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Antihepatotoxic Potential of *Cucumis sativus* and *Pogostemon patchouli* against Carbon tetrachloride induced Hepatotoxicity

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ABSTRACT

Cucumis sativus (Cucumber), a very commonly used plant in India and *Pogostemon patchouli* (Patchouli), a herb from traditional Chinese medicine have been screened earlier for antioxidant activity which is considered to be basic action required for an organ protective action. The present study was aimed to evaluate hepatoprotective potential of *Cucumis sativus* and *Pogostemon patchouli* against carbon tetrachloride (CCl_4) induced hepatotoxicity in rats. A single *i.p* injection of 1ml of CCl_4 /kg body weight in olive oil (1:1) induced liver marker enzymes such as alkaline phosphatase (ALP), serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), bilirubin (TBIL) and distorted the hepatic tissue architecture along with increased levels of lipid peroxides (LPO) and reduction of total protein (TPROT), catalase (CAT) and reduced glutathione (GSH) in liver tissue. Treatment groups receiving higher doses of both plant extracts significantly *($p<0.01$)* restored the levels of all biochemical parameters and antioxidant system of the body towards standard control. Extent of lipid peroxidation was also found to be less *($p<0.05$)*, restoring the structural integrity of hepatocytes as compared to disease group. Silymarin was used as standard drug. Result interpretation supported hepatoprotective role biochemically and histologically. Thus above plants can be further studied for isolation of responsible active components, deducing possible way of mechanism of action of these plants in liver protection.

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INTRODUCTION

Liver is referred to as body's 'chemical factory' carrying out hundreds of jobs that are vital to life. It is the first organ to metabolize all foreign compounds and hence it is susceptible to almost as many different diseases. In today's world liver is overwhelmed with toxin problems, alcohol abuse, and susceptibility to viral infections, immune disorders or problems of metabolism leading to serious liver disorders. Liver diseases such as cirrhosis, fatty liver and chronic hepatitis are important world health issues. In spite of tremendous advances in modern medicine, there are no reliable and effective drugs available which can stimulate liver function, offer protection to liver from damage or help in regeneration of hepatic cells. [1]

In Ayurvedic system of medicine, *Cucumis sativus* has been attributed to several medicinal properties. Plant is also used for jaundice, bleeding disorders and anuria. Seeds are highly nourishing. [2] Till date present study on plant represents variety of pharmacological activities like anticancer [3], anthelmentic [4], antimicrobial [5], hypolipidemic [6], antiulcer [7], analgesic and antioxidant. [8] Presence of isoorientin [9, 10], β amyrin [9, 11], α amyrin [9, 12], caffeine [9, 13], cucurbitacin-B [9, 14], cucurbitacin-E [9, 15] in leaves and seeds of *Cucumis sativus* is reported. It also contains α linolenic acid which is found to have anti-inflammatory effect. [9, 15] Thus it may have contributed to hepatoprotective effect. Compounds with anti-inflammatory activity may be responsible for reversing the inflammatory features associated with hepatic injury which may be responsible for hepatoprotective effect.

Pogostemon patchouli (Lamiaceae) is one of the delightful herbal Chinese medicines, traditionally used for biliousness. [16] In Asian countries, such as Japan and China, this herb has been used traditionally as an energizer, tonic. Phytochemical screening has revealed presence of azulene, eugenol, rhamnetin, which all known to have hepatoprotective activity. The plant has got economic importance too, due to its essential patchouli oil which is obtained mainly from distillation of leaves and found to have variety of pharmacological activities including analgesic, anti-inflammatory [17], antiemetic [18], antiallergic, immunomodulatory and antimicrobial , anti-IFV [19] and radical-scavenging actions. [20]. So based on their traditional use, phytochemical profile and related pharmacological reports, an attempt was made to screen hepatoprotective activity of Methanolic extract of leaves and seeds of *Cucumis sativus* (MECS) and hexane extract of leaves of *Pogostemon patchouli* (HEPP) against CCl_4 induced hepatotoxicity in rats.

MATERIALS AND METHODS

Chemicals and reagents

CCl_4 was purchased from Research Lab Fine Chemical Industries, Mumbai. ALP, SGPT, SGOT, TPROT, TBIL kits were purchased from CREST BIOSYSTEMS, Mumbai. Silymarin was obtained as gift sample from Micro Labs Limited, Mumbai. Solvents for extraction (methanol and ethanol), Glacial metaphosphoric acid, Thiobarbituric acid (TBA), trichloroacetic acid (TCA), potassium dihydrogen phosphate, disodium hydrogen phosphate were obtained from SD Fine Chemicals, Mumbai. Reduced glutathione, DTNB-5, 5'-dithiobis [2-nitrobenzoic acid], was obtained from Himedia Laboratories. Hydrochloric acid, Hydrogen peroxide and olive oil used were of LR grade.

Animals

Female Sprague Dawley rats, weighing 180-200g were obtained from Haffkine Institute, Mumbai. Animals were housed in environmentally controlled room (temperature 23-27 °C, 50-70% humidity with 12 h light and dark cycle). Animals were provided with sufficient 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.

Plant materials

Cucumis sativus was collected from local market whereas leaves of plant *Pogostemon patchouli* were collected from V.G. Vase Kelkar College, Mumbai. The plants *cucumis sativus* (ps/180912) and *Pogostemon patchouli* (pd/150912) were taxonomically identified and authenticated by Dr. H.M. Pandit, Department of botany, Guru Nanak Khalsa College, Mumbai.

Preparation of Test Drug Extract

Seeds and leaf of *Cucumis sativus* were extracted in Soxhlet apparatus using methanol as a solvent, while the leaves of *Pogostemon patchouli* were extracted using hexane in Soxhlet apparatus. The extracts were then concentrated using Rotary evaporator under reduced pressure and low temperature (40-50ºC). Later extracts were preserved and dried in dessicator. The extracts were administered in the form of emulsion using Tween 80 as emulsifying agent. A dose of 350 and 700 mg/kg body weight for MECS and 300 and 600 mg/kg body weight for HEPP was administered for evaluation of hepatoprotective activity of both the plants. Silymarin was suspended in 1% carboxy methyl cellulose (CMC) and was given at dose of 100 mg/kg body weight *p.o.*

Phytochemical analysis

Plant extracts were subjected to various phytochemical screening [21] to test the presence of secondary metabolites, which are responsible for various biological activities.

Experimental

***In Vivo* CCl_4 induced hepatotoxicity model** [22]

After 1 week of acclimatization period, rats were randomly divided into 7 groups, containing 6 animals each. All groups except group II received treatment daily for 9 days. Group I served as normal control (NC) and received 5ml/kg of 25% tween 80 body weight

emulsion orally. Group II served as CCl_4 control (disease control). Group III received standard drug, silymarin (100mg/kg body weight, *p.o*). Group IV and Group V received MECS (350, 700 mg/kg body weight respectively, *p.o*) whereas Group VI and VII received HEPP (300, 600 mg/kg body weight respectively, *p.o*). Groups II, III, IV, V, VI and VII were injected with a single dose of 1ml/kg body weight, *i.p* CCl_4: olive oil (1:1) on 9^{th} day of the study 30 min after treatment. After 24 h of CCl_4 treatment, blood was collected and serum was separated for the biochemical investigations. The liver was removed to investigate various liver antioxidant enzymes and histopathological alterations.

Biochemical studies

Determination of liver functional enzymes activities

In biochemical investigation of serum enzymes, ALP was determined by pNPP Kinetic Method [23], SGPT, SGOT were determined by Modified IFCC method [24]. TBIL was determined by Modified Jendrassik & Grof's method [25] whereas TPROT was determined by Biuret method. [26, 27]

Estimation of enzymatic and non-enzymatic anti-oxidants and lipid peroxidation

Liver homogenate was prepared by homogenizing liver in 10 mM potassium dihydrogen phosphate buffer (1:10) following centrifugation at 700g for 10 min.

Reduced glutathione assay [28]

Hepatic reduced glutathione (GSH) level was determined by improved method of Beulteret.

0.2 ml of liver homogenate was added to 3 ml of precipitating solution (1.67 g glacial meta phosphoric acid, 0.2 g EDTA and 30 g of sodium chloride in 100 ml distilled water) and final volume was made to 5 ml with distilled water. Above solution was allowed to stand for 5 min, and then filtered, 0.5 ml of filtrate was added to 2 ml of phosphate solution (0.3 M Disodium hydrogen phosphate) and 0.25 ml of 0.04% DTNB in 1% sodium citrate solution). Absorbance was immediately measured at 412 nm. Content of test samples was calculated from GSH standard curve and expressed as mM/g liver wt.

Catalase [29]

The CAT activity was measured according to method of Aebi. To 2 ml of 30 mM H_2O_2 hydrogen peroxide solution [0.34 ml 30% hydrogen peroxide in phosphate buffer (6.81 g potassium dihydrogen phosphate, 8.9 g disodium hydrogen phosphate each in 1000 ml D.W., mixed in proportion of 1:1.5)] in cuvette, 1 ml of tissue sample was added and the kinetics of the reaction was monitored for 60 sec spectrophometrically at 240 nm. Results were expressed in terms of mM H_2O_2 decomposed /min/g liver wt.

Lipid peroxidation [30]

The Extent of lipid peroxidation was estimated according to the method of Buege and Aust method. In brief, samples were mixed with TBARS reagent consisting of 0.375% TBA and 15% trichloroacetic acid in 0.25-N hydrochloric acid. The reaction mixtures were placed in a boiling water bath for 15 min and centrifuged at 1000g for 5 min. The absorbance of the supernatant was measured at 535 nm. MDA, a measure of lipid peroxidation, was calculated using an extinction coefficient of 1.56×10^5/M cm. The results were expressed as µM/mg wet tissue.

Histopathological examination

The histological appearance of the hepatocytes reflects their damaged condition. In brief, the autopsied livers were washed in normal saline to remove blood stains and were blotted. The livers were then fixed immediately in 10% formalin solution and were observed under microscope.

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 (p <0.01) highly significant.

RESULTS

Phytochemical constituents

The preliminary phytochemical screening of MECS showed presence of glycosides, alkaloids, tannins, fixed oils, carbohydrates, proteins, steroids and flavanoids. While preliminary phyochemical screening of HEPP revealed the presence of alkaloids, glycosides, phenolic acids, flavanoids, terpenoids, steroids and tannins. Major constituent of patchouli, Sesquiterpenoids present in patchouli oil are reported to be the constituents responsible for various biological activities.

Effect on biochemical parameters

The activities of various biochemical enzymes in vehicle control, disease control and treated groups are represented in Tables 1. The activities of ALP, SGPT, SGOT, and TBIL were significantly increased with a significant decrease in TPROT levels in CCl_4 control compared to normal control. The levels of the above enzymes were significantly reversed *($p<0.01$)* on treatment with different doses (350, 700 mg/kg body weight of MECS and 300, 600 mg/kg body weight of HEPP, *p.o.*). The activity of higher doses of both the extracts was comparable to that of the standard drug Silymarin.

Lipid peroxidation

MDA level is widely used as a marker of free radical mediated lipid peroxidation injury. We measured MDA levels in the liver and the results are shown in Table 2. MDA levels in the CCl_4 treated group (7.395±0.14 µMol/g wt. of wet liver tissue) were significantly higher than that in the control group (1.668±0.054 µMol/g wt. of wet liver tissue). % inhibition of lipid peroxidation of higher doses of MECS and HEPP was 81.63% and 81.63% respectively. Silymarin also inhibited the elevating MDA levels upon CCl_4 administration (95.25 % inhibition). These findings indicated that the free radicals being released in the liver were effectively scavenged when treated with MECS and HEPP extracts.

Hepatic antioxidant enzyme activities

CAT and GSH were measured as an index of antioxidant status of tissues. Significantly lower liver catalase and GSH activity were observed in rats from the CCl_4 treated group as compared to the normal control group. Higher doses significantly restored catalase (*p<0.01*) and GSH (*p<0.05*) towards normal level as compared to lower doses.

Table 1: Effect of CCl_4, silymarin, MECS and HEPP on serum blood parameters of CCl_4 induced hepatotoxicity rats,

Treatment Groups	ALP (IU/L)	SGPT (IU/L)	SGOT (IU/L)	TBIL (mg/dl)	TPROT (mg/dl)
Normal control	106.9±6.626	64.49±3.162	108.1±4.562	0.24±0.013	7.002±0.166
Disease control	437.2±23.29	174.3±6.086	308.1±10.81	1.872±0.079	4.628±0.139
Standard control	190.5±3.496** (74.69) ↓	107.2±3.982** (61.11) ↓	177.3±9.889** (65.40) ↓	0.917±0.062** (58.52) ↓	6.468±0.267** (77.51) ↑
MECS (350 mg/kg)	265.2±7.314** (52.07) ↓	149.1±5.575** (22.40) ↓	252.2±9.178** (27.95) ↓	1.473±0.047** (24.45) ↓	5.292±0.112^{ns} (27.97) ↑
MECS (700 mg/kg)	217.8±8.669** (66.42) ↓	128.1±9.083** (42.07) ↓	198.4±4.583** (54.85) ↓	1.052±0.047** (50.25) ↓	5.942. ±0.25** (55.35) ↑
HEPP (300 mg/kg)	271.9±5.94** (50.05) ↓	145±4.963** (26.68) ↓	250.2±5.975** (28.95) ↓	1.513±0.059** (19.78) ↓	5.393±0.01^{ns} (32.22) ↑
HEPP (600 mg/kg)	220.3±7.79** (65.66) ↓	127.1±8.912** (42.98) ↓	202.7±5.985** (52.70) ↓	1.055±0.028** (48.64) ↓	5.775±0.302** (48.31) ↑

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

Table 2: Effect of MECS and HEPP on Lipid Peroxidation, Reduced Glutathione and Catalase levels of CCl_4 induced hepatotoxicity rats,

Treatment Groups	Lipid Peroxidation (µMol/g wt. of wet liver tissue)	Reduced Glutathione (mMol/g wt. of wet liver tissue)	Catalase (µMol/g wt. of wet liver tissue)
Normal control	1.668±0.054	21.76±0.658	3.152±0.351
Disease control	7.395±0.14	15.82±1.359	0.624±0.094
Standard control	1.94±0.189** (95.25) ↓	19.19±0.569* (56.73) ↑	2.584±0.572** (77.50) ↑
MECS (350 mg/kg)	3.387±0.271** (69.98) ↓	17.64±0.525 ^{ns} (30.64) ↑	1.081±0.2387** (14.11) ↑
MECS (700 mg/kg)	2.72±0.129** (81.63) ↓	18.72±0.256* (48.82) ↑	2.428±0.2668** (71.20) ↑
HEPP (300 mg/kg)	3.438±0.155** (69.09) ↓	15.74±0.3507^{ns} No increase	0.74±0.101^{ns} (4.59) ↑
HEPP (600 mg/kg)	2.561±0.288** (84.40) ↓	18.60±0.1851** (46.80) ↑	1.879±0.130** (48.43) ↑

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

Histopathology

The histopathological studies of the liver showed centrilobular necrosis with mononuclear cell infiltration and spotty pyknosis in CCl_4 control rats (**Figure 2**) in comparison with normal control (**Figure 1**). Groups administered with low doses of extracts exhibited mild to moderate centrilobular necrosis with mild to moderate degree mononuclear cell infiltration, minimal degree lobular disarray with multiple spotty pyknosis. The groups receiving higher doses showed minimal degree lobular disarray, normalization of fatty changes as well as normalization of necrosis of the liver. The animals treated with silymarin showed almost normalization of fatty accumulation and necrosis. The maximum protection against hepatic damage was achieved with the 700 mg/kg and 600 mg/kg body weight of MECS and HEPP respectively (**Figure.5** and **Figure 7**).

Figure 1: Group 1 (Vehicle Control)

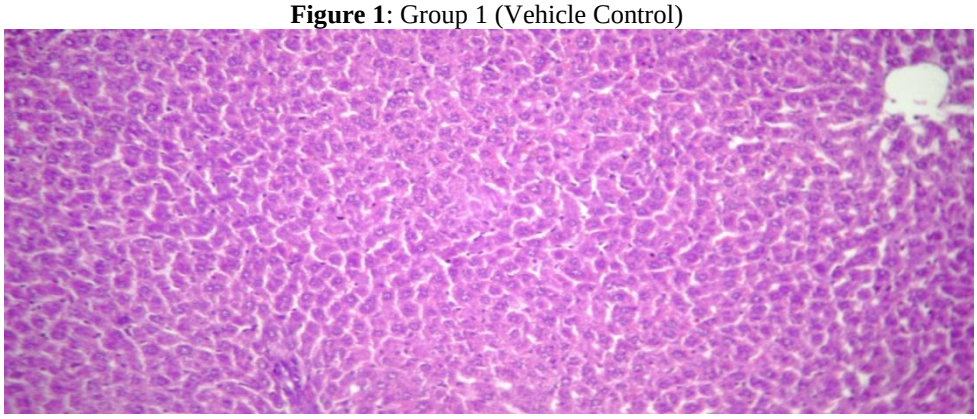


Figure 2: Group 2 (Disease Control, 1ml/kg CCl_4)

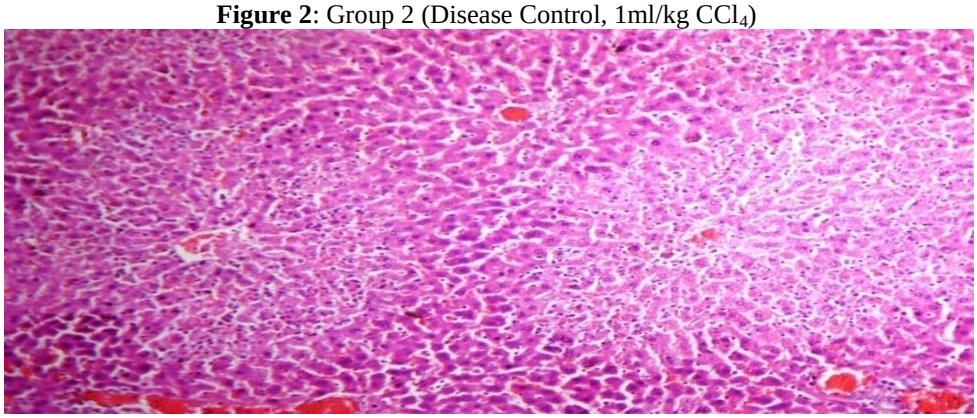


Figure 3: Group 3 (Standard Control, 100 mg/kg Silymarin)

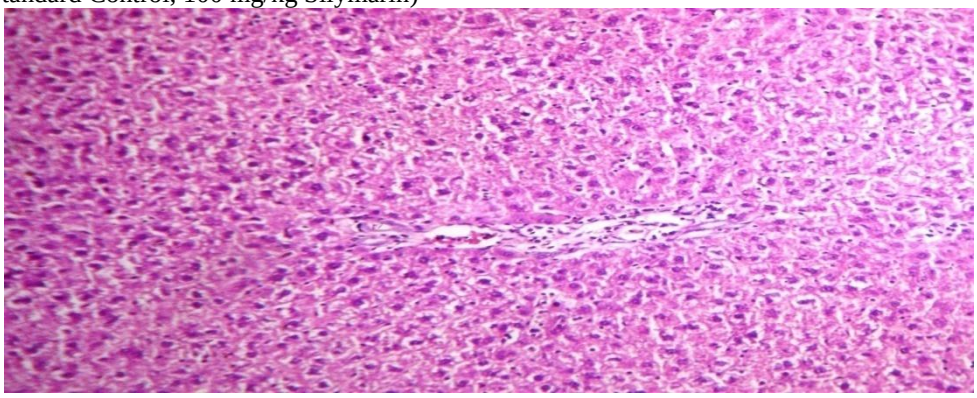


Figure 4: Group 4 (MECS 350 mg/kg)

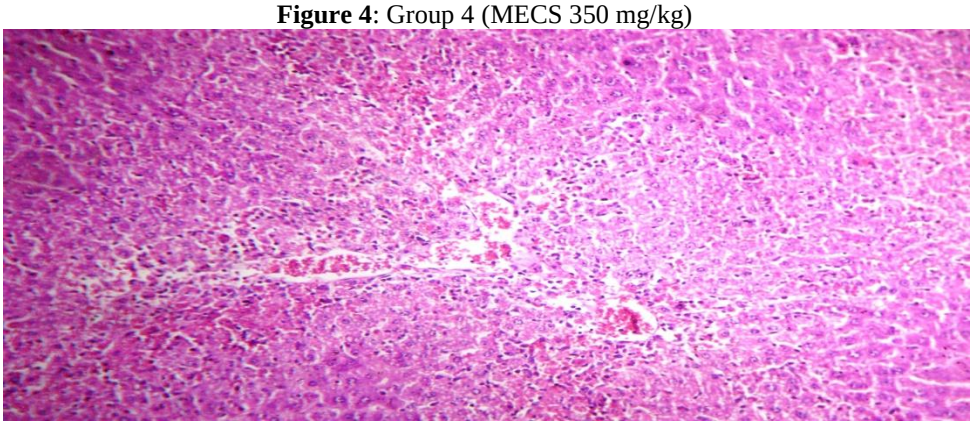


Figure 5: Group 5 (MECS 700 mg/kg)

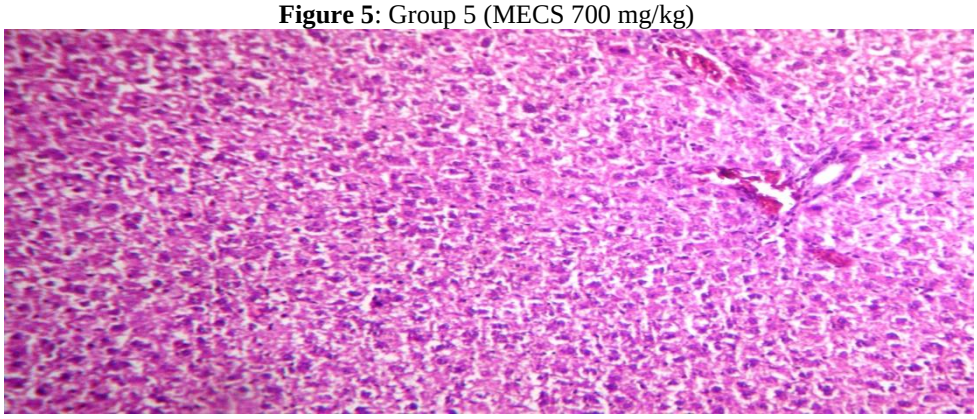


Figure 6: Group 6 (HEPP 300 mg/kg)

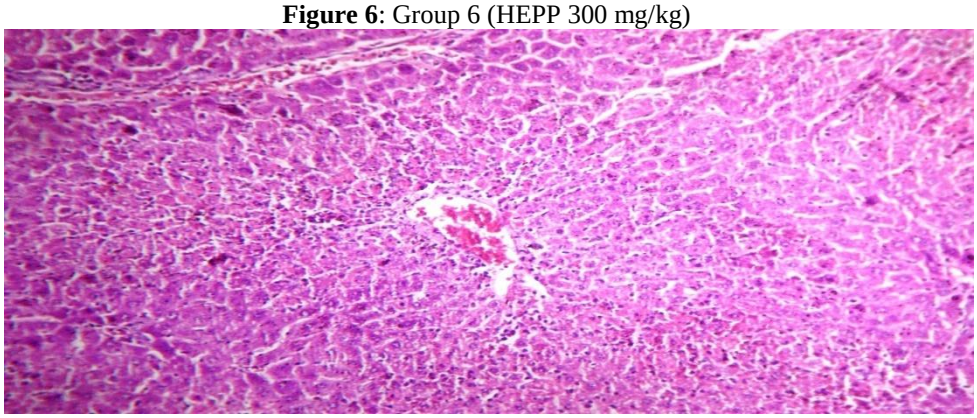
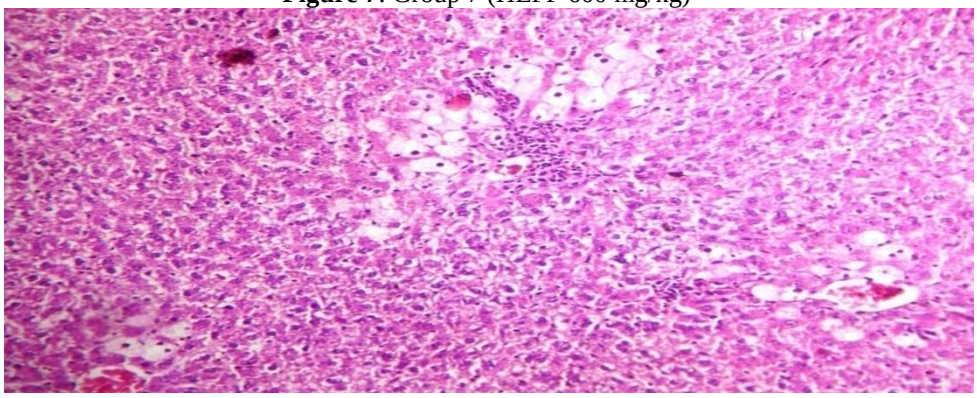


Figure 7: Group 7 (HEPP 600 mg/kg)



DISCUSSIONS

The present study reports the potential hepatoprotective activity of *Cucumis sativus* and *Pogostemon patchouli* against hepatic injury produced by carbon tetrachloride in rats. CCl_4 is a well known hepatotoxic agent and the preventive action of liver damage by CCl_4 has been widely used as an indicator of liver protective activity of drugs in general. This is the best-characterized chemical to study xenobiotic induced free radical mediated acute liver injury in rats. Since, the pathological lesions developed in CCl_4 treated animals closely resemble the symptoms of acute viral hepatitis and cirrhosis in human; it serves as an excellent model to assess the efficacy of hepatoprotectants. [31]

Hepatic damage can be assessed by level of released cytosolic transaminases including SGPT and SGOT in circulation. [32] A high level of SGOT indicates liver damage that may be due to viral hepatitis as well as cardiac infarction or muscle injury. SGPT is released in similar manner but is mostly present in hepatocytes. Therefore SGPT is more reliable as it is more specific to liver and thus a better parameter to detect liver injury. CCl_4 administration caused marked elevation in serum enzymes SGOT and SGPT. [33] Administration of both extracts significantly lowered the increased serum enzymes induced by CCl_4, indicating improvement of the functional status of the liver, which was also supported by the histopathological findings.

Alkaline phosphate is excreted normally via bile by liver. Prolong destruction of the hepatic cells results in more hepatic releases to exacerbate hepatic dysfunction and also causes elevation in the serum levels of ALP and bilirubin. [34] Hyperbilirubinaemia is a very sensitive test to substantiate the functional integrity of the liver. Decrease in elevated bilirubin level together with the suppression of activity of ALP in serum of rats treated with MECS and HEPP, suggested stabilization of biliary dysfunction of rat liver during acute injury with CCl_4.

Reactive oxygen species (ROS), such as superoxide anions and H_2O_2, are produced throughout cells during normal aerobic metabolism or it is the result of chemical induced toxicity. The intracellular concentration of ROS is a consequence of both their production and their removal by various antioxidants. The endogenous antioxidant system in mammalian cells consists of catalase (CAT) and reduced glutathione. These enzymes work in concert to detoxify superoxide anion and H_2O_2 in cells. Our results indicated that pretreatment of MECS and HEPP caused an increase in the activity of antioxidant enzymes.

Most of the hepatotoxic chemicals including CCl_4 damaged liver mainly by inducing lipid peroxidation directly or indirectly. In higher animals peroxy radicals are important agents that mediate lipid peroxidation thereby damaging cell membrane, leading to liver injury, artherosclerosis and kidney damage. In the present study, MECS and HEPP extracts were effective in reducing the production of TBARS. They reported the inhibition of malondialdehyde (MDA) which was comparable with silymarin; MDA is a major reactive aldehyde that appears during the peroxidation of biological membrane polyunsaturated fatty acid. Therefore, the hepatic content of MDA is used as an indicator of liver tissue damage involving a series of chain reactions.

CCl_4 is a hepatotoxin which upon administration causes liver fibrosis, necrosis and cirrhosis sequentially. CCl_4 administration also results in ballooning, infiltration of lymphocytes and hydropic degeneration with a clear cytoplasm and vacuolization. [35] Fatty changes due to CCl_4 revealed accumulation of lipid droplets in cytoplasm of hepatocytes. In the present study, pyknosis was seen in the group treated with CCl_4. Pyknosis refers to the irreversible condensation of chromatin in the nucleus of a cell undergoing programmed cell death or apoptosis and it is followed by fragmentation of the nucleus. [36] Concerning DNA fragmentation, CCl_4 may have role in increasing DNA degeneration in rat liver.

CONCLUSION

In conclusion, it may be mentioned that the altered biochemical profiles due to CCl_4 exposure is reversed towards normalization by *Cucumis sativus* and *Pogostemon patchouli* extract and the effect was more pronounced with the high doses of extracts. The contents of the extract not only protect the integrity of plasma membrane but at the same time restored bodily antioxidant system. Beneficial effect of MECS and HEPP may be due to the presence of high antioxidant activity. The results suggests that the compound present in the plant extracts efficiently work on the liver to keep it normally functioning so components responsible for hepatoprotective action needs to be identified and isolated for future work.

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ABBREVATIONS

CCl_4: Carbon tetrachloride
ALP: Alkaline phosphate,
SGPT: Serum glutamic pryuvic transaminase
SGOT: Serum glutamic oxaloacetic transaminase,
TBIL: Total Bilirubin,
TPROT: Total Proteins,
LPO: Lipid peroxides,
GSH: Reduced Glutathione,
Catalase: CAT,
H_2O_2: Hydrogen peroxide
HEPP: Hexane extract of *Pogostemon patchouli*,
MECS: Methanolic extract of seeds and leaves of *Cucumis sativus*,
TBA:Thiobarbituric acid,
TCA: Trichloroacetic acid,
CMC: Carboxy methyl cellulose,
DTNB: 5, 5'-dithiobis [2-nitrobenzoic acid],
MDA: Malondialdehyde