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ANALYSIS OF LIPID PEROXIDATION AND HEPATOPROTECTIVE ACTIVITY OF THE METHANOLIC EXTRACT OF *MICROCOS PANICULATA* FRUITS ON CARBON TETRACHLORIDE-MEDIATED HEPATOTOXICITY IN RATS

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Abstract

The study was designed to investigate the lipid peroxidation (LPO) and hepatoprotective activity of the methanolic extract of *Microcos paniculata* fruits (FME) against CCl_4 mediated hepatotoxicity in rats. Lipid peroxidation of FME was determined by estimating the thiobarbituric acid reactive substances (TBARs). After that hepatoprotective activity of FME was evaluated by checking the alterations in serum biochemical indicators (SGPT, SGOT, ALP, TP and TB) and histopathological structures of the Sprague Dawley rats. In case of LPO of FME, the CCl_4 control group showed significant acute hepato cellular damage and biliary obstruction that was indicated by the elevated level of LPO (20.85±0.64 nmoL/mg of protein) (*P<0.05, *vs.* control). But the groups which received standard drug sylimarin and FME at 100 mg/kg as well as 400 mg/kg body weight orally showed significant (*P<0.05, *vs.* control) decrease in the elevated level of LPO (9.02±0.78 nmoL/mg of protein and 8.25±0.59 nmoL/mg of protein respectively). Moreover, during the evaluation of hepatoprotective activity of FME, the CCl_4 treated group revealed significant acute hepato cellular damage that was indicated by not only elevated levels of SGPT, SGOT, ALP and TB but also by reduced level of TP (*P<0.05, *vs.* control). Both FME 200 and 400 mg/kg restored the altered levels of these parameters indicated by CCl_4. Results of the study were supported by the histopathological observations also. From the results, it can be concluded that FME exerted protective activity in rats against the CCl_4-induced hepatotoxicity.

Keywords: *Lipid peroxidation, Hepatoprotective, Microcos paniculata, Carbon tetrachloride*.

Introduction

Being the most crucial organ of the body, liver accomplishes several biochemical and physiochemical tasks including metabolism, detoxification, scavenging free radicals etc. Currently human health is affected by acute hepatic failure which can be due to toxins, drugs, hepatitis virus infection and so on [1, 2, 3]. Though liver has elevated regeneration capability but severe inflammation arises from continued liver damage which can start several events consecutively such as fibrosis, cirrhosis as well as the liver failure of the final step. To determine the hepatoprotective activity of a drug, CCl_4 is commonly used as hepatotoxicant. Cytochrome P450 2E1 can promptly transform CCl_4 into $\cdot CCl_3$. This $\cdot CCl_3$ is quickly converted into $CCl_3O_2\cdot$ [4]. As a result, acute hepatic injury occurs that is marked by hepatitis, deep necrosis as well as fatty degeneration [2]. Medicinal plants provide natural products, which are thought as secured as well as efficient therapeutic agents for liver diseases [1].

Microcos paniculata belongs to the family Tiliaceae having the local name as not only Kathgua but also Fattashi, which grows as like as shrub or small tree. It can grow naturally and distributed also in Bangladesh. By tradition, hepatitis, diarrhea, heat stroke, fever, dyspepsia, wounds and colds are treated by this plant. However, it is active against the digestive system, along with its insectisidal property. Several studies were performed for knowing some activities of this plant such as, antipyretic, larvicidal, cytotoxic, insecticidal, neuropharmacological, free radical scavenging, antidiarrheal, analgesic, anthelmintic, cytotoxic, α-glucosidase inhibition and antimicrobial. Besides, reports were found having its anti-inflammatory, brine shrimp lethality as well as nicotinic receptor antagonistic properties. In addition to, *M. paniculata* can also prevent both the angina pectoris and coronary heart disease. Additional reports revealed about its acute toxicity study [5, 6, 7, 8, 9, 10, 11, 12].

Till now, no animal work has been carried out to reveal the liver lipid peroxidation and hepatoprotective activity of the methanolic extract of *M. paniculata* fruits. Therefore, the present study was designed to evaluate the liver lipid peroxidation (LPO) and hepatoprotective activity of the methanolic extract of *M. paniculata* fruits (FME) by using animal models.

Methods

Chemicals and reagents

Silymarin and olive oil were purchased from the Bangladeshi manufacturer Square Pharmaceuticals Ltd.; various kits like SGPT, SGOT and ALP were purchased from the German manufacturer Human but TP and TB kits were purchased from the Czech Republic manufacturer Erba Lachema. Again, hematoxylin and eosin were obtained from BDH Chemicals Ltd, England and Merck, Germany respectively. All solvents (Methanol, formalin, alcohol, xylene, CCl_4, thiobarbituric acid, sodium dodecylsulphate, acetic acid, n-butanol and pyridine) used were of analytical grade and obtained from Merck, Germany. ketamine was purchased from Incepta Pharmaceutical Ltd. Bangladesh. Paraffin wax was collected from Shijiazhuang Lanyang Chemicals Co. Ltd. China. Moreover, hematoxylin and eosin were bought from Merck, Germany.

Plant material

M. paniculata fruits were collected from the Jahangirnagar University campus (23.8791° N, 90.2690° E), Savar, Dhaka, Bangladesh in November, 2013. Species identification was verified by Sarder Nasir Uddin, Principal Scientific Officer at the Bangladesh National Herbarium (accession number 35348). A dried specimen was deposited in the herbarium for future reference.

Preparation and extraction of plant material

Methanolic extraction was carried out by using 200 g of powdered fruits of *M. paniculata*. Fresh fruits were rinsed 3–4 times

successively with running water and once with sterile distilled water. Washed plant material was then dried in the shade for a period of 7 d. The dried plant material was then ground by using a laboratory grinding mill (MACSALAB 200 Cross Beater, Eriez, Erie, Pennsylvania, U.S.A.) and passed through a 40-mesh sieve to get fine powder. Powdered fruits (200 g) were extracted in 2 L of methanol, using a soxhlet apparatus and a hot extraction procedure. Whatman No.1 filter papers were used to filter the liquid extract. The filtrate was then dried in a hot air oven (BST/HAO-1127, Bionics Scientific Technologies Pvt. Ltd., Delhi, India) at 40 ℃. The extraction yield of FME was 11.08% (w/w), which was stored at 4 ℃ for additional studies.

Experimental animals

Fifty Sprague Dawley rats, 6–7 weeks old, weighting 150-190 g were collected from the Department of Pharmacy, Jahangirnagar University, Savar, Dhaka, Bangladesh, which were used in these experiments. These animals were kept under standard environmental conditions, having relative humidity 55 %–65 %, 12 h light/12 h dark cycle and (27.0±1.0) ℃ temperature. Proper supply of foods and water *ad libitum* were ensured. Before the experiment, animals were adapted to the laboratory conditions for 1 week. The Institutional Animal Ethical Review Committee of Jahangirnagar University, Savar, Dhaka, Bangladesh approved the protocol used in the experiment conducted with these animals.

Analysis of liver lipid peroxidation

Lipid peroxidation of liver tissue was determined by estimating the thiobarbituric acid reactive substances (TBARs) according to the method of Ohkawa *et al.* 1979 [13] with slight modification. The tested samples including sylimarin as standard, normal saline (0.9% NaCl solution) as contol, carbontetrachloride treated control & and plant extracts were given orally to the rats, except CCl_4 treated control which were given intraperitonially & investigated for their biochemical parameter. Twenty five experimental animals were randomly selected and divided into five groups denoted as group I, group II, group III, group IV and group V consisting of five rats in each group. Each group received a particular treatment. Prior to any treatment, each rat was weighed properly and the doses of the test samples and control materials were adjusted accordingly. A total of 25 rats were divided into 5 groups of 5 animals each.

Group I: Vehicle control and received orally with normal saline 5mL/kg body weight daily for 7 days.

Group II: Carbontetrachloride treated control and received orally normal saline (5mL/kg body wt/day) and also received CCl_4 (1 mL/kg i.p.) diluted in olive oil (1:1) on fifth and seventh day.

Group III: Animals served as standard received silymarin (100 mg/kg/day, p.o) for seven days.

Group IV: Served as test and received methanolic extract of fruit of *Microcos paniculata* (200 mg/kg/p.o) daily for seven days.

Group V: Served as test and received methanolic extract of fruit of *Microcos paniculata* (400 mg/kg/p.o) daily for seven days.

On day 5 & 7, Carbontetrachloride diluted in olive oil (1:1) was given intraperitoneally (i.p) at a dose of 1ml/kg body wt/day to all rats except the rats in Group I. After 24 hours of the second dose of CCl_4, all the rats were sacrificed by cervical decapitation and hepatic tissues were carefully excised and homogenized. Tissue homogenates (0.1 mL) from each rat of all groups were distributed into separated culture tubes. Then, 0.1 mL of 8.1% (w/v) sodium dodecylsulphate, 2 mL of 0.4% thiobarbituric acid in 20% acetic acid (pH 3.5) and 0.1mL distilled water were added to the test tubes. Each tube was tightly capped and heated in a boiling water bath at 95 ℃ for one hour. After cooling the tubes with tape water, 1.5 mL of n-butanol-pyridine (15:1, v/v)

was added and shaken vigorously for about 10 minutes. The tubes were then centrifuged at 2325 x g in room temperature (Digital centrifuge**;** DSC-1512SD) and the absorbance of the supernatant fraction was measured at 532nm.

Hepatoprotective activity evaluation

This test was performed with slight modifications as described by Porchezhian *et al.* [14]. A total of 25 rats were divided into 5 groups of 5 animals each. Group I served as vehicle control group and orally received normal saline at a dose of 5mL/kg body weight daily for 7 days. Group II was designed as CCl_4 treated control group and orally received normal saline (5mL/kg body weight /day for seven days) and also received CCl_4 (1 mL/kg; i.p.) diluted in olive oil (1:1) on fifth and seventh day. Group III animals served as standard group which received silymarin orally (100 mg/kg/day for seven days). Group IV and Group V served as test groups which received FME orally at a dose of 200 mg/kg and 400 mg/kg daily for seven days. On day 5 & 7, CCl_4 diluted in olive oil (1:1) was given (at a dose of 1mL/kg body weight; i.p.) to all rats except the rats in Group I. After 24 h of the second dose of CCl_4, all the rats were sacrificed by cervical decapitation under mild ketamine anesthesia, blood samples were collected separately from the retro orbital plexus and allowed to stand for 30 min at room temperature. The clear serum was separated by centrifugation at 2325 x g for 10 min (80-2 Electronic Centrifuge, Green point Co., Ltd., China) and serum samples were stored at -40°C. Biochemical parameters (SGPT, SGOT, SALP, TP and TB) were determined by using the serum samples through different assay kits [15, 16, 17, 18, 19].

Histopathological study

Quickly excised livers of the sacrificed rats were rinsed in ice cold normal saline and fixed in 10 % (v/v) neutral formalin solution for 12 h. The excised livers were then dehydrated through a graded series of alcohol (50%-100%), processed in xylene and fixed in paraffin wax. The paraffin blocks were cut into 5 µm thickness using a rotary microtome (Leica RM 2145- Rotary Microtome, Leica, Germany) and were mounted on slides and stained by using hematoxylin and eosin. The slides were then examined by using an Exacta-Optech microscope (Biostar B3 Bioculare, Exacta Optech+Labcenter SpA, San Prospero, Italy) at 40X, which was connected with a digital camera (Canon DS126181, Tokyo, Japan) [20, 21, 22, 23, 24].

Statistical analysis

All results are expressed as mean ± standard error (S.E.). All tests were analyzed statistically by one-way ANOVA followed by Dunnett's t test. $P<0.05$, *vs.* control was considered to be statistically significant. All data were analyzed using SPSS software (version 16; IBM Corporation, New York, USA).

Results

Analysis of liver lipid peroxidation

The results of investigation of LPO activity of FME in CCl_4 induced hepatotoxic rats were shown in figure 1.

In the CCl_4 control group, the significant acute hepato cellular damage and biliary obstruction was indicated by the elevated level of LPO (20.85±0.64 nmoL/mg of protein.*$P<0.05$ *vs.* control). But the FME 400mg/kg body weight p.o showed a significant decrease in the elevated level of LPO than standard silymarin and FME 200 mg/kg respectively (LPO of silymarin, FME 200 mg/kg and FME 400mg/kg are 9.02±0.78 nmoL/mg of protein, 12.08±0.62 nmoL/mg of protein and 8.25±0.59 nmoL/mg of protein consecutively.*$P<0.05$ *vs.* control).

Hepatoprotective activity evaluation

The result of FME on CCl_4 induced hepatotoxicity in rats are presented in table 1. In the CCl_4 treated group, the significant acute hepato cellular damage was indicated by not only elevated levels of SGPT, SGOT, ALP and TB but also by reduced level of TP (*$P<0.05$, *vs.* control). Besides, the administration of silymarin restored the altered level of these parameters indicated by CCl_4 and the values of these parameters of silymarin were close to

the values of these parameters of normal control group. Again, both FME 200 and 400 mg/kg restored the altered levels of these parameters indicated by CCl_4. However, except TB values of both lower and higher dose of FME and SGPT value of FME 400 mg/kg, other values of them were significant when compared to control (*P<0.05, *vs.* control).

Histopathological study

Results of the histopathological study provided supportive evidence for biochemical analysis. Histology of the liver section of normal control animals exhibited normal hepatic cells each with well-defined cytoplasm, prominent nucleus and nucleolus and well brought out central vein (Figure 5), whereas the CCl_4 intoxicated group animals showed hepatic necrosis with dilated blood vessels and infiltration of inflammatory cells (Figure 6). However administration of silymarin (100 mg/kg) almost normalized these defects in the histopathological architecture of the liver nearly to the level of the control treated group (Figure 7). On the other hand, the liver section of the rat treated with FME 400mg/kg showed less necrosis and more activity than FME 200 mg/kg in protecting the liver cells from CCl_4 injury (Figure 8 & 9).These observations are depicted below in the photomicrographs (Figure: 5-9).

Discussion

The thiobarbituric acid (TBA) assay is the most commonly used assay to study lipid peroxidation. Addition of iron or copper salts to biological molecules causes site-specific formation of oxygen-derived free radicals such as peroxyl radical ($ROO^{\bullet}$) and hydroperoxyl radical ($HOO^{\bullet}$) which can trigger lipid peroxidation chain reactions. This reaction occurs by abstracting a hydrogen atom from a side chain methylene carbon of polyunsaturated fatty acids and transforms it into lipid hydroperoxides. These lipid hydroperoxides easily decompose to secondary products, such as aldehydes and MDAs, which can be detected by thiobarbituric acid reactive substances (TBARS) method. Level of lipid peroxidation products in vivo is determined by the balance between their formation, metabolism, secondary reactions, and excretion. These biomarkers are useful for evaluating the beneficial effects of antioxidant foods, spices, beverages, supplements, and drugs. Several antioxidants protect biomolecules against oxidation [25]. The diminished LPO activity after treatment with the extract may be attributed to the antioxidant activity of the plant by scavenging the $CCl_3\bullet$ radical generated due to the metabolic transformation of CCl_4 in the liver. This increased level of serum biomarker reduced significantly and restored near normal levels by the treatment of fruit methanolic extract of *Microcos paniculata*. In addition, it was seen by assessing antioxidant status that oxidative stress effect of CCl_4 was significantly reduced (*P<0.05 *vs.* control).

This study demonstrates the hepatoprotective effect of FME against CCl_4-induced liver injury in rats. Results indicated that FME was able to suppress the hepatotoxicity of CCl_4 and thus exhibited its hepatoprotective effect. Free radicals that are produced metabolically from CCl_4 are responsible for creating necrosis through covalent binding with cell protein as well as membrane disintegration prompted by increased lipid-peroxidation [4, 26]. However, administration of CCl_4 initiates too much discharge of inflammatory mediators that worsen hepatic injury. Possibly Kupffer cells were stimulated by CCl_4. As a result, hepatic inflammation may occur by producing several proteins as like as IL-1β, IL-6 and TNF-α. Besides, the expression of hepatic mRNA may occur which is also responsible for hepatic inflammation. CCl_4 induces apoptosis of hepatocytes [3]. Appropriate organ homeostasis is maintained in healthy organisms through apoptosis and mitosis, where the number of cells removed by apoptosis become identical with the number of cells developed by mitosis. But, different liver diseases occur through the homeostatic

imbalance of apoptosis and mitosis. However, acute liver injuries such as fulminant hepatitis as well as reperfusion damage can be happened by extreme and/or continued apoptosis. So, one of the therapeutic approaches of liver injury is to prevent apoptosis [27].

SGPT and SGOT are used as biomarkers for determining hepatic damage. Both of their levels are increased when parenchymal and non-parenchymal liver cells injury occur [2]. Not only SGPT and SGOT but also ALP, TP and TB act as determinant for hepatotoxicity and hepatic damage [28, 29]. In this study, hepatotoxic control (CCl_4 treated) group significantly increased the levels of SGPT, SGOT, ALP, TB and decreased TP level when compared to control. Again this group showed hepatic necrosis and infiltration of inflammatory cells. As silymarin can protect liver and shows antioxidant activity against several toxic materials such as CCl_4, alcohol and thioacetamide, so it was used as standard drug [2]. Moreover, due to ROS as well as lipid peroxidation of the liver cell membrane and subsequent rise in membrane permeability, the liver enzymes are leaked out from the liver cells into the circulation ensuing their elevated serum level [29].

In the present study, FME (200 mg/kg and 400 mg/kg) attenuated the levels of SGPT, SGOT, ALP, TB which were found to be lower than the CCl_4 treated group. On the other hand, FME increased the levels of TP that were higher than CCl_4 treated group and indicated the protective effect of the extract against CCl_4 induced hepatic damage. Here, the results of liver function tests were correlated with the histopathological changes taken from photomicrographs. Histopathological results of the present study not only confirmed the hepatotoxic effect of CCl_4 on hepatic cells but also the hepatic recovery after treatment of intoxicated rats with FME. All the results indicated that there was a re-stabilization of the cell membranes and repair in the hepatic tissue. Colak *et al.* reported that phenolic acids and flavonoids were useful against liver diseases [4]. El-bakry *et al.* stated that the antioxidant can prevent CCl_4 induced hepatic damage [30]. In addition, alkaloids, glycosides and tannins have antioxidant property [31, 32, 33]. Previous study showed that the methanolic extract of *M. paniculata* fruits contained alkaloids [10].

Nevertheless, FME may act as hepatoprotective agent due to its antioxidant effect of phytochemical alkaloids present in it or the inhibition of cytochrome P450 2E1 or through the suppression of mRNA, inflammatory proteins such as IL-1β, IL-6 and TNF-α as well as the apoptosis of hepatocytes.

Conclusion

From this study, we can conclusively state that fruit methanolic extract of *Microcos paniculata* (FME) significantly reduced LPO level and restored to near normal level in CCl_4 induced hepatotoxic experimental rats. Moreover, the FME might possess hepatoprotective activity. This plant can be, further subjected to isolation of the hepatoprotective agent. Future studies will provide more insight into the molecular mechanism of how the extract acts as antioxidant and hepatoprotective agent.

Abbreviations

CCl_4 = Carbon Tetrachloride
SGPT = Serum Glutamic Pyruvic Transaminase
SGOT = Serum Glutamic Oxaloacetic Transaminase
ALP = Alkaline Phosphatase
TP = Total Protein
TB = Total Bilirubin
$^{\cdot}CCl_3$ = Trichloromethyl Free Radical
$CCl_3O_2\cdot$ = Trichloromethyl Peroxide Free Radical
i.p. = Intraperitoneally
IL = Interleukin
TNF = Tumor Necrosis Factor
ROS = Reactive Oxygen Species

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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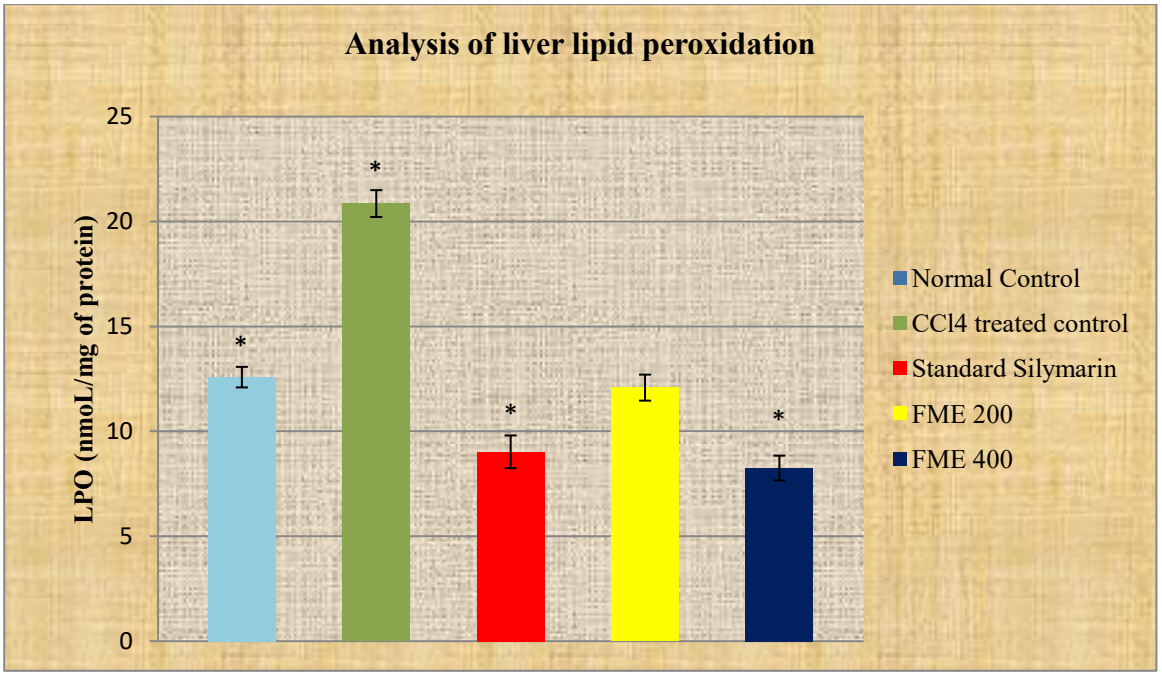

Figure 1: Analysis of LPO of FME [Values are presented as mean ± standard error. n = 5 rats in each group. *P<0.05, *vs.* control (Dunnett's t test)].

Figure 2: Sacrifice of rat by cervical decapitation.

Figure 3: Perfusion of rat's liver.

Figure 4: Perfused liver of rat.

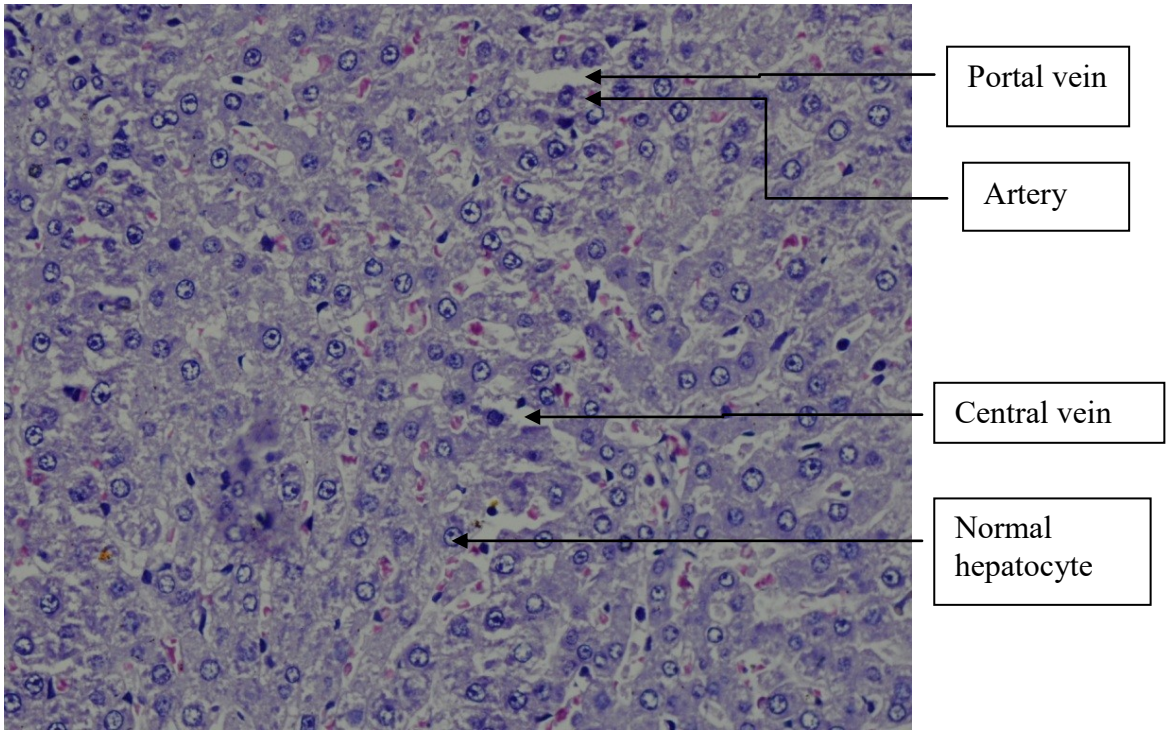

Figure 5: Photomicrograph of control rat liver, showing normal architecture of hepatic cell.

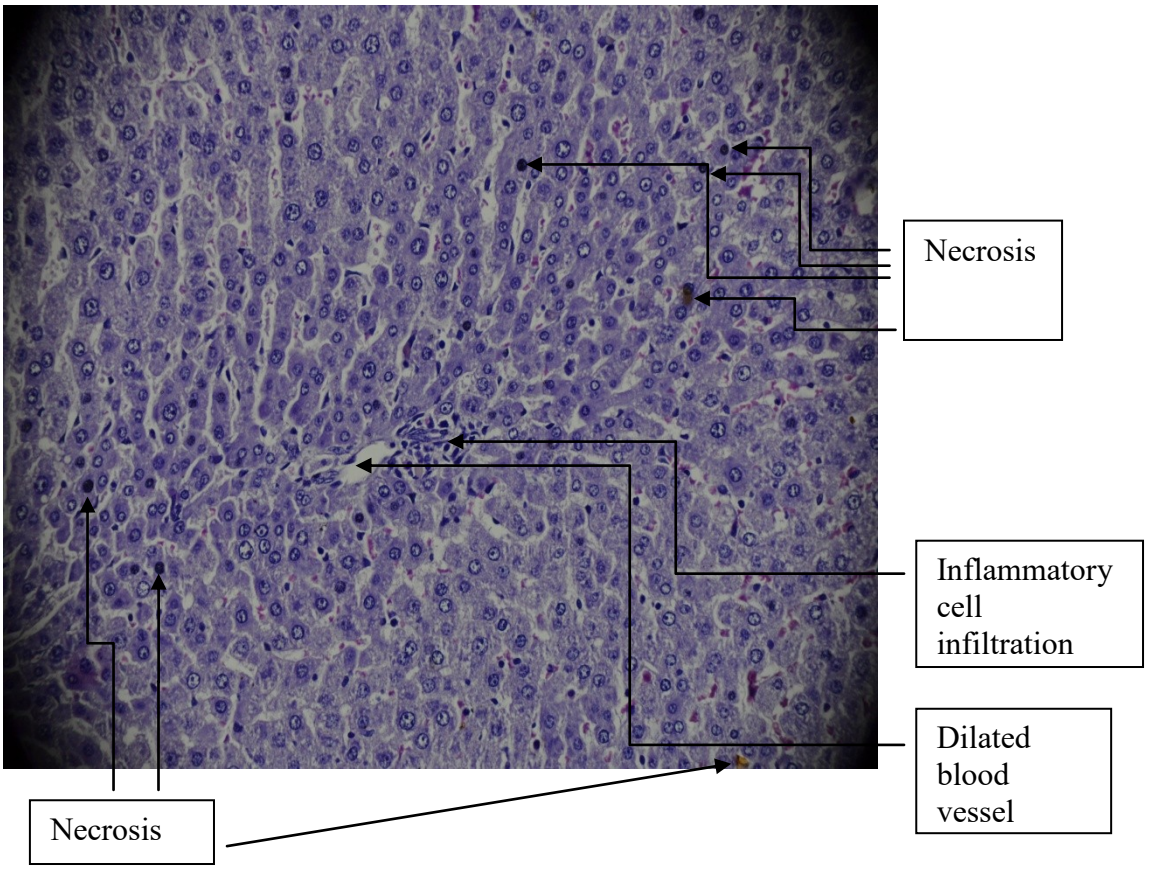

Figure 6: Photomicrograph of CCl_4 treated rat liver, showing hepatic necrosis with dilated blood vessels.

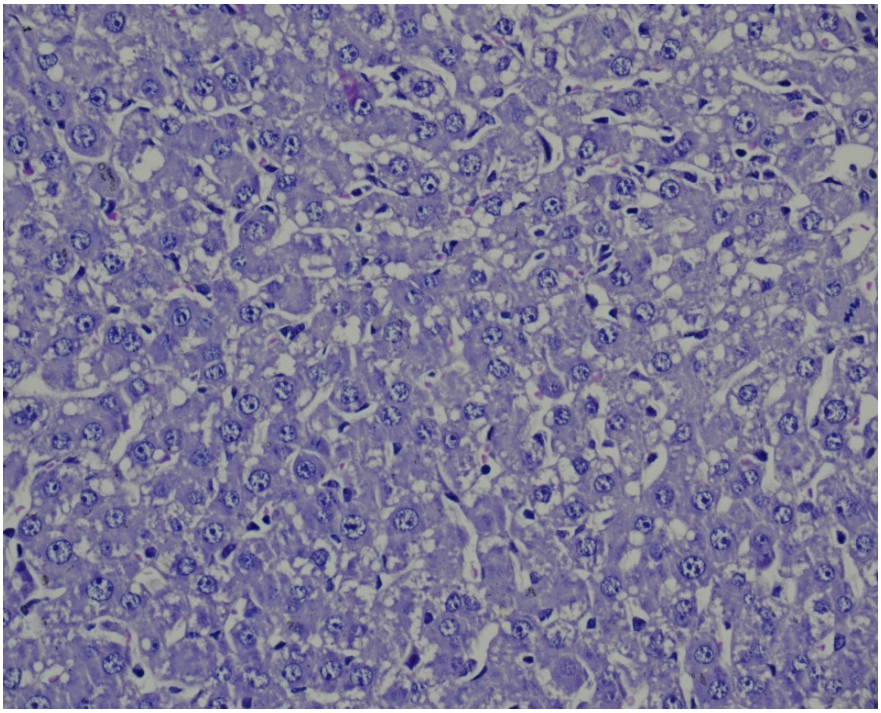

Figure 7: Photomicrograph of silymarin treated rat liver, showing improvement over CCl_4 treated group.

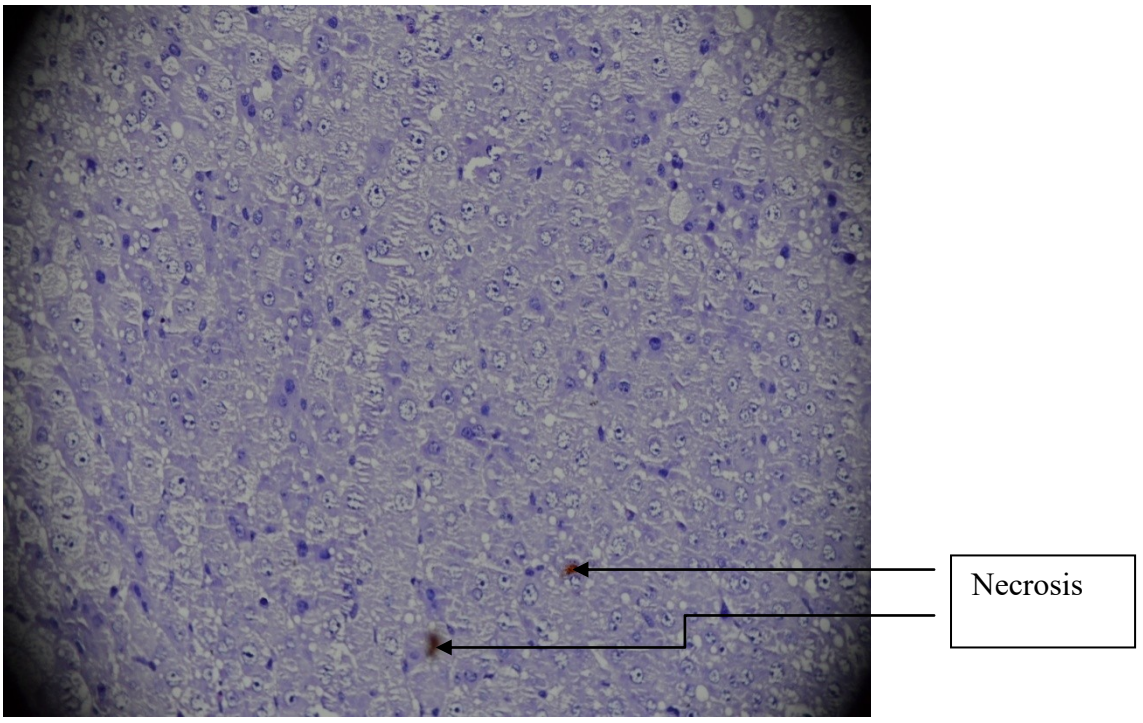

Figure 8: Photomicrograph of FME 200 mg/kg treated rat liver, showing reduced necrosis, over CCl_4 treated group.

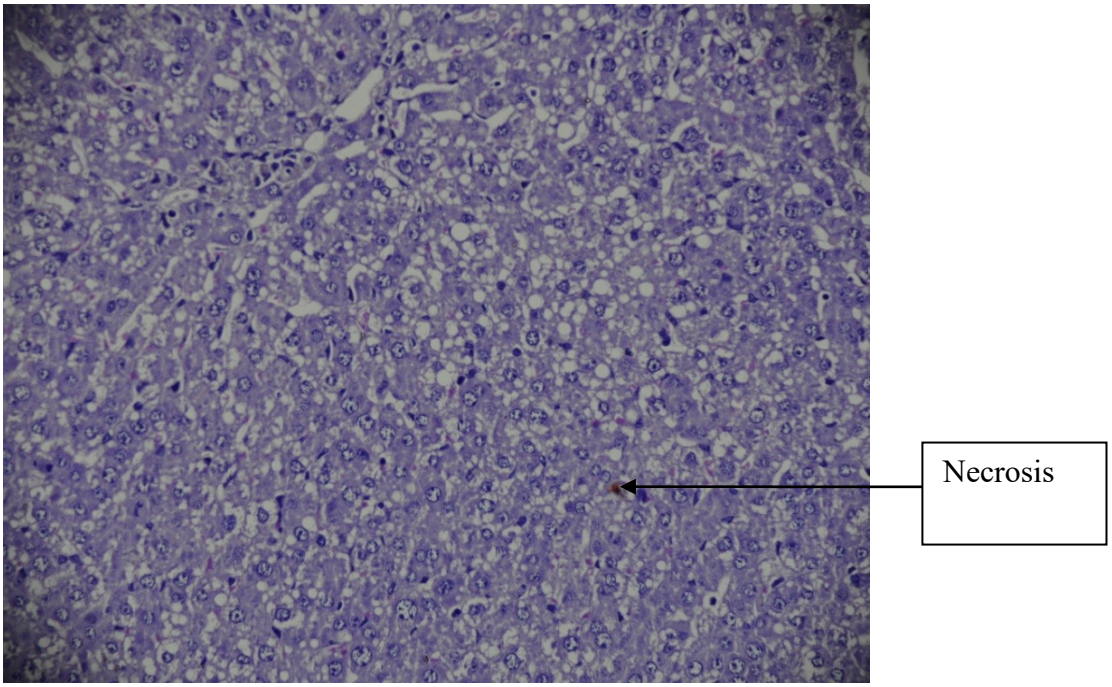

Figure 9: Photomicrograph of FME 400 mg/kg treated rat liver, showing better reduction of necrosis than FME 200 mg/kg treated rat liver, over CCl_4 treated group.

Table 1: Effect of FME on biochemical parameters of rats.

Treatment	SGPT (IU/L)	SGOT (IU/L)	ALP (IU/L)	TP (g/L)	TB (µmol/L)
Normal Control (Vehicle treated)	30.15±1.64	41.80±1.74	119.75±3.08	63.80±2.82	15.60±0.25
Hepatotoxic Control (CCl_4 Treated)	118.62±3.28*	145.00±6.83*	220.38±4.72*	34.40±2.25*	75.00±2.76*
Silymarin	32.87±1.90	44.80±1.85	116.11±4.11	59.00±2.35	18.73±0.35
FME 200	52.18±2.37*	72.00±1.48*	154.14±2.67*	46.40±2.50*	13.58±0.34
FME 400	33.89±2.15	58.60±1.75*	136.62±3.54*	54.80±2.03*	14.84±0.21

Values are presented as mean ± standard error. n = 5 rats in each group. *P<0.05, *vs.* control (Dunnett's t test).