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RESEARCH ARTICLE

Antihepatotoxic activity of *Pogostemon patchouli* against alcohol-induced hepatotoxicity in rats.

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Abstract

Alcohol induced disorders affect millions of individuals worldwide. Alcoholic liver disease (ALD) represents a spectrum of clinical illness and morphological changes that range from fatty liver, alcoholic hepatitis to cirrhosis. The present study aimed to investigate the effectiveness of *Pogostemon patchouli*, one of the Chinese traditional medicinal herbs in alleviating alcohol-induced hepatotoxicity as a model of clinical liver illness. Oxidative stress has been considered as one of the reasons for etiology of ALD, Thus *in vitro* and *ex vivo* antioxidant activity evaluation before *in vivo* studies was the prime objective of the present study. For *in vivo* model, female rats were grouped into five groups from which one group served vehicle control. Groups from 2 to 5 received a daily oral dose of 56% ethanol for 5 weeks. Group 2 served as untreated control. Group 3 received Silymarin and served as standard control while groups 4 and 5 were respectively treated with n-hexane extract of *Pogostemon patchouli* (HEPP) in a curative approach. Alanine transaminases (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), total bilirubin (TBIL), as well as total protein (TPROT) levels were estimated in the serum. Malondialdehyde (MDA), reduced glutathione (GSH) activity was determined in liver tissue homogenate. A histopathological analysis of liver tissue was also achieved. Results showed amelioration of all serum *($p<0.01$)* and liver parameters *($p<0.05$)* following administration of HEPP. Conclusively, treatment with plant extract alleviated alcohol-associated peroxidative injury biochemically and histologically preventing clinical problems associated with liver injury.

Introduction

Liver is a versatile organ of the body that regulates internal chemical environment. Liver injury induced by various hepatotoxins has been recognized as a major toxicological problem for years. Since liver is the first organ of metabolism it is an important target of toxicity to xenobiotics, toxic chemicals, oxidative stress and ethanol. Alcoholic liver disease (ALD), the most extensively investigated aspect of ethanol on health is considered as one of the major causes of illness and death worldwide (Liu et al., 2010). In the last several decades, significant progress has been made in understanding the cellular and molecular mechanisms contributing to the pathogenesis of ALD (O'Shea et al., 2010). These mechanisms include direct hepatotoxicity, production of reactive oxygen species (ROS) induced by ethanol and its metabolites, activation of innate immunity and complement system with subsequent production of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) (Arteel, 2003; Breitkopf et al., 2009; Zhao et al., 2008).

Wide cultural acceptability of traditional medicines is nowadays growing exponentially due to the increased incidence of the adverse drug reactions and economic burden on the modern system of medicine. Several medicinal plants have been screened from Ayurveda or different systems of medicine. Since herbs serve excellent source of antioxidants which is inversely associated with lipid peroxidation and cytotoxicity, an attempt was made to study the effects of the n-hexane extract *Pogostemon patchouli* (HEPP). The leaves of this plant are used in Chinese

traditional medicinal system for biliary disorders (Nadkarni, 1976). Other activities of *Pogostemon patchouli* include a wide range of biological activities like antioxidant or ROS scavenger (Kim et al., 2010), analgesic and anti-inflammatory (Lu T, et al., 2011), antimicrobial, antiallergic and immuno-modulatory effect (Li et al., 2011). Since the plant is tonic and used in the treatment of biliousness and inflammatory swellings in traditional medicine; also the presence of mono and sesquiterpenes, flavonoids, phenolic acids and tannins in the plant inspired us to study its hepatoprotective activity. The plant has got economic importance too, for its use as an essential oil.

Materials and methods

Chemicals and reagents:

Ethanol, gallic acid, thiobarbituric acid, sodium nitroprusside (SNP), Ferrous sulphate (iron solutions were prepared just before use), sodium dodecyl sulphate (SDS), glacial metaphosphoric acid, trichloroacetic acid (TCA), potassium dihydrogen phosphate, disodium hydrogen phosphate were obtained from SD Fine Chemicals, Mumbai. 2, 2-diphenyl, 1-picrylhydrazine (DPPH), reduced glutathione, DTNB-5, 5'-dithiobis [2-nitrobenzoic acid], malondehyde bis-(dimethyl acetal) (MDA), was obtained from Himedia Laboratories, Mumbai. Silymarin was obtained as gift sample from micro labs Limited, Mumbai. Hydrochloric acid, Hydrogen peroxide and olive oil used were of LR grade. Commercial kits used for determination of all biochemical parameters (ALT, AST, ALP, TBIL, and TPROT) were purchased from Crest Biosystems, Mumbai. All other reagents were of LR grade.

Collection of Plant material and extraction:

Plant material:

Dried leaves of plant *Pogostemon patchouli* (commonly known as patchouli) were collected from V.G. Vase Kelkar College, Mumbai and authenticated by Dr. H.M. Pandit, Department of Botany, Guru Nanak Khalsa College, Mumbai. A voucher specimen (pd/150912) has been deposited at the Herbarium unit for future reference.

Extraction:

Leaves of patchouli were stored for 60 days. Leaves were then made into a coarse powder. Powder was extracted with n-hexane using Soxhlet apparatus (23 h). The solvent was completely removed by rotary vacuum evaporator under reduced pressure and low temperature (40-50ºC). Later extract was preserved in desiccator. The % yield of the extract was found to be 7.49%. The extract was administered in the form of emulsion using 25% Tween 80 as emulsifying agent. Silymarin was suspended in 1% carboxy methyl cellulose (CMC) and was given at dose of 100 mg/kg body weight *p.o*.

Animals:

Female Sprague Dawley rats, weighing 180-200g were obtained from, Bharat serum & vaccines Ltd. Thane. Animals were housed in environmentally controlled room (temperature 23-27℃, 50-70% humidity with 12 h light/dark cycle). Animals were provided with food and water *ad libitium*. All experiments in this study were carried out with the prior approval of the Institutional Animal Ethics Committee (IAEC/PR/2012/03) and were conducted at Bharati Vidyapeeth's College of Pharmacy, strictly adhering to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division of Government of India.

Experimental Design:

Phytochemical analysis:

Plant extract was subjected to various phytochemical screening (Khandelwal, 2004) to test the presence of secondary metabolites, which are responsible for various biological activities.

***In Vitro* antioxidant activity:**

DPPH radical scavenging assay:

The DPPH radical scavenging activity was assayed according to the method of Shimada *et al*. (1992) with some modifications. Briefly, 2 ml of DPPH solution (0.2 mM DPPH in methanol) was mixed with 2 ml of test sample (100-1000 µg/ml).The mixture was shaken to ensure complete mixing and allowed to stand at room temperature for 30 min. The absorbance of the mixture was then measured at 517 nm. (Jun et al., 2013; Ohkawa et al., 1979).Silymarin and Gallic acid were used as the positive control. The DPPH radical scavenging activity was calculated by the following formula:

$$Scavenging\ activity\ (\%) = 1 - \frac{(\mathrm{A1} - \mathrm{A2})}{\mathrm{A0}} \times 100$$

Where A0 is the absorbance of the control (DPPH mixed with methanol only), A1 is the absorbance of methanolic solution of DPPH mixed with test sample and A2 is the absorbance of the test sample only.
EC_{50} value for HEPP, Silymarin and Gallic acid was also calculated using linear regression equation using Graph pad prism software.

***Ex vivo* antioxidant activity of HEPP against hepatotoxins in rat liver homogenate:**
In the present study, *Patchouli* was studied *ex vivo* for its effect on the formation of thiobarbituric acid reactive substances (TBARS) induced by pro-oxidants (Ferrous sulphate and sodium nitroprusside)

Production of TBARS:
Production of TBARS was determined using a modified method of Ohkawa *et al*., with some modifications (1979). The rats were sacrificed by carbon dioxide overdose and the livers were then removed rapidly, cleaned of extraneous tissue by washing with saline and placed on ice. They were homogenized in cold 100 mM Tris buffer pH 7.4 (1:10 w/v). The homogenate was centrifuged for 10 min at 3500 rpm to yield a pellet that was discarded and supernatant was separated. The supernatant was used for the assay.
The homogenates (100 µl) were incubated with or without 50 µl of the pro-oxidants ($FeSO_4$ and sodium SNP) and 50 µl plant extract together with an appropriate volume of distilled water to give a total volume of 300 µl at 37ºC for 1 h. The color reaction was carried out by adding 200, 250, 500 µl each of the 8.1% SDS, acetic acid pH 3.4 and 0.6 % TBA, respectively. The above reaction mixtures, including those of serial dilutions of 0.03 mM standard MDA, were incubated at 95-100ºC for 1 h. The absorbance was read after cooling at a wavelength of 532 nm (Olalye MT et al., 2007).

Calculation: MDA content of test samples was calculated by extrapolation method using standard graph. The results were expressed in µmol/g liver. Inhibition (%) of MDA formation was also calculated using negative control (SNP and $FeSO_4$ control).

***In vivo* alcohol-induced hepatotoxicity model: Curative approach**
Rats were divided into 5 groups, containing 6 animals each. Group 1 (vehicle control group) received distilled water. Group 2 (disease group) received a daily oral dose of 56% ethanol. The initial loading dose of 56% ethanol was 6 g/kg/day. The dose was progressively increased during week 1 to a maintenance dose of 8 g/kg/day and that was continued for another 4 weeks. (Hebatallah AD et al., 2012) Group 3, 4, 5 (therapeutic groups) were concomitantly given Silymarin (100 mg/kg body weight), Low dose of HEPP (300mg/kg body weight), High dose of HEPP (600 mg/kg body weight) along with ethanol administration 30 min after herbal extracts treatment respectively. The animals of all groups were weighed thrice/week.

Blood and tissue collection:
On 36^{th} day, rats were sacrificed by carbon dioxide over dose, blood samples were withdrawn terminally and allowed to clot, and serum was separated by centrifugation at 6500 rpm for 15 min. It was kept at -20 º C till determination of liver function enzyme activities (ALT, AST, ALP, TBIL, and TPROT). Meanwhile, the abdominal cavity was dissected immediately; the liver was separated, washed and divided into two portions: First portion was homogenized in 10 mM potassium phosphate buffer (1: 10) for the determination of reduced glutathione content and extent of lipid peroxidation estimation. Second portion fixed in 10% formalin and was given for histopathological evaluation.

Estimation of biochemical parameters:
Serum ALT and AST was determined by Modified IFCC method (Bergmeyer H-U et al., 1986). ALP was established by pNPP Kinetic Method (McComb RB et al., 1972) whereas TBIL was estimated following the Modified Jendrassik & Grof's method (Jendrassik et al., 1938) and serum TPROT was determined by Biuret method (Doumas, 1975; Gornall et al., 1949).

Estimation of lipid peroxidation and endogenous antioxidant:
The levels of lipid peroxidation and contents of non-enzymatic anti-oxidant, reduced glutathione were estimated (Ohkawa et al., 1979; Ramachandra et al., 2013). Liver tissue homogenates were prepared in (10 mM) potassium phosphate buffer (pH 7.4) using a homogenizer and centrifuged at 3000 rpm for 10 min at 25ºC. The supernatant was used immediately for assay of all parameters.

Statistical analysis:
The values were expressed as mean ± SEM. Statistical analysis and comparison between the groups was performed by one way analysis of variance (ANOVA) followed by Dunnett's test. Statistical difference between unexposed and exposed (with or without treatment) was considered to be significant.

Results

Phytochemical constituents:
Preliminary phyochemical screening of HEPP revealed the presence of alkaloids, glycosides, phenolic acids, flavanoids, terpenoids, steroids and tannins. Major constituent of patchouli are Sesquiterpenoids that are reported to be the constituents responsible for various biological activities.

Antioxidant activity:
In DPPH antioxidant assay, the radical inhibitory activity of extract is expressed as a relative or absolute decrease of concentration or absorbance of DPPH or as EC_{50} (required concentration of a compound decreasing the absorbance of a DPPH solution by 50 %) value (Gunars T et al., 2010), suggesting that the antioxidant activity of n-hexane extract is due its proton donating ability. HEPP exhibited a significant dose dependent inhibition of DPPH activity with 50% inhibition at the concentration of 504.75 µg/ml. Silymarin and Gallic acid showed EC_{50} value of 324.21 and 267.45µg/ml respectively. Basically, a higher DPPH radical-scavenging activity is associated with a lower EC_{50} value.
As shown in Fig.1, the scavenging activities (%) of n-hexane extract of patchouli and standard antioxidants on DPPH radical increased with the increase of concentrations. At the highest concentration of 1000 µg/ml, the scavenging activity for n-hexane and standard drugs Silymarin and Gallic acid were 74.86%, 94.22% and 98.02% respectively.

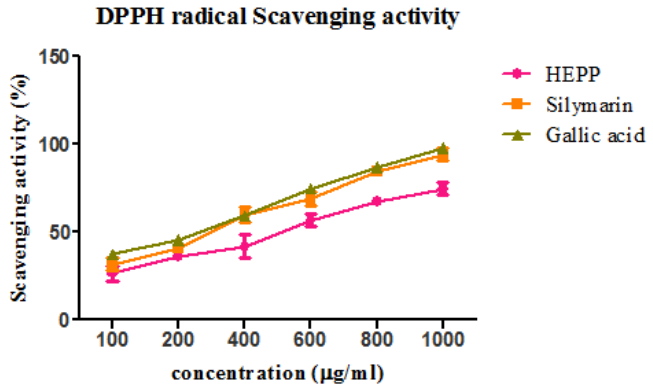


Figure 1: Comparison of DPPH free radical scavenging activity (%) of HEPP, Silymarin and Gallic acid. Results are expressed as mean ± SEM.

TBARS Results:
There was a statistically significant increase in the formation of TBARS in sodium nitroprusside (5 µM) and ferrous sulphate (10µM) induced oxidative stress. At a concentration of 1000 µg/ml , HEPP caused a decrease *($p<0.05$)* in MDA formation as compared to control as shown in Fig. 2 and Fig. 3. At the concentration of 1000 µg/ml, inhibition of MDA formation (%) in SNP induced toxicity was found to be 70.78%, 79.17% and 85.35% for HEPP, Silymarin and Gallic acid respectively whereas in $FeSO_4$ induced toxicity % inhibition of MDA formation was found to be 60.41%, 66.49% and 79.79% for HEPP, Silymarin and Gallic acid respectively

***In vivo* Results:**
Plasma markers of hepatotoxicity:
In the present study, we assessed the therapeutic effect of HEPP against 56% ethanol induced hepatotoxicity in rats. The activity levels of serum ALT, AST, ALP, TBIL and TPROT were taken as indices for hepatotoxicity induced by ethanol. Significant increase *($p < 0.01$)* in the plasma levels of AST, ALT, ALP and TBIL were observed in ethanol treated group as compared to the normal control group. However, the low dose HEPP (300 mg/kg body weight) treated groups showed significantly lesser activity, whereas the higher dose of HEPP (600 mg/kg body weight) and Silymarin (100 mg/kg body weight) recorded comparable levels of protection. Total hepatic protein content was

significantly lowered after ethanol treatment while, HEPP and Silymarin treated groups showed significant *($p < 0.01$)* increase as shown in Table 1.

Endogenous antioxidant and lipid peroxidation:
Animals exposed to ethanol showed decrease *($p < 0.01$)* in the levels of non-enzymatic antioxidant, reduced glutathione and increase in lipid peroxidation compared to vehicle control animals. However, HEPP treated groups depicted significantly lesser restoration of GSH and LPO with the low dose (300 mg/kg body weight). The levels of GSH *($p<0.05$)* and indices of LPO *($p<0.01$)* in HEPP (600 mg/kg body weight) group were very much comparable to those in the Silymarin group as shown in Table 2.

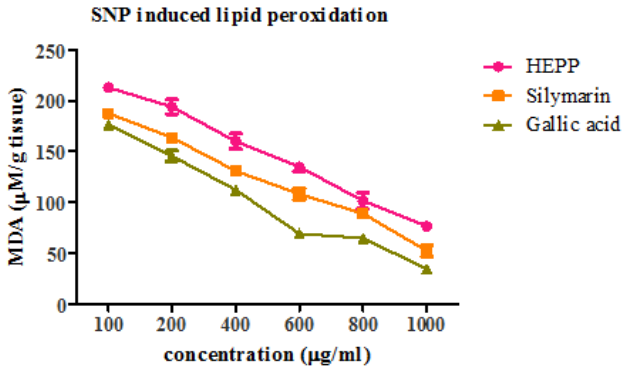


Figure 2: Comparison of Effect of HEPP, Silymarin and Gallic acid on SNP induced elevation of MDA levels in rat liver. SNP control value was 279.32±1.5713. Results are expressed as mean ± SEM.

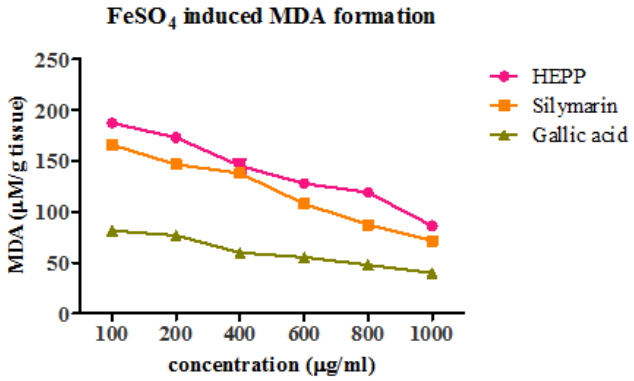


Figure 3: Comparison of Effect of EEPP, HEPP, Silymarin, Gallic acid on $FeSO_4$ induced elevation of MDA levels in rat liver.$FeSO_4$ control value was 227.64±2.5303. Results are mean ± SEM

Table 1: Effect of HEPP on serum blood parameters of 56% ethanol induced hepatotoxicity in rats. Results are expressed as mean ± SEM (n=6) Values are compared using one way ANOVA followed by Dunnett's test. *(p*<0.05; p**<0.01; p***<0.001)* when compared with Disease control groups, ns indicates non significant.

Treatment Groups	ALP (IU/L)	ALT (IU/L)	AST (IU/L)	TBIL (mg/dl)	TPROT (mg/dl)
Normal control	128.5±4.059	41.13±1.749	110.8±3.841	0.238±0.014	6.722±0.226
Disease control	475.6±24.28	92.53±4.696	232.7±10.69	0.663±0.041	4.502±0.093
Standard control	250.9±12.66** (90.93) ↓	58.42±2.993** (66.36) ↓	153.8±6.597** (64.73) ↓	0.398±0.031** (62.35) ↓	6.433±0.205** (85.07) ↑
HEPP (300mg/kg)	378.6±14.38** (39.25) ↓	73.59±3.075** (36.84) ↓	190.2±5.743** (34.86) ↓	0.5233±0.0362** (32.94) ↓	4.478±0.322** (42.99) ↑
HEPP (600mg/kg)	280.6±6.736** (78.91) ↓	63.06±2.385** (57.33) ↓	161.4±7.535** (58.49) ↓	0.3850±0.0158** (53.71) ↓	5.675±0.177** (51.67) ↑

Values in parenthesis indicate % increase or decrease in respective biochemical parameter.

Table 2: Effect of HEPP on Lipid Peroxidation and Reduced Glutathione levels of 56% ethanol induced hepatotoxicity in rats. Results are expressed as mean ± SEM (n=6) Values are compared using one way ANOVA followed by Dunnett's test. *(p*<0.05; p**<0.01; p***<0.001)* when compared with Disease control groups, ns indicates non significant.

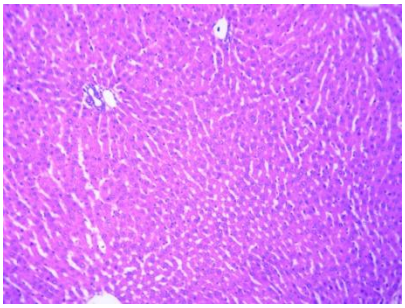
Treatment Groups	Lipid Peroxidation (µMol/g wt. tissue)	Reduced Glutathione (µMol/g wt. tissue)
Normal control	0.765±0.031	96.36±1.827
Disease control	5.195±0.041	64.31±3.718
Standard control	1.395±0.018** (85.78) ↑	82.31±1.921* (56.16) ↑
HEPP (300mg/kg)	2.763±0.040** (54.89) ↑	$62.67±3.760^{ns}$ (No increase)
HEPP (600mg/kg)	2.295±0.004** (65.46) ↑	76.17±3.644* (37.00) ↑

Values in parenthesis indicate % increase or decrease in respective biochemical parameter.

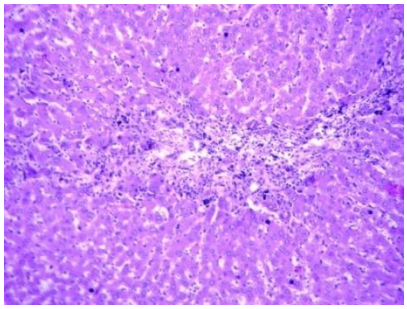
Histological observations:

Sections of liver of normal control rats showed intact central vein and hepatic cords with healthy hepatocytes and thin sinusoidal spaces. However, ethanol treated rats showed central vein disruption, lobular disarray, multifocal moderate degree mononuclear lobular inflammation, some sections showed spotty vacuolar degeneration of hepatocytes, Kupffer cell hypertrophy and multiple spotty pyknosis. Multifocal minimal to mild degree necrosis and loss of periportal hepatocytes was seen. However, HEPP (300mg/kg body weight) treated rats showed mild degree lobular disarray and granular degeneration, occasional spotty pyknosis and spotty necrosis of hepatocytes. HEPP (600mg/kg body weight) treated rats showed intact central vein, moderate to marked degree diffuse granular degeneration and minimally multifocal mild degree periportal mononuclear cell infiltration as shown in Fig. 4

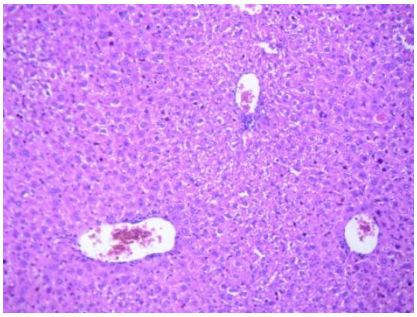
Figure 4. Photograph of liver tissue. (magnification 100 x, Hematoxylin and eosin staining). Vehicle control group showing normal histological structure. Ethanol control group showing hepatic degenerative changes and inflammatory cell infiltration along with necrosis and spotty pyknosis. Standard and therapeutic groups showing minimized hepatocytes degeneration, mononuclear cell infiltration and necrosis.



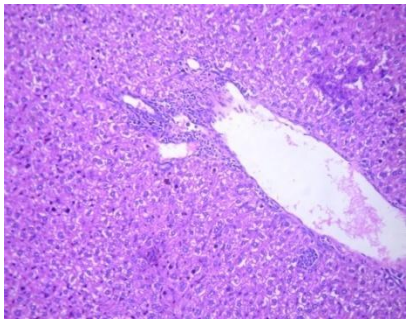
Group 1- Normal control



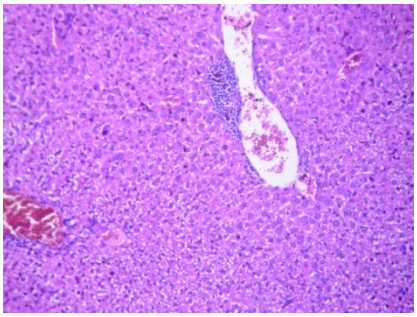
Group 2-Disease control (ethanol)



Group 3- Standard control (Silymarin)



Group 4- Low dose (300 mg/kg) of HEPP



Group 5- High dose (600 mg/kg) of HEPP

Discussion

The data obtained after ethanol administration in albino rats revealed that chronic ethanol administration caused acute oxidative stress along with necrosis in the liver, which corresponded to the deviations in all tested biochemical parameters. The above effect is attributed to the toxicity produced by chronic administration of ethanol that resembles clinical symptoms in humans.

Liver is the main organ for alcohol metabolism. Alcohol is metabolized in liver by three different ways: (1) by enzyme alcohol dehydrogenase (ADH), (2) by cytochrome P-450 2E1 (CYP2E1); and (3) by mitochondrial Catalase. ADH and CYP2E1 both convert alcohol to acetaldehyde, which is in part responsible for liver damage. However, the process of liver injury is much more complex resulting from biochemical, genetic, cellular, immunological changes in connection with the intake and metabolism of excessive quantities of alcohol.

The model of DPPH radical scavenging activity is a widely used method to evaluate the free radical scavenging activities of antioxidants. In the DPPH assay, the antioxidants are able to reduce the stable DPPH radical (purple) to the non-radical form DPPH-H (yellow). The DPPH scavenging activities of antioxidants are attributed to their hydrogen donating abilities.

In *ex vivo* TBARS assay, SNP produces nitric oxide radicals. Chronic exposure of hepatocytes to reactive nitrogen species causes inactivation of methionine synthase which is related to cytotoxic and cytostatic activity leading to functional and morphological alterations. Increase in the formation of MDA induced by $FeSO_4$ is associated with an overload of iron. Free iron in the cytosol and in the mitochondria can cause considerable oxidative damage by increasing superoxide production, which can react with Fe^{3+} to generate Fe^{2+} that participates in the Fenton reaction. Iron overload results in the lipid peroxidation products, such as MDA. The possible mechanism of iron toxicity includes free radical mediated peroxidative reactions, which are readily catalyzed by iron. Decreased MDA formation or increased inhibition (%) of MDA is relative to decreased extent of lipid peroxidation.

Hepatic cells consist of different enzymes to carry out variety of metabolic activities. Aminotransferases are the sensitive indicators of hepatocellular (necroinflammatory) injury and loss of functional integrity of hepatic cells. Abnormal increase in ALP is related to cholestatic injury and is due to abnormal synthesis of bile in presence of increased biliary pressure. It is well known that toxicants like ethanol produce sufficient injury to biliary tree to cause elevation in serum bilirubin level. Bilirubin is one of the useful clinical clues to substantiate the functional integrity of liver and severity of necrosis and its accumulation is a measure of binding, conjugation and excretory capacity of hepatocytes. Hepatotoxicity also affects the synthesizing capacity of liver where, depression in TPROT level is observed due to the defect in protein biosynthesis. This is due to disruption and dissociation in polyribosomes from endoplasmic reticulum. The above all facts are consistent with the disease group showing deviations in all biochemical parameters.

Oxidative stress has been reported to play a role in the pathogenesis of alcohol-induced liver injury (Arteel, 2003). Total antioxidant capacity depicts the synergistic interaction among different antioxidants; maintaining balance between oxidants and endogenous antioxidants (Serafini et al., 2000). The liver is the primary organ of oxidative drug metabolism, so it is susceptible to oxidative damage caused by increased reactive oxygen species (ROS). The role of ROS in liver tissue following ethanol treatment affects membrane permeability and fluidity causes production of lipid-derived radicals, lipid peroxidation, and depletion of antioxidant defences (Ronis et al., 2004; Sampey et al., 2003).

Patchouli has also been evaluated for various pharmacological activities scientifically. Studies have reported some of the findings which can be correlated with present study to best predict the mechanism of action of this plant preventing hepatotoxicity. Some of these are; aqueous extract of *Pogostemon patchouli* effectively protected human neuroglioma cell line A172 against both the necrotic and apoptotic cell death induced by hydrogen peroxide radicals (H_2O_2). Another study revealed two possible mechanisms associated with its anti-inflammtory activity are, reducing the amount of arachidonic acid transformation to prostaglandins by suppressing protein and mRNA expression of inflammatory mediators and another includes cleaning of free radical increasing the activity of antioxidant enzymes such as GSH.

Restoring effect of all serum enzymes suggest possible role of HEPP at the stage of repair as well as its role as a membrane stabilizer. It could be postulated that HEPP exerted its effect against ethanol-induced hepatotoxicity via modulating the extent of lipid peroxidation and augmenting the antioxidant defense system. The protective effect of HEPP against alcohol-induced oxidative stress demonstrated in the present study can be attributed to its antioxidant properties and possible chelating effects on free radicals.

Conclusion

Taken together, all the above-mentioned benefits were demonstrated upon administering the alcohol-treated rats with HEPP in a therapeutic approach. In conclusion, the present study suggests that HEPP administration may play an important role in ameliorating alcoholic liver injury.

Acknowledgement

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