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Hepatoprotective and antioxidant activities of methanol extract of *Michelia champaca* on carbon tetra chloride induced liver damage

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ABSTRACT

Michelia champaca, a healthful plant and it's normally utilized in people medication to treat varied diseases. The aim of the study was to evaluate the hepatoprotective & invivo antioxidant activities of *Michelia champaca* against carbon tetrachloride induced liver injury in rats. The methanol extract of *Michelia champaca* at dose of 250 and 500 mg/kg were administered orally once daily for seven days. Serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), total bilirubin (TB), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and total protein (TP) were estimated from serum. In-vivo antioxidant activity of methanol extract of *Michelia champaca* was evaluated by various assays including catalase (CAT), superoxide dismutase (SOD), reduced glutathione, glutathione peroxidase (GPx), glutathione reductase (GRD) and malondialdehyde (MDA) levels in liver tissues. Histopathological examination of the liver sections was carried out to support the induction of hepatotoxicity. The results of the study indicated that, methanol extract of *Michelia champaca* 500 mg/kg showed a major decrease in SGPT, SGOT, TB, ALP, LDH levels that were all elevated within the carbon tetra chloride treated group. The *Michelia champaca* extract showed considerably redoubled the degree of CAT, SOD, GSH, GPx, GRD and reduce the degree of MDA. *Michelia champaca* leaves extract therapy also protective effects against histopathological alterations. From the above study it can be concluded that methanol extract of *Michelia champaca* had hepatoprotective & antioxidant activities against carbon tetra chloride induced hepatic damage in experimental animals.

Keywords: *Michelia champaca*, carbon tetra chloride, hepatoprotective, antioxidant activity.

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INTRODUCTION

Michelia champaca Linn known as *champaca* is belonging to the family of Magnoliaceae. It consists of 12 genera and 220 species of evergreen trees and shrubs, native to tropical and subtropical South and Southeast Asia (Indomalaya), including southern China. It is a very tall tree that grows up to 30m tall. The young branches are covered with grey hairs. The leaves are ovate in shape and are up to 30.5cm long and 10.2cm wide narrowing to a fine point at the apex. Conventionally it is widely used in both Ayurveda and Siddha medicine. It is being used in fever, colic, leprosy, post-partum protection and in eye disorders[1]. Juice of the leaves of *Michelia champaca* is given with honey in cases of colic. The flower oil is useful in cephalalgia, opthalmia and gout [2]. The flowers and fruits are considered stimulant, antispasmodic, tonic, stomachic, bitter and cool remedies and are used in dyspepsia, nausea and fever. Flower, flower buds and fruits are useful in ulcers, skin disease wounds[3]. The flowers mixed with sesamum oil forms an external application in vertigo[4] and also applied to foetid discharges from the nostrils. Various pharmacological activities of *Michelia champaca* were reported in literature such as antitumor[5], anti-inflammatory[6], antidiabetic[7], leishmanicidal[8], wound healing[9], antiinfective[10], antioxidant[11], diuretic[12], antiulcer[13], antibacterial[14], antifertility[15], anthelmintic[16] and cardio protective[17]. *Michelia champaca* consists of various secondary metabolites such as alkaloids, saponins, tannins, sterols, flavonoids and triterpinoids[18, 19]. Despite remarkable advances in modern medicine, hepatic disease remains a worldwide health problem, thus the search for new medicines is still ongoing. Hepatic cells participate in a variety of metabolic activities; therefore the development of liver protective agents is of paramount importance in the protection from liver damage. The literature has constantly shown that hepatoprotective effects are associated with plant extracts rich in antioxidants. Many compounds and extracts from plants have thus been evaluated for hepatoprotective and antioxidant effects against chemically-induced liver damage. Moreover, research on hepatoprotective medicinal plants as a major indicator of the general screening systems can trigger the safety evaluation in the early phase of drug discovery because most of the toxic compounds are metabolized in liver. To the best of our knowledge, the hepatoprotective effect of *Michelia champaca* leaves against CCl_4-induced liver injury in rats has not been demonstrated. Hence, the present study focused on evaluating the hepatoprotective and antioxidant effects of methanol extract from *Michelia champaca* leaves on carbon tetra chloride -induced liver injury in rats.

MATERIALS AND METHOD

Chemicals

All chemicals used in the study were of analytical grade. CCl_4 was procured from Krishna Chemicals Pvt. Ltd., Gujarat, and Silymarin from GVK Bio sci, Hyderabad.

Collection of plant material

Michelia champaca was collected from Tirupati (Andhra Pradesh) and further identified, confirmed & authenticated by Dr. Madavchetty, Professor, Botany department, Sri Venkateswara University, Tirupati. Voucher specimen No (GIP-Plant No-005) has retained in GITAM Institute of Pharmacy, GITAM University. The collected leaves of *Michelia champaca* were washed with tap water. The leaves were cut in to small pieces and air-dried thoroughly under shade (at room temperature) for 2months to avoid direct loss of phytoconstituents from sunlight. The shade dried material was powdered using the pulverizer and sieved up to 80 meshes. It was then homogenized to fine powder and stored in air tight container for furthers analysis.

Preparation of *Michelia champaca* extract

The leaves of *Michelia champaca were* refluxed with methanol in a soxhlet extractor for 48 hrs. The excess solvent was removed from the extract by vacuum rotary flash evaporator and concentrated over the hot water bath. Finally dried extract was stored in desiccators for hepatoprotective and antioxidant studies.

Preliminary phytochemical screening

The methanol extract was subjected to various phytochemical studies to identify the presence of various phytoconstituents like alkaloids, glycosides, steroids, flavonoids, tannins, carbohydrates, proteins and terpenes[20].

Safety Evaluation

The toxicity study was carried out using OECD guide lines No. 423. Three female rats of the same age group and weight were taken in a single dose up to the highest dose of 2000 mg/kg B/W orally. The animals were observed for 1hr continuously and then hourly for 4hr, and finally after every 24hr up to 15 days for any mortality or gross behavioral changes[21].

IN-VIVO HEPATOPROTECTIVE ACTIVITY

Experimental animals

Albino rats of either sex, weighing 200-250gm were procured and maintained in standard laboratory conditions. The animals were fed with standard pellet diet and water ad libitum. The

study was approved by the Institutional Animal Ethical Committee (IAEC) and experiments were conducted as per the guidelines of CPCSEA.

Experimental design

A total of 30 rats were divided into5 groups of 6 rats each. Group I served as normal control and received only the vehicle (1mL/kg/day orally). Group II received CCl_4 1mL/kg (1:1 of CCl_4 in olive oil) i.p. once daily for 7 days. Group III received CCl_4 1mL/kg (1:1 of CCl_4 in olive oil) i.p. and silymarin 100 mg/kg orally (p.o.) for 7 days. Groups IV, V were administered with methanol extract of leaves of *Michelia champaca* at 250, and 500 mg/kg body weight p.o., respectively. Additionally, Groups IV, V intraperitoneally received a dose of the CCl_4–olive oil mixture (1:1, 1 mL/kg body weight) once daily for seven days. All rats were sacrificed by cervical dislocation 24hrs after the last treatment.

Biochemical parameters

The blood samples were subjected to clot for 45 min at room temperature. Serum was separated by centrifugation at 3500 rpm at 37^0C for 15 min and analyzed for various biochemical parameters such as Serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), total bilirubin (TB), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and total protein (TP).

Antioxidant parameters

For estimating antioxidant activity, animals were sacrificed and liver was excised, rinsed in ice – cold normal saline followed by 0.15M Tris-HCl (pH-7.4) blotted dry and weighed. A10% w/v of homogenate was prepared in 0.15M Tris-HCl buffer and processed for the estimation of lipid peroxidation (TBARS). A part of homogenate after precipitating proteins with trichloroacetic acid (TCA) was used for estimation of glutathione. The remaining homogenate was centrifuged at 1500 rpm for 15 min at 4^0C. The supernatant thus obtained was used for the estimation of catalase (CAT), superoxide dismutase (SOD), reduced glutathione, glutathione peroxidase (GPx), glutathione reductase (GRD) and malondialdehyde (MDA).

Estimation of hepatoprotective parameters

SGOT & SGPT

Serum transaminases (GOT and GPT) were determined by the method of Reitman and Frankel[22]. Each substrate (0.5mL) [either α -L-alanine (200mM) or L-aspartate (200mM) with 2mM α -ketoglutarate] was incubated for 5 min at 37°C. A 0.1mL of serum was added and the volume was adjusted to 1.0mL with sodium phosphate buffer (pH 7.4; 0.1M). The reaction mixture was incubated for 30 and 60 min for GPT and GOT, respectively. A 0.5mL of 2, 4-dinitrophenyl

hydrazine (1mM) was added to the reaction mixture and left for 30 min at room temperature. Finally, the color was developed by the addition of 5mL NaOH (0.4 N) and the product formed was read at 505nm. Data was expressed as IUL^{-1}.

Bilirubin

Bilirubin content was estimated by method of Malloy and Evelyn[23]. The two test tubes were taken and each into was added 0.2ml of serum sample and 1.8 ml of distilled water. To the unknown, 0.5 ml of diazo reagent and to the blank, 0.5 ml of 1.5% hydrochloric acid was added. Finally, to each tube, 2.5 ml of methanol was added and then allowed to stand for 30 minutes in ice and absorbance was read at 540nm. For a standard curve, the above standard was diluted 1in 5ml methanol. The amount of direct reacting bilirubin was determined similarly by substituting 2.5ml of water for 2.5ml of methanol. The values were expressed as mg/dl.

Alkaline phosphatase

Alkaline phosphatase (ALP) was assayed by the method of Kind and King[24]. The reaction mixture of 3.0 ml containing 1.5 ml of buffer (carbonate-bicarbonate buffer, 0.1M, pH 10.0), 1 ml of substrate and requisite amount of the enzyme sources was incubated at 37^oC for 15 minutes. The reaction was arrested by the addition of 1.0 ml of Folins phenol reagent. The control tubes were received the enzyme after arresting the reaction. The contents was centrifuged and to the supernatant, 1.0 ml of 15% sodium carbonate solution, 1.0ml of substrate and 0.1ml of magnesium chloride (0.1M), was added and mixture was incubated for 10 minutes at 37^0C. The colour was read out 640 nm against the blank.

Lactate dehydrogenase activity

Lactate dehydrogenase (LDH) activity was estimated in serum by the standard method [25, 26]. The reaction mixture consisted of 0.1mL of nicotinamide adenine dinucleotide (NADH)-reduced disodium salt (0.02 M), 0.1mL of sodium pyruvate (0.01 M), 0.1mL of serum, and made up to 3mL with sodium phosphate buffer (0.1M; pH 7.4). The changes in the absorbance was recorded at 340nm at 30s interval each for 3 min and the enzyme activity was calculated using a molar extinction coefficient of $6.220M^{-1}$ cm^{-1} and it was expressed as nanomoles NADH oxidized min^{-1} mg^{-1} protein.

Estimation of antioxidant parameters

Catalase assay

Catalase activity (CAT) was measured by the method of Aebi[27]. A 0.1mL of supernatant was added to cuvette containing 1.9mL of 50mM phosphate buffer (pH 7.0). Reaction was started by

the addition of 1.0mL of freshly prepared 30mM H_2O_2. The rate of the decomposition of H_2O_2 was measured spectrophotometrically at 240 nm. Activity of CAT was expressed as Umg^{-1} of protein.

Superoxide dismutase assay

Superoxide dismutase (SOD) activity was analyzed by the method described by Rai et al [28]. Assay mixture contain 0.1mL of supernatant, 1.2mL of sodium pyrophosphate buffer (pH 8.3; 0.052M), 0.1mL of phenazine methosulfate (186 mM), 0.3mL of nitroblue tetrazolium (300 mM), and 0.2mL of NADH (750 mM). Reaction was started by the addition of NADH. After incubation at 30^{o}C for 90s, the reaction was stopped by the addition of 0.1mL of glacial acetic acid. Reaction mixture was stirred vigorously with 4.0mL of n-butanol. Color intensity of the chromogen in the butanol was measured spectrophotometrically at 560nm and the concentration of SOD was expressed as Umg^{-1} of protein.

Reduced glutathione assay

Reduced glutathione (GSH) was measured according to the method of Ellman[29]. The equal quantity of homogenate was mixed with 10% trichloroacetic acid and centrifuged to separate the proteins. To 0.01 ml of this supernatant, 2ml of phosphate buffer (pH 8.4), 0.5 ml of 5'5-dithio, bis (2-nitrobenzoic acid) and 0.4ml double distilled water was added. Mixture was vortexed and the absorbance read at 412nm within 15 min. The concentration of glutathione was expressed as µ g/mg of protein.

Glutathione reductase assay

Glutathione reductase (GRD) activity was assayed by the method of Mohandas et al [30]. The assay system consist of 1.65mL sodium phosphate buffer (0.1M; pH 7.4), 0.1mL EDTA (0.5 mM), 0.05mL oxidized glutathione (1mM), 0.1mL NADPH (0.1 mM), and 0.05mL supernatant in a total mixture of 2mL. The enzyme activity was quantified by measuring the disappearance of NADPH at 340nm at 30s intervals for 3min. The activity was calculated using a molar extinction coefficient of 6.22 X $10^3M^{-1}cm^{-1}$ and was expressed as nanomoles of NADPH oxidized min^{-1} mg^{-1} protein. Protein content in the tissue was determined by earlier method reported, using bovine serum albumin (BSA) as the standard.

Glutathione peroxidase assay

Glutathione peroxidase (GPx) activity was determined by the method described by Wendel[31]. The reaction mixture consist of 400µL of 0.25M potassium phosphate buffer (pH- 7.0), 200 mL supernatant, 100 µL GSH (10 mM), 100 µL NADPH (2.5mM), and 100µL GRD ($6UmL^{-1}$).

Reaction was started by adding 100µL hydrogen peroxide (12mM) and absorbance was measured at 366nm at 1min intervals for 5 min using a molar extinction coefficient of 6.22X 10^3 $M^{-1}cm^{-1}$. Data was expressed as mU mg^{-1} of protein.

Lipid peroxidation assay

Lipid peroxidation (LPO) was measured by the method of Liu et al [32]. Acetic acid 1.5mL (20%; pH 3.5), 1.5 of TBA (0.8%), and 0.2mL of sodium dodecyl sulfate (8.1%) was added to 0.1ml of supernatant and heated at 100^{o}C for cooled and 60 min. Mixture was cooled, and 5mL of n-butanol:pyridine (15:1) mixture and 1mL of distilled water was added and vortexed vigorously. After centrifugation at 1200g for 10min, the organic layer was separated and the absorbance was measured at 532nm using a spectrophotometer. Malonyldialdehyde (MDA) was an end product of LPO, which reacts with TBA to form pink chromogen–TBA reactive substance. It was calculated using a molar extinction coefficient of 1.56 X $10^5 M^{-1}$ cm^{-1} and it was expressed as nanomoles of TBARS mg^{-1} of protein.

Histopathological studies

Paraffin sections of buffered formalin-fixed liver samples were stained with hematoxyline-eosin to study the histological structure of control and treated (toxicant, *Michelia champaca* leaf extract, silymarin) rats liver.

Statistical analysis

The data were presented as mean ± SEM and analyzed using the one-way analysis of variance (ANOVA) procedure of Statistical Analysis System (Graph Pad Prism Version 5.0) followed by Dunnett's Multiple Comparison test to identify significant differences of treated groups from the controls ($p < 0.001$).

RESULTS AND DISCUSSION

Percentage yield

The percentage yield (Table 1.) of the methanol extract of *Michelia champaca* was found to be 32.4%.

Table 1: Percentage yield of methanol extract of *Michelia champaca*

Extract Name	% Yield (w/w)
Methanol extract of *Michelia champaca*	32.4

Preliminary phytochemical screening of *Michelia champaca*

It was found that the preliminary phytochemical screening of *Michelia champaca* showed the presence of alkaloids, steroids, triterpinoids, tannins, flavanoids, phenolics, and saponins in

methanol extract, where as absence carbohydrates, proteins, amino acids, and glycosides. The preliminary phytochemical screening for various secondary metabolites was tabulated in Table 2.

Table 2: Qualitative analysis (group tests) of methanol extract of *Michelia champaca*

Name of Phytochemical test	Observation report of methanol extract of *Michelia champaca*
Alkaloids	+
Amino Acids	-
Carbohydrates	-
Flavonoids	+
Glycosides	-
Phenolics	+
Proteins	-
Saponins	+
Steroids	+
Triterpinoids	+

"+" indicates positive; "-" indicates negative

Toxicity study

When rat fed with methanol extract of *Michelia champaca* up to 2000 mg kg^{-1},p.o. exhibited no mortality or any sign of gross behavioral changes when observed initially for 24hrs, and finally up to 15 days.

Carbon tetrachloride induced hepatotoxicity

The impact of CCl_4 on the levels of SGPT, SGOT, bilirubin, ALP, LDH, and total protein in the serum were summarized in Table 3. The hepatotoxicity induced by CCl_4 is due to its metabolite $CCl_3^{\bullet}$, a free radical that alkylates cellular proteins and other macromolecules with a simultaneous attack on polyunsaturated fatty acids, in the presence of oxygen, to produce lipid peroxides, leading to liver damage. Hepatocellular necrosis leads to elevation of the serum marker enzymes such as SGPT, SGOT, TB, ALP, and LDH which are released from the liver into blood [33]. The present study revealed a significant increase in the activities of SGOT, SGPT, bilirubin, ALP, and LDH levels on indicating considerable hepatocellular injuries [34, 35]. Administration of *Michelia champaca* methanol extract at different doses level (250 and 500 mg/kg) attenuated the increased levels of the serum enzymes, produced by CCl_4 and caused a subsequent recovery towards normalization comparable to the control groups animals.

Table 3: Hepatoprotective parameters of methanol extract of *Michelia champaca* against CCl_4 induced damaged

Biochemical Parameters	Control group	CCl_4 treated group	*Michelia champaca* methanol extract (mg/kg)		Silymarin (25 mg/kg)
			250	500	
SGPT (IUL^{-1})	46.9±74	186.8±11.4	119.3±4.2	65.7±10.5	52.7±76
SGOT (IUL^{-1})	106±9.5	254±15.4	191 ±9.06	139±6.21	102±9.5
Total bilirubin (mg/dl)	2.7±0.04	4.24±0.14	1.8±0.56	3.12±1.24	2.2±0.05
ALP (KA Units)	42.8±1.18	74.6±0.99	43.2±0.55	41.4±1.28	45.2±1.25
LDH (nanomoles NADH oxidized min^{-1} mg^{-1} protein)	375.6±11.4	524.8±14.5	439.8±2.1	445.2±1.15	404.7±0.13
Total protein (mg/dl)	9.7±0.15	4.28±0.06	5.9±0.62	7.91±0.55	8.2±0.13

Data are given as mean SD of six animals. * Significant difference ($p < 0.05$) from control or CCl_4 -treated rats. ALP = alkaline phosphatase; BIL=bilurubin, LDH= Lactate dehydrogenase; SGOT = serum glutamic oxaloacetic transaminase; SGPT = serum glutamic pyruvic transaminase.

***In vivo* antioxidant assays**

In CCl_4 induced hepatotoxicity, the balance between ROS production and these antioxidant defenses may be lost, 'oxidative stress' results, which through a series of events deregulates the cellular functions leading to hepatic necrosis. Hence result showed (Table 4) that the activities of CAT, SOD, GRD, GSH and GPx in group treated with CCl_4 declined significantly along with significantly increased lipid peroxidation level (expressed as MDA) than that of normal group. Co-administration of methanol extract of *Michelia champaca* at a dose of 250 and 500 mg/kg for 7 days markedly prevented these CCl_4 induced alteration and maintained enzymes level near to normal values. Standard (silymarin) treated group also significantly increased the level of CAT, SOD, GRD, GSH and GPx in CCl_4 induced toxic rats.CAT considered as most important H_2O_2 removing enzyme and also a key component of anti oxidative defense system. Here CAT activity was increased and then restored to normal levels on administration of methanol extract of *Michelia champaca*. SOD plays an important role in the elimination of ROS and protects cells against the deleterious effects of super oxide anion derived from the peroxidative process in liver and kidney tissues and the observed increase in SOD activity suggests that the methanol extract of *Michelia champaca* has an efficient protective mechanism in response to ROS. The reduced activities of GRD and GPx observed point out the hepatic damage in the rats administered with CCl_4 but the treated with, 250 and 500mg/kg of *Michelia champaca* methanol extract groups showed significant increase in the level of these enzymes, which indicates the antioxidant activity of the *Michelia champaca*. Regarding non enzymic antioxidants, GSH is a critical determinant of tissue

susceptibility to oxidative damage and the depletion of hepatic GSH has been shown to be associated with an enhanced toxicity to chemicals, including CCl_4. Furthermore, a decrease in hepatic tissue GSH level was observed in the CCl_4 treated groups [36]. The increase in hepatic GSH level in the rats treated with, 250 and 500 mg/kg of *Michelia champaca* methanol extract may be due to de novo GSH synthesis or GSH regeneration.vThe level of lipid peroxide is a measure of membrane damage and alterations in structure and function of cellular membranes. In the present study, elevation of lipid peroxidation in the liver of rats treated with CCl_4 was observed. The increase in LPO levels in liver suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defence mechanisms to prevent the formation of excessive free radicals. Treatment with *Michelia champaca* methanol extract significantly reversed all the changes. Hence, it is possible that the mechanism of hepatoprotection of *Michelia champaca* may be due to its antioxidant activity.

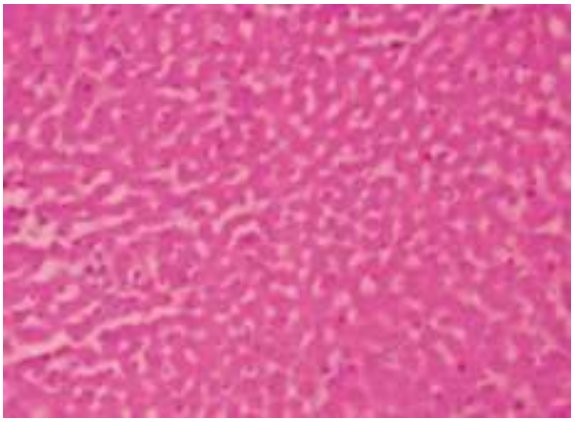
Table 4: Effect of methanol extract of *Michelia champaca* on biochemical parameters

S.no	Treatment	CAT (Umg^{-1} of protein)	SOD (Umg^{-1} of protein)	GSH (µg/mg of protein)	GRD (nanomoles of NADPH oxidized min^{-1} mg^{-1} protein)	GPx (mU mg^{-1} of protein)	MDA (nanomoles of TBARS mg^{-1} of protein)
1	Normal	189.6±0.06	59.4±6.5	9.2±07	18.6±1.39	276.6±0.99	4.6±03
2	Toxic Control	46.5±07	24.6±0.34	3.4±09	7.4±1.16	167.4±0.85	13.6±02
3	Standard	179.4±15	59.8±0.45	8.8±08	15.2±0.04	259.2±18	5.8±03
4	MEMC 250 mg/kg	41.5± 0.3	131.5±1.05	9.4±2.7	207.4±1.7	6.1±3.4	8.9±1.05
5	MEMC 500 mg/kg	53.7±0.5	157.9±1.1	11.9±1.2	219.8±0.11	6.2±5.9	6.4±04

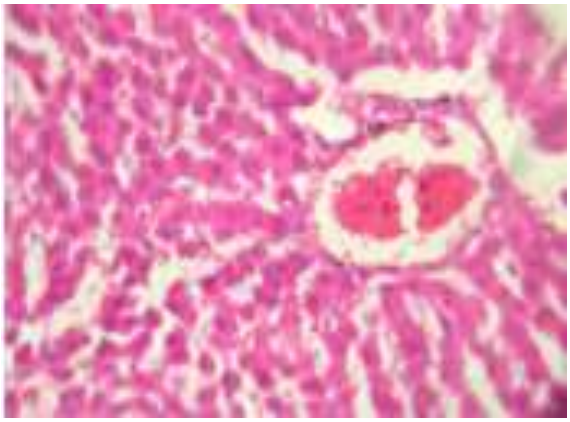
Data are given as mean SD of six animals. * Significant difference ($p < 0.05$) from control or CCl_4 -treated rats. SOD= Super oxide dismutase; CAT= Catalase, GPx= Glutathione peroxidase; GRD = glutathione reductase; MDA = Malonyldialdehyde; RD = Reduced glutathione

Histopathological observations

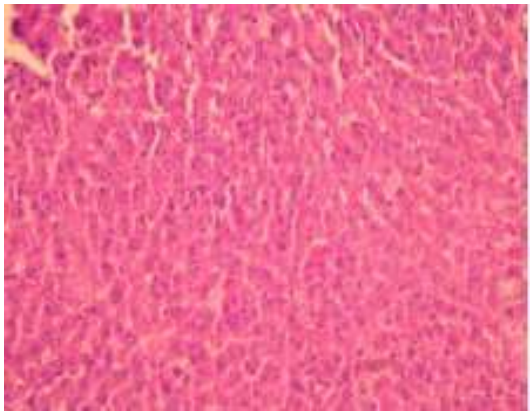
The hepatoprotective effect of the *Michelia champaca* methanol extract was further accomplished by the histopathological examinations. *Michelia champaca* methanol extract at different dose levels offers hepatoprotection, but 500mg/kg was much more effective than the lower dose. The histopathological studies of the liver showed fatty changes, swelling, necrosis, cell vacuolization, degenerated nuclei and inflammatory, infiltration with loss of hepatocytes in CCl_4 intoxicated rats (II) in comparison with normal rats (I). The liver sections of rats treated with the lower (IV) and higher (V) of the extract showed reduced degeneration of hepatocytes, normalization of fatty changes, decrease in vacuolization and necrosis of the liver. Silymarin treated group showed considerable reduction in necrosis and damage of liver cells (III).



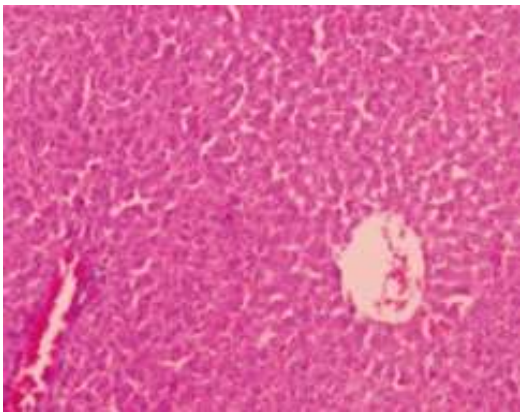
I-Normal



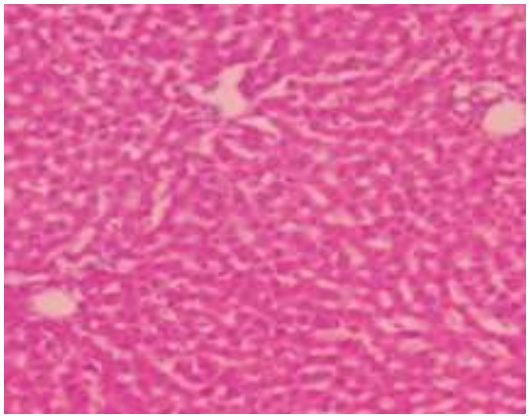
II- CCl4- Olive oil (1:1, 1 ml/kg)



Standard (Silymarin 25mg/kg)



V-High dose (500 mg/kg)



IV-Low dose (250 mg/kg)

CONCLUSION

As methanol extract of *Michelia champaca*, in the dose of 500 mg/kg, p.o., has improved the biochemical (SGPT, SGOT, TB, ALP, LDH and TP) and antioxidant parameters (CAT, SOD, GRD, GSH, GPx and MDA) levels significantly, which were comparable with silymarin. On the basis of the study it can be concluded that methanol extract of *Michelia champaca* possesses both

hepatoprotective and invivo antioxidant activities and can be employed in protecting tissue from oxidative stress.

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