated during alcohol metabolism. Accumulated evidence has demonstrated that both oxidative stress and abnormal cytokine production are important factors in the development of alcoholic liver damage (Bailey & Cunnigham, 1998; Bautista, 2001). Although much progress has been made in understanding the pathogenesis of alcoholic liver disease, there remains no effective therapy for this disease. In the absence of reliable drugs to protect the liver, herbs may play role in treating liver disorders.

In recent years, there has been a global trend toward the use of natural phytochemicals present in natural resources, such as fruits, vegetables, oilseeds, and herbs, as antioxidants and functional foods (Wang *et al.*, 1997; Kitts *et al.*, 2000; Farr, 1997). Many plants and herbal extracts demonstrate hepatoprotective activity; hence, many attempts have been made to formulate herbal preparations as functional foods (Choi *et al.*, 2006). Silymarin is an antihepatotoxic substance isolated from fruits of *Silybum marianum*. Possibly due to

their antioxidant and membrane stabilizing properties, the compounds have been shown to protect different organs and cells against a number of insults (Kvasnicka *et al.*, 2003).

The plant *Casuarina equisetifolia* Forst (in Bangladesh known as Jhau gachh, Hari) belongs to the family Casuarinaceae. Extracts of the leaves exhibited anticancer properties (Ahsan *et al.*, 2009). The bark is astringent and used against stomachache, diarrhea, dysentery, and nervous disorders (Jain & Dam, 1979). Seeds are anthelmintic, antispasmodic and antidiabetic (Chevallier, 1996). To the best of our knowledge there is no report on the biological activity of methanolic extract of *Casuarina stricta* and *Casuarina suberosa*. Thus the purpose of the present study is to investigate the protective effects of methanolic extract of *Casuarina stricta* and *Casuarina suberosa* leaves on alcohol induced hepatotoxicity in rats and also the phytochemical screening was carried out.

MATERIALS AND METHODS

Preparation of the extract

Samples of *Casuarina stricta* and *Casuarina suberosa* were purchased from El-Orman Garden, Ministry of Agriculture, Egypt. The dried leaves of *Casuarina stricta* and *Casuarina suberosa* (2 kg) were finely

powdered and exhaustively extracted with 100% methanol, by maceration at room temperature. The crude methanolic extract was evaporated to dryness under reduced pressure. The process of maceration and evaporation was repeated till exhaustion of the plants powder, and then the residues were combined and weighed.

Phytochemical screening of the extracts

Preliminary phytochemical screening for alkaloids, steroids, carbohydrates, tannins, fixed oils, proteins, triterpenoids, deoxysugar, flavonoid, cyanogenetic and coumarin glycosides carried out on the extract according to the procedures of Khandelwal (2006).

Experimental design

Animals and experimental protocol

Swiss albino mice (20-25 g) of either sex were used for the acute study LD50 of methanolic extract of the plant under investigation was determined according to Behrens & Karber (1970). For the acute toxicity study, it was found that the tested extracts were not lethal even at a dose of 4000 mg/kg and consequently the dose 250 mg/kg was selected for the study.

Forty eight male Wister albino rats weighing (150-200) g were used for this study. The animals were housed in a temperature (25 ± 1°C), humidity controlled room and a 12-hour light-dark cycle (lights on at 0600 h). Rats were allowed free access to tap water and standard pellet diet. The institutional Animal Ethics Committee approved all experimental protocols. The animals were classified into eight groups of six as follows: Control group (1): Rats received distilled water; Ethanol-treated group (2): Rats received 40 % ethanol (1-ml/100g body weight) for 6 weeks; Ethanol and *Casuarina stricta* extract treated group (3): Rats received 40% ethanol (1 ml/100g body weight) and *Casuarina stricta* extract at a dose of 250 mg/kg for 6 weeks; *Casuarina stricta* extract treated group (4): Rats received *Casuarina stricta* extract at a dose of 250 mg/kg for 6 weeks; Ethanol and *Casuarina suberosa* extract treated group (5): Rats received 40% ethanol (1ml/100 g body weight) and *Casuarina suberosa* extract at a dose of 250 mg/kg for 6 weeks; *Casuarina suberosa* extract treated group (6): Rats received *Casuarina suberosa* extract at a dose of 250 mg/kg for 6 weeks; Ethanol and Silymarin treated group (7): Rats received 40 % ethanol (1ml/100 g body weight) and Silymarin at a dose of 50 mg/Kg for 6 weeks; Silymarin treated group (8): Rats received Silymarin at a dose of 50 mg/kg for 6 weeks. In each of the treatment groups, all treatments were orally administered.

Blood collection and biochemical assays

Fasting blood samples were withdrawn from the retro-orbital vein of each animal using a glass capillary tube after fasting period of 12 hours. The blood samples allowed to coagulate and then centrifuged at 3000 rpm for 20 min. The separated sera were used for the estimation of serum levels of ALT, AST, albumin, total protein, γ glutamyl transferase (γ GT), by using commercial kits (Quimica Clinica Aplicada, Spain). Total serum antioxidant capacity was determined using commercial kits purchased from Biodiagnostic, Egypt. Serum hydroxyproline was assayed using the

method described by Dabev & Struck (1971). Serum bile acid was determined according to the method of Mashige *et al.* (1976).

Liver was removed immediately after decapitation and rinsed with cold ice saline to remove excess blood. A portion of liver was quickly weighed, minced and homogenized in ice cold medium as 10 % W/V homogenate according to the measured parameter. The homogenates were then centrifuged at 5000 x g and below zero temperature for 10 minutes and the supernatant were used for the assays of SOD, GSH, catalase and The MDA level, the end product of lipid peroxidation, was measured in the homogenates using commercial kits purchased from Biodiagnostic, Egypt.

Statistical analysis

The data are represented as Mean ± S. E.M., and statistical significance was done using student's 't' test and one way analysis of variance (ANOVA) followed by LSD test. P-values <0.05 were considered as statistically significant.

RESULTS

Table 1 shows the summary of the phytochemical analysis of methanolic extract of *Casuarina stricta* and *Casuarina suberosa*.

Effect of extracts on serum ALT, AST, γ GT, hydroxyproline and bile acids

Extracts of *Casuarina stricta* and *Casuarina suberosa* had no effect either on serum enzyme activities, or hydroxyproline and bile acids compared to control rats indicating its safety. Ethanol administration significantly elevated the enzymatic activities as well as hydroxyproline as compared to control animals. Treatment with both extracts as well as Silymarin (reference drug) together with ethanol notably decreased the enzyme activities and hydroxyproline when compared to ethanol -treated group, p <0.05. Neither extract nor ethanol administration had effect on serum bile acids concentration.

TABLE 1 Phytochemical analysis of methanolic extract of *Casuarina stricta* and *Casuarina suberosa*

Chemical group	<i>Casuarina stricta</i>	<i>Casuarina suberosa</i>
Tannins	+++	++++
Flavonoids	+++	++++
Saponins	-	-
Alkaloids	++	+++
Phenol	+++	++++
Terpenoids	++	++
Steroids	++	++

Effect of extracts on serum albumin, total protein, A/G ratio and total antioxidant capacity

Extracts of *Casuarina stricta* and *Casuarina suberosa* had no effect on serum albumin and total serum protein. Both extracts significantly increased total serum antioxidant capacity. In ethanol-treated group there was a significant decrease in serum albumin, total serum protein and total serum antioxidant capacity when compared to their corresponding control values, $p < 0.05$. Treatment with both extracts as well as Silymarin (reference drug) revealed a significant rise in serum albumin, total serum protein and total serum antioxidant capacity when compared to ethanol-treated group, $p < 0.05$ (Table 2 and Table 3).

Effect of extracts on liver tissue SOD, catalase, GSH and MDA

In ethanol-treated group there was a significant decrease in SOD, catalase activities and GSH level and a significant rise in MDA level when compared to their corresponding control values, $p < 0.05$. Treatment with both extracts as well as Silymarin (reference drug) revealed a significant rise in SOD, catalase activities and GSH level and a significant decrease in MDA level when compared to ethanol-treated group values, $p < 0.05$ (Table 4).

DISCUSSION

The present study was conducted to evaluate the protective effect of alcoholic extract of *Casuarina stricta* and *Casuarina suberosa* leaves against ethanol induced hepatic disorders in rats. Results suggest that both extracts possess protective and antioxidant action against hepatic dysfunctions induced by alcohol when administered once daily at a dose of 250 mg/kg body weight for 6 weeks together with the alcohol.

In the present study, the administration of alcohol increased the mean values of liver enzymes (ALT, AST and γ GT). These results were in agreement with Rajakrishnan and Menon (2001). The authors reported that exposure of hepatocytes to ethanol alters the membrane structure and functions by increasing the leakage of enzymes into the circulation. It has been reported that excess alcohol consumption has been linked with altered liver metabolism and liver damage, with leakage of cytoplasmic liver enzyme γ GT into blood (Das *et al.*, 2005). Also, in this study, there was a significant decrease in serum total proteins, albumin and A/G ratio in ethanol group. These results were in agreement with Ahmed *et al.* (2002) who found a decrease in serum total proteins and albumin in ethanol-administered rats and the author suggested that was due to the decrease in the functional ability of liver in ethanol-administered rats. Also, the decrease in A/G ratio is a predictor of a bad outcome and poor health.

Chronic intoxication with ethanol is probably the most common cause of liver fibrosis. Chronic alcohol consumption has hepatotoxic effects on human beings. It causes steatosis, alcoholic hepatitis, fibrosis and cirrhosis (Bankowski, 1994). Experimental and clinical data suggest that acetaldehyde, which is an intermediary product of ethanol oxidation, is responsible for stimulation of collagen biosynthesis in the liver. Liver fibrosis is accompanied by a significant increase in collagen content in this

organ (Bankowski, 1994; Matsuda *et al.*, 1997). Free radicals are involved in various human diseases that can possibly be prevented by antioxidants (Chatterjee *et al.*, 2005).

Exposure of living organisms to a constant generation of reactive oxygen species (ROS) resulting in the development of antioxidative defense systems which protect cells and tissues against their harmful effects. The efficiency of enzymatic and non-enzymatic antioxidative systems could be detected by the determination of single components of this system or by so-called total antioxidant capacity (TAC) (Kankofer *et al.*, 2005). In the present study, a significant decrease in serum total antioxidants in ethanol treated rats was observed. This is in agreement with Masalkar *et al.* (2005). The author found a decrease in antioxidant status in alcoholic patients and showed that increased generation of free radicals and deficiencies of dietary antioxidants can be important etiological factor in alcoholic liver disease.

The results of the present study revealed a significant increase in serum level of hydroxyproline in ethanol treated group. The alteration of hydroxyproline levels in serum is considered as an index of collagen metabolism and provides valuable information about the biochemical and pathologic events of hepatic fibrosis. Significantly increased levels of serum hydroxyproline have been reported in patients with alcoholic liver cirrhosis (Mata *et al.*, 1975) and chronic liver diseases (Yamada & Hiyarima, 1983). The results of the present study revealed a significant decrease in tissue GSH level, SOD activity and a significant increase in liver MDA level. These results were in agreement with Singanan *et al.* (2007). The level of SOD was decreased in ethanol intoxicated rats. This is due to the low level of Zinc (a metal constituent of the enzyme SOD) in plasma and liver tissues (Reding *et al.*, 1984) The low level of zinc was also found in alcoholic liver cirrhosis (Henkin & Smith, 1972). The GSH depletion in hepatic mitochondria is considered the most important sensitizing mechanism in the pathogenesis of alcoholic liver injury (Singanan *et al.*, 2007).

The MDA assay is the most popular method of estimation of malondialdehyde level, which is an indication of lipid peroxidation and free radical activity. The increase in lipid peroxidation, a degradative process of membrane polyunsaturated fatty acid has been suggested by the increase in malondialdehyde in ethanol induced toxicity in the liver. The increased lipid peroxidation results in changes in cellular metabolism of the hepatic and extra hepatic tissues, which ultimately leads to the whole cell deformity and cell death (Winrow *et al.*, 1993).

In the present study, there was a significant decline in catalase activity in ethanol-treated group as compared to their corresponding normal controls. This result was in agreement with that of Krishnamoorthy (2012). The author found that catalase activity was found to attain a near normal level in liver of ethanol and plant extract combined treated rats. Also the author reported that decline in the catalase activity of liver in ethanol-administered rats revealed the oxidative stress elicited by ethanol intoxication. The supplementation of plant extract to the alcohol-fed animals recorded an elevated level of activities and revealed a better antioxidant defense status in the liver tissue when compared to that of control animals (Krishnamoorthy, 2012).

TABLE 2 Effect of treatment of methanolic extracts of *Casuarina stricta* and *Casuarina suberosa* on serum ALT, AST, γ GT, albumin and total protein.

Group	SALT U/l	SAST U/l	γ GT U/l	Albumin g/dl	Total protein g/dl	Globulin	A/G Ratio
(1)	28.0 \pm 1.35	46.4 \pm 2.6	3.03 \pm 0.29	3.50 \pm 0.22	6.45 \pm 0.25	2.95 \pm 0.076	1.18 \pm 0.092
(2)	39.0 \pm 3.38*	65.1 \pm 2.63*	10.47 \pm 0.64*	2.35 \pm 0.19*	4.98 \pm 0.26*	2.63 \pm 0.038*	0.89 \pm 0.051*
(3)	26.7 \pm 1.99 @	51.6 \pm 1.3 @	4.82 \pm 0.32* @	3.43 \pm 0.12 @	6.29 \pm 0.22 @e	2.86 \pm 0.042	1.19 \pm 0.087 @
(4)	24.5 \pm 2.60 @	50.8 \pm 0.75 @	2.98 \pm 0.34 @b	3.85 \pm 0.37 @	6.80 \pm 0.24 @	2.95 \pm 0.3	1.30 \pm 0.046 @
(5)	30.4 \pm 2.27 @	52.0 \pm 1.89 @	3.00 \pm 0.35 @ b	3.24 \pm 0.14 @	5.92 \pm 0.3 @e	2.68 \pm 0.17	1.20 \pm 0.016 @
(6)	28.7 \pm 2.5 @	46.5 \pm 3.1 @	2.89 \pm 0.26 @ b	3.4 \pm 0.1 @	6.27 \pm 0.3 @e	2.87 \pm 0.14	1.18 \pm 0.026 @
(7)	24.0 \pm 1.39 @	49.5 \pm 2.2 @	3.66 \pm 0.12 @ b	4.00 \pm 0.28 @	6.96 \pm 0.16 @d	2.96 \pm 0.146	1.35 \pm 0.031 @* f d
(8)	28.6 \pm 1.92 @	47.2 \pm 2.9 @	2.89 \pm 0.26 @ b	3.89 \pm 0.29 @	7.00 \pm 0.23 @d	3.11 \pm 0.02 * @	1.25 \pm 0.018 @* f d

TABLE 3 Effect of treatment of methanolic extracts of *Casuarina Stricta* and *Casuarina Suberosa* on total serum antioxidant capacity, hydroxyproline and bile acids.

Group	Total antioxidant capacity	Hydroxyproline	Bile acids
	mM / L	µg/ml	µmol/L
(1)	1.7 ± 0.03	1.13 ± 0.065	14.12 ± 1.08
(2)	1.09 ± 0.06*	1.91 ± 0.051*	16.13 ± 1.57
(3)	2.11 ± 0.07* [@] _c	1.3 ± 0.027* [@]	15.79 ± 0.4
(4)	2.21 ± 0.04* [@] _c	1.16 ± 0.013 [@] bc	14.67 ± 0.9
(5)	2.2 ± 0.02* [@] _c	1.13 ± 0.059 [@] bc	14.85 ± 0.72
(6)	2.18 ± 0.035* [@] _c	1.25 ± 0.008 [@] c	15.2 ± 0.27
(7)	1.91 ± 0.093 [@]	1.41 ± 0.059* [@] b	15.7.13 ± 1.24
(8)	2 ± 0.06* [@]	1.22 ± 0.019 [@] c	13.78 ± 0.52

Results are expressed as mean ± S.E.

*Significantly different from control group

[@] Significantly different from ethanol treated group (2)

b: Significantly different from ethanol + *Casuarina stricta* treated group (3).

c: Significantly different from ethanol + Silymarin treated group (7).

TABLE 4 Effect of treatment of methanolic extracts of *Casuarina Stricta* and *Casuarina Suberosa* on liver homogenate SOD, catalase activities, GSH and MDA levels.

Group	SOD activity	Catalase activity	GSH level	MDA level
	U/mg protein	U/mg protein	µg/g Wt tissue	mg/gWt tissue
(1)	28.2 ± 1.29	0.68 ± 0.048	3.42 ± 0.24	17 ± 1
(2)	19.2 ± 1.56*	0.41 ± 0.034*	2.69 ± 0.15*	24.8 ± 1.6*
(3)	36.3 ± 1.0 * [@]	0.58 ± 0.037 [@]	4.85 ± 0.5* [@]	16.46 ± 0.8 [@] _{dl}
(4)	34.7 ± 1.8 * [@]	0.76 ± 0.032 [@] _{cdjfg}	4.59 ± 0.45* [@] _{et}	13.65 ± 0.65* [@]
(5)	34.3 ± 2.1 * [@]	0.53 ± 0.05 [@]	4.78 ± 0.43* [@]	12.18 ± 1.7* [@] _b
(6)	36.3 ± 1.8* [@]	0.64 ± 0.013 [@]	6.62 ± 0.62* [@] _{bd}	12.88 ± 1.2* [@]
(7)	38.3 ± 1.7* [@]	0.61 ± 0.035 [@]	4.91 ± 0.32* [@]	13.27 ± 1.23* [@]
(8)	34.7 ± 1.7 * [@]	0.6 ± 0.049 [@]	6.2 ± 0.74* [@] _d	13.68 ± 0.59* [@]

Results are expressed as mean ± S.E.

*Significantly different from control group.

[@] Significantly different from ethanol treated group (2).

b: Significantly different from ethanol + *Casuarina stricta* treated group (3).

d: Significantly different from ethanol + *Casuarina suberosa* treated group (5).

e: Significantly different from Silymarin treated group (8).

f: Significantly different from *Casuarina suberosa* treated group (6).

g: Significantly different from ethanol and Silymarin treated group (7).

Preliminary phytochemical screening of *Casuarina stricta* and *Casuarina suberosa* extract revealed the presence of alkaloids, terpenoids, flavonoids, polyphenols, steroids and tannins. The antioxidant activity of *Casuarina stricta* and *Casuarina suberosa* extracts could be attributed to its flavonoidal content. Flavonoids act as scavengers of various oxidizing species i.e. super oxide anion ($O_2^{\bullet-}$), hydroxyl radical or peroxy radicals, they also act as quenchers of singlet oxygen (Das & Ratty, 1986). Numerous plant constituents have proven to show free radical scavenging or antioxidant activity (Aruoma & Cuppett, 1998). Phenols are very important plant constituents. There is a highly positive relationship between total phenols and antioxidant activity of many plant species, because of the scavenging ability of their hydroxyl groups. It was also reported that phenolic compounds are effective hydrogen donors, making them very good antioxidants (Vinson, 1998).

Casuarina stricta and *Casuarina suberosa* extracts contain a group of phenolic compounds known as tannins are significant plant secondary metabolites. These compounds are greatly varied in their structures and concentration within and among plant species. Various studies have demonstrated that tannins from plants exhibit hepatoprotective efficacy (Bagchi *et al.*, 2000; Shimoda *et al.*, 2008). *Casuarina suberosa* extract appears to be superior in that successfully prevented the alterations of studied parameters in the treated groups.

In conclusion, *Casuarina suberosa* extract greatly changed the biochemical parameters in the ethanol intoxicated rats and maintained well to the normal level. These results clearly suggest that, the *Casuarina suberosa* have enormous hepatoprotective value. The herbal extract has equivalent therapeutic value with the standards drug Silymarin. Further, this study creates a hope on new drug discovery in controlling liver diseases using *Casuarina suberosa* as precursor.

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