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Research Article

PROPHYLACTIC EFFECTS OF *FLAVERIA TRINERVIA* EXTRACT AGAINST ETHANOL INDUCED HEPATOTOXICITY USING RATS

JOY HOSKERI H.[1], KRISHNA V.[1,*] RAMESH BABU K.[2], BADARINATH D. K.[3]

[1]P.G. Department of Studies and Research in Biotechnology and Bioinformatics, Kuvempu University, Shankaraghatta 577451, Karnataka, India, [2]Department of Pathology, Shimoga Institute of Medical Sciences, Shimoga 577201, Karnataka, India, [3]Department of Biotechnology, The Oxford College of Science, Bangalore 560102, India. Email: krishnabiotech2003@gmail.com

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ABSTRACT

The prophylactic effects of methanol extract of *Flaveria trinervia* was evaluated against ethanol induced hepatotoxicity using rats. Methanol extract at three different doses (50, 75 and 100 mg/kg) was administered orally to the ethanol treated animals during the last week of the 7 weeks study. Silymarin was used as the standard reference. Methanol extract up to the dose of 500 mg/kg did not show any sign of mortality. Biochemical tests with respect to the hepatotoxicity markers in support with the histopathological examination of rat the liver sections were performed. The substantially elevated serum enzymatic levels of serum aspertate transaminase, serum alanine transaminase, alkaline phosphatase and total bilirubin in ethanol treated animals were restored towards normalcy by the treatment of methanol extract. *In vivo* antioxidant and *in vitro* free radical scavenging activities were also positive for all the three doses of methanol extract. However, 100 mg/kg of methanol extract showed significant activity when compared to the other two doses. Biochemical observations in support with histopathological examinations revealed that the *F. trinervia* plant possesses a potent hepatoprotective effect against ethanol induced hepatic damage in rats.

Keywords: *Flaveria trinervia*, Antioxidant activity, Hepatoprotective activity, Histopathology, Alcoholic hepatotoxicity

INTRODUCTION

Alcoholic liver disorders are the major cause of morbidity and mortality in the world. Although current therapies offer a tantalizing glimpse into a future that may include therapies that directly alter the process of liver injury or repair. None has been shown consistently improve the course of alcoholic liver damage. Consequently, liver transplantation remains an ultimate option for selected patients with liver failure due to chronic alcoholic consumption[1]. Much of the cell damage that occurs during liver degeneration is believed to be caused by the free radicals or highly reactive oxygen species liberated during alcohol metabolism. These free radicals react with the cell membrane and induced lipid peroxidation that has been implicated as an important pathological mediation in many of the clinical disorders [2].

Plants are the richest source of novel chemical compounds. In indigenous system of medicine, several plants are known to act as potent hepatoprotective drugs for jaundice and their therapeutic property were evaluated using animal models by many investigators *viz*, *Berberis aristata* [3]; *Luffa echinata* [4]; *Cassia angustifolia* [5]; *Cnidium monnieri* [6]; *Emblica officinalis* [7]; *Pergularia daemia* [8].

Several herbal products are available all over the world with an acclaimed hepatoprotective activity that are considered to be less toxic and free from side effects. *Flaveria trinervia* Spring C. Mohr (Asterecae) plant that grow only in alkaline soil [pH 7.2-8.2], mainly in the marshy land near Chitradurga Dist, Karnataka State, India. This plant is locally referred as Bellary halabu or katthe kivi gida. Traditionally it is used as a promising drug for curing alcoholic liver disorders in Karnataka state, India [9]. To justify the ethnomedical claims, methanol extract of *F. trinervia* plant was screened for its prophylactic effects against ethanol induced hepatotoxicity.

MATERIAL AND METHODS

Drugs and chemicals

Distilled ethanol was obtained from Shamsons Distilleries Pvt. Ltd., Duggavathi, Davanagere Dist, Karanataka, India. Methanol (Merck, India). Silymarin (Micro Labs, India).

Plant resource

Flaveria trinervia herb was collected from the agricultural fields near by Chitradurga city of Karnataka State, India. This plant was authenticated by Dr. Manjunatha by comparing with the voucher specimen deposited at the Kuvempu University herbarium specimen FDD-No. 53 [9].

Isolation and phytochemical investigation

The fresh whole plant material was shade dried, porously powdered mechanically and was subjected to soxhlet extraction for about 48 h using methanol as the solvent system. Extract was filtered and concentrated in vacuum under reduced pressure using rotary flash evaporator (Buchi, Flawil, Switzerland) and was allowed for complete evaporation of the solvent and vacuum dried. The yield of methanol crude extract for 1 kg of powdered whole plant material was 32.5 g.

Animals

Male Wistar albino rats weighing 150-200 g were procured from Venkteshwara traders, Bangalore and were maintained at standard housing condition. Animals were fed with commercial diet (Pranav Agro Industries Ltd., Sangli) and water *ad- libitum* during the experiment. The institutional animal ethical committee (Reg.No.SETCP/IAEC/2010-2011/165) permitted us to carry out this study.

The staircase method was adopted for the determination of the acute toxicity [10]. Healthy Wistar albino rats of either sex weighing 150-200 g were used to determine the safer dose. 5% DMSO (v/v) was used as a vehicle to suspend the extract and was administered orally.

Experimental design for *in-vivo* antioxidant and hepatoprotective activity

Thirty six female rats were randomly divided into six groups of six animals in each group. All the group animals expect group 1 were fed with the ethanol containing liquid diet by incrementing the ethanol content from 0 - 30% for one week, this dose was progressively increased to 35% ethanol for one week followed by 40% of the caloric content for 5 more weeks [11]. Group 1 animals were adapted to ethanol free liquid diets over the same period. Thereafter, animals were maintained on 0% or 40% ethanol-containing diets throughout the study. Rats were monitored daily to

ensure adequate nutritional intake and maintenance of body weight [11-16].

During the last week of the study, group 1 rats were maintained as control by administering 5% DMSO orally. Group 2 rats were fed with only ethanol containing diet daily. Group 3, 4 and 5 rats were treated with suspension of methanol extract at the dose of 50 mg/kg (ME1), 75 mg/kg (ME2) and 100 mg/kg (ME3) respectively, prepared in 5% DMSO. Group 6 rats were treated with the standard drug silymarin at the dose of 250 mg/kg, b. w. orally once daily. Both, methanol extract and the drug were administered to the animals after an interval of 3 h after the administration of ethanol during the last week of 7 week study. This treatment was slightly modified as reported by Billy et al., 2009 [11]. Animals were sacrificed 24 h after the last treatment. Blood was collected into a sterilized centrifuge tube, allowed to clot and serum was separated at 2500 rpm for 15 min.

Estimation of biochemical parameters

The activity levels of hepatospecific marker enzymes *viz.*, serum aspertate transaminase (AST), serum alanine transaminase (ALT) were estimated by the method reported by Reitman and Frankel (1957) [17]. The activity level of alkaline phosphatase (ALP) in serum was estimated by the method of King (1965) [18] and total bilirubin by method reported by Malloy et al., 1937 [19].

1,1-diphenyl 2-picryl hydrazyl (DPPH) scavenging activity

The free radical-scavenging activity of methanol extract was measured in terms of hydrogen donating or radical-scavenging ability using the stable radical DPPH [20]. Solution of DPPH (0.1 mmol) in ethanol was prepared and 1.0 mL of this solution was added to 3.0 mL of solution with different concentrations of methanol extract solution (100–1000 µg/mL) in distilled water. After thirty minutes of reaction absorbance was measured at 517 nm. Lower the absorbance of the reaction mixture indicates higher the free radical-scavenging activity. Ascorbic acid was used as the standard drug. The percentage inhibition of DPPH was calculated. The results were presented graphically (percentage inhibition values *vs* concentration) and the IC_{50} values were estimated.

Estimation of lipidperoxidase, peroxidases, SOD, CAT and TBARS

Liver samples were dissected out of each animal and was washed immediately with ice cold phosphate buffer saline to remove as much blood as possible. Liver homogenates (5% w/v) were prepared in cold 50 mM potassium phosphate buffer (pH 7.4) using a glass homogenizer. The unbroken cells and cell debris were removed by refrigerated centrifugation at 3000 rpm for 10 min. The supernatant was used for the estimation of lipid peroxidases [21], peroxidase [22], superoxide dismutase (SOD) [23] and catalase activity [24]. The antioxidant status was assessed from the levels of malondialdehyde (MDA) an end product of lipid peroxidation [25]. As 99% of the thiobarbituric acid reactive substance is malondialdehyde (MDA), thiobarbituric acid reactive substance concentrations of the samples were expressed as nmol of MDA/g of wet tissue using molar extinction coefficient of the chromophore (1.56×10^{-5} M^{-1} cm^{-1}) [26].

Histopathological studies

The liver tissue was dissected out from the animals of each group after draining the blood and washed with the normal saline and fixed in 10% formalin, dehydrated in gradual ethanol grades (50–100%), cleared in xylene and embedded in paraffin. Sections of 5 µm thickness were prepared, processed in alcohol-xylene series and were stained with alum-haematoxylin and eosin (H–E) dye for photomicroscopic observation for the evaluation of histological changes, including cell necrosis, fatty change, tissue architecture [27].

Statistical analysis

The data of biochemical estimations were expressed as mean ± S.E.M. of six animals in each group. The statistical analysis was carried out using one way ANOVA followed by Tukey's *t*-test. The difference in values at $P \leq 0.01$ was considered as statistically significant.

RESULTS

Hepatoprotective studies

After 72 h observation, methanol extract up to the dose of 500 mg/kg did not show any sign of mortality. One tenth of this dose was considered as safer dose for administration. Administration of the toxic dose of ethanol to the animals resulted in a marked increase in the levels of serum AST (421.78 ± 5.95 IU/L), ALT (209.02 ± 7.38 IU/L), ALP (417.67 ± 7.43 IU/L), total bilirubin (11.42 ± 0.97 mg/dL) and direct bilirubin (1.95 ± 0.09 mg/dL). On the contrary, the blood samples of the animals treated with the three different doses of methanol extract exhibited significant hepatoprotective activity by ameliorating the increase in serum AST and ALT levels (Table 1). Among the treated groups the extent of liver damage was lesser in magnitude in the animals treated with methanol extract at the dose of 100 mg/kg.

Table 1: *In vivo* hepatoprotective effect of extract of *Flaveria trinervia* on ethanol induced hepatotoxicity

Group	Treatment	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	Total bilirubin (mg/dL)	Direct bilirubin (mg/dL)
Group I	Control	186.15 ± 2.24**	80.98 ± 3.56**	165.22 ± 4.98**	1.5 ± 0.17**	0.47 ± 0.15**
Group II	Ethanol-intoxicated	421.78 ± 5.95	209.02 ± 7.38	417.67 ± 7.43	11.42 ± 0.97	1.95 ± 0.09
Group III	ME1	270.4 ± 19.83**	172.33 ± 2.89**	389.82 ± 4.33**	8.7 ± 0.35*	1.52 ± 0.13*
Group IV	ME2	283.58 ± 2.29**	159.92 ± 3.27**	363.03 ± 8.22**	9.55 ± 0.52ns	1.25 ± 0.13**
Group V	ME3	217.17 ± 4.8**	154.33 ± 4.05**	359.78 ± 6.08**	5.62 ± 0.57**	1.47 ± 0.14*
Group VI	Silymarin + Ethanol	201.37 ± 2.71**	133.5 ± 4.27**	231.08 ± 2.36**	2.4 ± 0.25**	1.17 ± 0.13**

Values are the mean ± S.E.M. of six rats. Symbols represent statistical significance. *$P < 0.05$, ** $P < 0.01$, ns: not significant, as compared to ethanol-intoxicated group.

ME1 = 50 mg/kg methanol extract; ME2 = 75 mg/kg methanol extract; ME3 = 100 mg/kg methanol extract.

DPPH-scavenging activity in cell free system

Figure 1 depicts the significant decrease in the concentration of DPPH radical due to scavenging ability of methanol extract. A plot of percentage inhibition versus concentration showed that the IC_{50} value of methanol extract was 706.67 µg/mL. Where as, the IC_{50} value of ascorbic acid was found to be 183.33 µg/mL.

Effects of extract on lipidperoxidase, peroxidases, SOD, CAT and TBARS levels

The lipidperoxidase, superoxide dismutase, catalase and peroxidase levels have been significantly increased in the animals groups treated with the different doses of methanol extract (Table 2). Whereas, ethanol treated group showed decrease in levels of antioxidant enzymes when compared to the control group. All the doses of methanol extract showed significant *in vivo* antioxidant activity by increasing the levels of antioxidant enzymes. However, among the treated groups, animals treated with 100 mg/kg of methanol extract showed maximum protection than 50 mg/kg and

75 mg/kg treated animals. In order to probe the possible mechanism by which methanolic extract prevented the hepatic damage caused by ethanol administration, investigation on levels of thiobarbituric acid reactive substance revealed that the ethanol treatment caused a significant increase in thiobarbituric acid reactive substance. This effect was significantly (P<0.01) reversed by the treatment of methanol extract. There was a significant depletion in the level of the thiobarbituric acid reactive substance (3.05 ± 0.17). The effect of methanol extract at the dose of 75 mg/kg was comparable to that of the standard drug silymarin. Table 2 depicts the changes in the levels of thiobarbituric acid reactive substance due to the effects of methanolic extract.

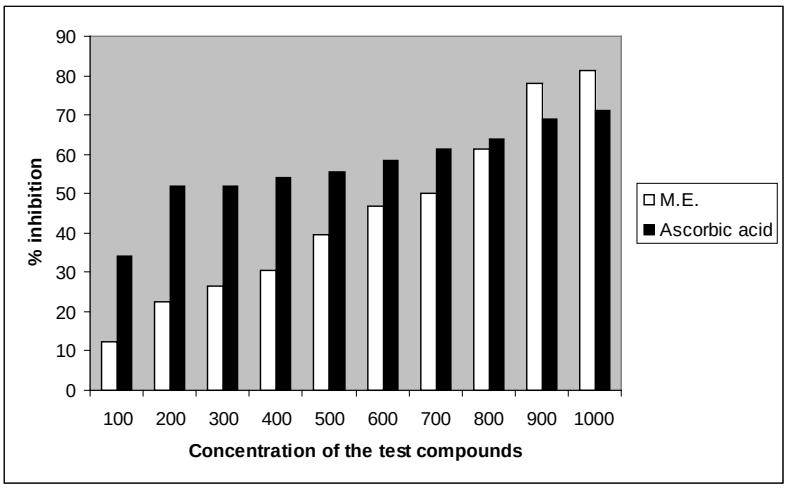


Fig. 1: *In vitro* antioxidant assay using DPPH radical

In vitro antioxidant activity was measured as IC_{50} by comparing with the ascorbic acid. M.E. = Methanol extract.

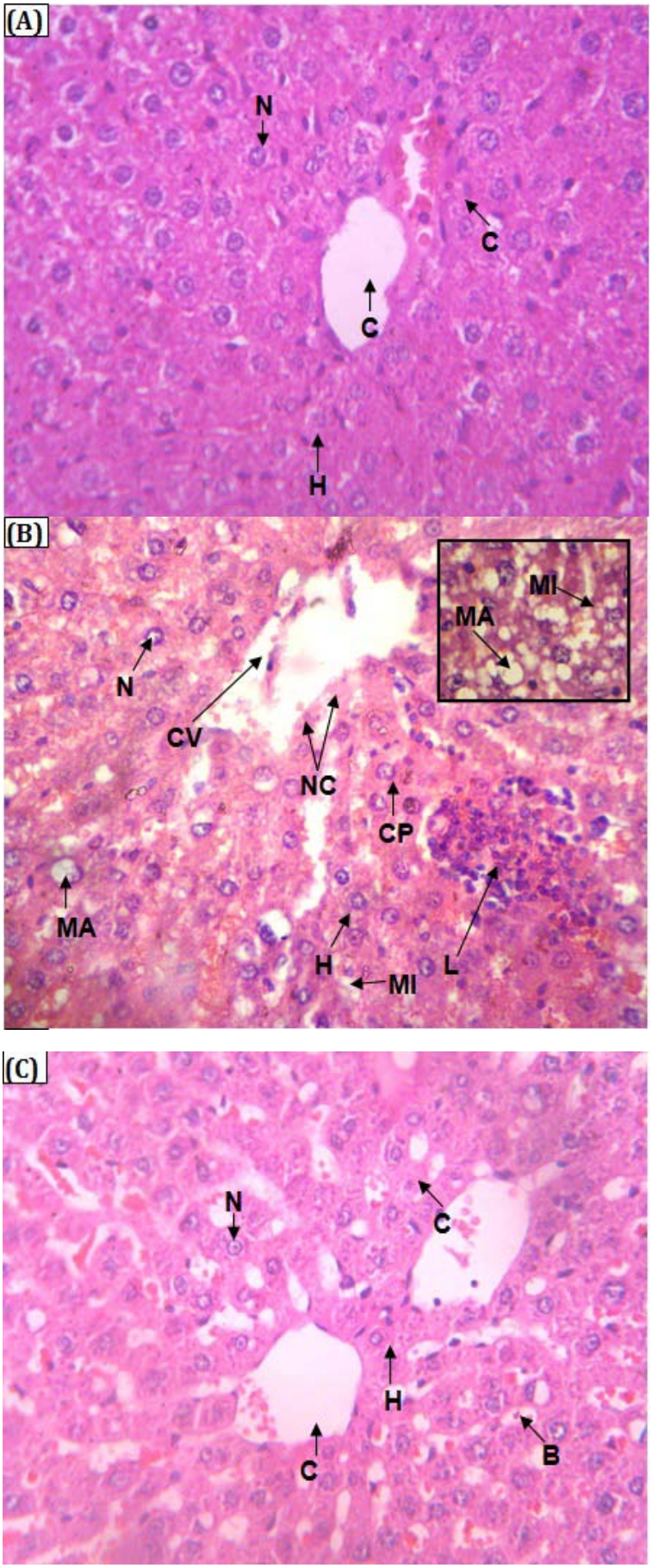
Table 2: *In vivo* antioxidant activity of extract of *Flaveria trinervia* on ethanol induced hepatotoxicity

Group	Treatment	Lipid peroxidases (IU/mg)	SOD (IU/mg)	CAT (IU/mg)	Peroxidases (IU/mg)	TBARS(nmol MDA/g of wet tissue)
Group I	Control	44.4 ± 3.61**	15.13 ± 1.47**	93.18 ± 5.81**	18.32 ± 6.49**	2.98 ± 0.19**
Group II	Ethanol	22.83 ± 2.84	6.62 ± 1.32	39.22 ± 5.79	4.95 ± 3.03	4.35 ± 0.13
Group III	ME 1	26.08 ± 1.54*	7.77 ± 0.13*	46.53 ± 1.52**	7.3 ± 0.26**	3.39 ± 0.03*
Group IV	ME 2	27.73 ± 0.73*	7.37 ± 1.5*	47.57 ± 2.22**	6.58 ± 1.23*	3.05 ± 0.17**
Group V	ME 3	33.15 ± 3.85**	7.92 ± 0.25**	47.93 ± 3.89**	10.28 ± 0.64**	3.69 ± 0.11*
Group VI	Silymarin + Ethanol	33.85 ± 0.94**	9.55 ± 1.2**	67.85 ± 7.24**	15.7 ± 5.72**	3.15 ± 0.9**

Values are the mean ± S.E.M. of six rats. Symbols represent statistical significance. *P < 0.05, ** P < 0.01, ns: not significant, as compared to ethanol-intoxicated group. ME1 = 50 mg/kg methanol extract; ME2 = 75 mg/kg methanol extract; ME3 = 100 mg/kg methanol extract.

Histopathological observations

Histopathological study of the liver sections of control animals (group 1) showed intact tissue architecture with normal hepatocytes and well-preserved cytoplasm with no lymphocyte infiltration. Prominent nucleus, nucleolus and well formed clearly visible central veins were observed. The liver sections of ethanol-intoxicated rats exhibited centrilobular necrosis with deformed tissue architecture, micro and macro vesicular fatty changes with lymphocyte infiltration were observed. The liver sections of ME1 treated animals showed slight ballooning in the tissue, lack of necrotic cells, normal hepatic architecture with moderate accumulation of fat droplets was observed in the hepatocytes.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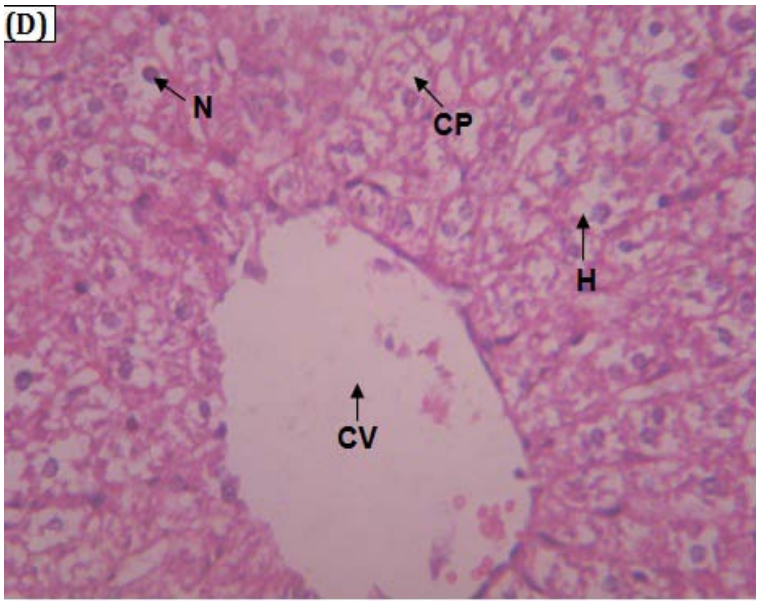
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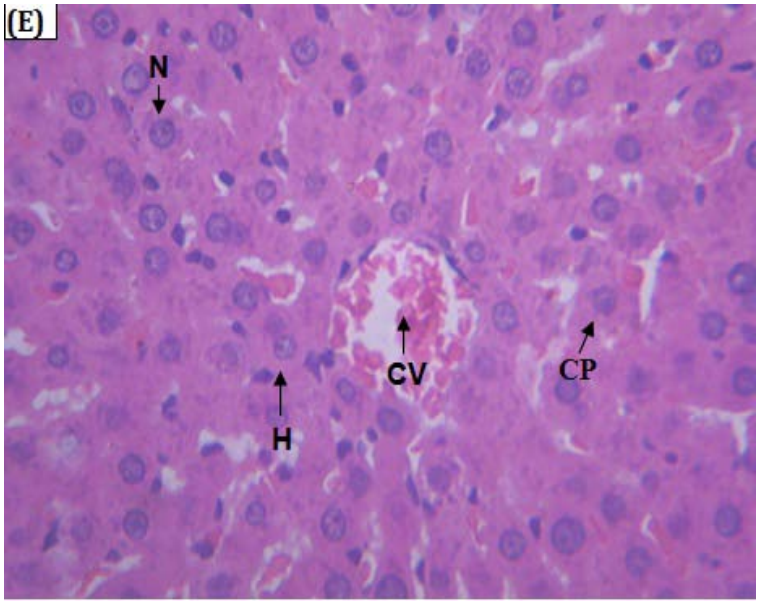
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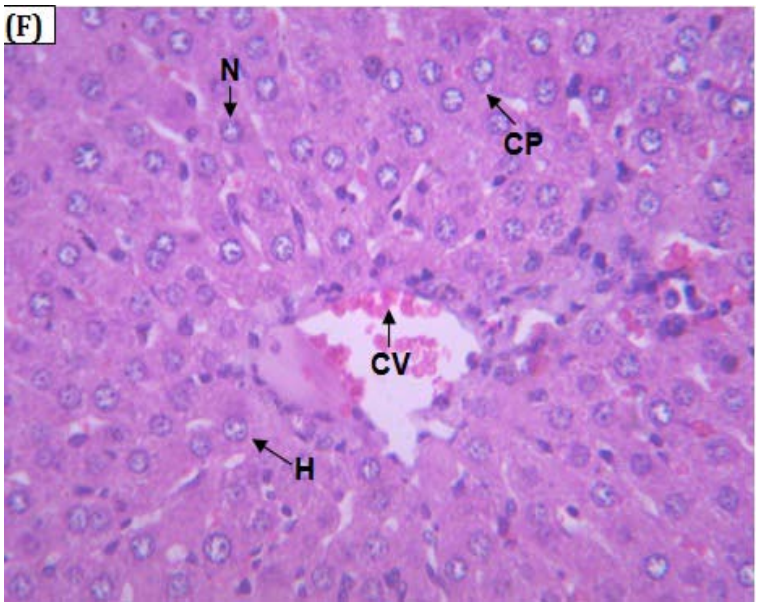

Fig. 2: (A) Liver sections of normal control rats showing: Intact tissue architecture with normal hepatocytes and a well-preserved cytoplasm; well brought out central vein; prominent nucleus and nucleolus. (B) Liver section of the ethanol treated rats showing: infiltration of the lymphocytes, centrilobular necrosis with a deformed tissue architecture due to the loss of cellular boundaries with micro and macrovesicular fatty change (inset at the upper right of figure B is showing the micro and macrovesicular fatty change) (C) Liver section of the rats treated with ethanol and ME1 showing: partially brought out central vein, ballooning in the tissue, hepatocytes with well-preserved cytoplasm, prominent nucleus and nucleolus, with a decrease in the fatty lobules (D) Liver section of rats treated with ethanol and ME2 showing: well brought out central veins, hepatocytes with well-preserved cytoplasm with complete reduction of fatty lobules, prominent nucleus and nucleolus and well formed tissue architecture. (E) Liver section of rats treated with ethanol and ME3 showing: a normal hepatic architecture, absence of necrotic cells, with complete reduction of fatty lobules, well brought out central vein, hepatic cell with well-preserved cytoplasm, prominent nucleus and nucleolus (F) Liver section of rats treated with ethanol and silymarin showing: well brought out central veins, hepatic cell with well-preserved cytoplasm, prominent nucleus and without fatty lobules, lack of necrotic cells, well formed tissue architecture (H, heptocytes; N, nucleus; CV, central vein; CP, cytoplasm; MI, Microvesicular Fatty change; MA, Macrovesicular fatty change; NC, necrosis; L, Lymphocyte).

Whereas in the liver sections of ME2 treated animals showed lack of ballooning in the tissue, lack of necrotic cells, normal hepatic architecture with lack of fatty change in hepatocytes. The histological architecture of the rat's liver treated with ME3 exhibited significant liver protection against ethanol induced hepatotoxicity evident by the presence of normal hepatic architecture, with normal hepatic cords and visible central vein, absence of necrosis, fatty infiltration, normal lobular pattern and lack of lymphocyte infiltration almost comparable to the control and silymarin treated groups (Figure 2).

DISCUSSION

Traditionally *Flaveria trinervia* herb is used as a promising drug for alcoholic liver disorders in Karnataka state, India. So far few reports are available on the preliminary phytochemical and pharmacological aspects of this plant. Literature survey revealed that there are no reports available on the hepatoprotective and antioxidant activity of *F. trinervia* against ethanol induced hepatotoxicity. In the present study, methanol extract of *F. trinervia* at three different doses was evaluated for its hepatoprotective effects against alcohol induced hepatotoxicity in rat model to find out the therapeutic efficacy of *F. trinervia.*

Alcohol (ethanol) is extensively metabolized to acetaldehyde in the liver by the enzyme alcohol dehydrogenase [28]. Acetaldehyde is further oxidized to acetate by acetaldehyde dehydrogenase/oxidase, leading to the generation of reactive oxygen species (ROS) [29]. These reactive species oxidize cellular biomolecules, such as proteins and DNA and initiate membrane peroxidative degradation in the adipose tissue resulting in fatty infiltration of the hepatocytes [30]. The increase in the levels of serum bilirubin reflects the depth of jaundice and the increase in transaminase and alkaline phosphatase as the clear indication of cellular leakage and loss of functional integrity of cell membrane. Bilirubin is the conventional indicator of liver diseases, restoration total bilirubin levels may be due to the inhibitory effects on cytochrome P450 resulting in the hindrence of the formation of hepatotoxic free radicals [31]. These hepatotoxicity marker enzymes were found to be increased in the animals treated with ethanol. The decrease of these hepatotoxic serum marker enzymes levels towards a near- normalcy in the animals treated with ME1, ME2 and ME3 confirms the hepatoprotective effect of *F. trinervia* plant against ethanol induced hepatotoxicity. Results were found comparable to a commercial hepatoprotective drug silymarin. This suggests that the *F. trinervia* extracts could be able to repair the probable hepatic injury and/or restore the altered cellular permeability, thus reducing the toxic effect of ethanol in the liver tissue.

Experimental studies in a rat model using a different mode of alcohol administration also showed variable results [32-34]. However, the cell is endowed with an elaborate antioxidant defense system to protect the cells against free radical damage. These include antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase, and lipidperoxidase.

The decreased levels of tissue antioxidant enzymes due to ethanol administration were found to be significantly elevated in the methanol extract treated groups indicating that the *F. trinervia* plant extract could reduce the free radical generation and increase in the free radicals scavenging mechanism. This significant increment in the tissue antioxidant enzymes is in corroboration with the increased free radicals scavenging mechanism. Similar study was reported by Maruthappan and Sakthi, 2009 [35], where the significant decrease in the activity of liver SOD, CAT in ethyl alcohol intoxicated rat was observed and the therapeutic treatment of *Azadirachta indica* herbal drug promoted the hepatoprotection by elevating free radical scavenging activity. In the present study, chronic sub lethal

doses of ethanol was elevated and hence decreased the levels of MDA content in liver homogenate respectively, whereas the methanol extract treatment markedly reversed these effects. It is conceivable that the effect of tested extract concentration may be due to a reduction in hepatic peroxidative activities thereby leading to restoration of the MDA content in ethanol induced hepatotoxicity.

Histopathological studies revealed that among all the treated groups, animals treated with ME2 and ME3 exhibited significant liver protection against ethanol induced hepatotoxicity as evident by the normal hepatic tissue architecture, absence of fatty infiltration, lack of necrotic cells, presence of normal hepatic cords with normal lobular pattern, and well preserved cytoplasm, decreased lymphocyte infiltration which was almost comparable to the silymarin treated groups.

Although various pharmaceutical industries are coming with antioxidant, but most of them are associated with side effects. Several herbal products are available all over the world with an acclaimed medicinal value and pharmacological property free from side effects [36,37]. Many phytoconstituents present in the plant extract including phenolics also show antioxidant activity [38]. Sterols and glycosides display significant inhibitory effects on superoxide anion generation and elastase release by human neutrophils [39]. Terpenes are potent anti-inflammatory agents [40]. This indicates that the hepatoprotective effect of the *F. trinervia* extract is due to the presence of these phytoconstituents in the extract. Many investigators have evaluated hepatoprotective property of the herbal extracts using the experimental rats. This significant effect of the phytoextract is due to the presence of a single active constituent in higher levels or due to the combined effect of more than one phytoconstituent.

CONCLUSION

The results of this investigation strongly support the ethnomedical uses of *F. trinervia.* All the three doses of methanol extract of *F. trinervia* afforded significant protection against ethanol induced hepatotoxicity by decreasing the hepatotoxic serum marker enzymes levels towards a normalcy and acting as a free radical scavenger by intercepting those radicals evolved by ethanol metabolism by the microsomal enzymes and hindering the interaction of oxygen related free radicals with polyunsaturated fatty acids and would abolish the enhancement of lipid peroxidative processes. This investigation suggests that extract of *F. trinervia* can be used a promising drug for acute cases of alcoholic liver disorders.

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