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Effect of Partially Purified Fractions from *Pterocarpus mildbraedi* Extracts on Carbon Tetrachloride Intoxicated Rats

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Effect of Partially Purified Sub-fractions from *Pterocarpus mildbraedi* Extract on Carbon Tetrachloride Intoxicated Rats

Abstract

***Background*:** This study was designed to determine the hepatoprotective effect of partially purified fractions from *P. mildbraedii* extract on carbon tetrachloride (CCl_4) intoxicated rats**.**

Methods: The methanol extract of *P. mildbraedii* was subjected to solvent partitioning using n-hexane, chloroform, ethylacetate and water. Separation of fractions with proven antioxidant activity was achieved by chromatographic techniques. Acute toxicity and hepatoprotective studies of the methanol sub-fraction 6 (Me 6), methanol sub-fraction 7 (Me 7) and methanol sub-fraction 8 (Me 8) from *P. mildbraedii* extract on carbon tetrachloride (CCl_4) intoxicated Wister rats.

Results: Intoxication of rats with CCl_4 resulted in significant ($p < 0.05$) increase in the activities of Aspartate Transferase (AST), Alanine Transferase (ALT), Alkaline Phosphatase (ALP), level of Malondialdehyde (MDA) while glutathione (GSH) concentration, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities were decreased. Administration of Me6, Me7 and Me8 sub-fractions of *P. mildbraedii* caused a significant reduction ($p > 0.05$) in the activities of the liver enzymes, MDA level, total and direct bilirubin in dose-dependent manner. There were significant ($p < 0.05$) increases in GSH concentration, SOD, CAT, and Gpx activities in the treated groups. The subfractions also restored the damaged hepatic-architecture in the treated groups.

***Conclusions*:** Therefore, Me6, Me7 and Me8 leaf sub-fractions of *P. mildbraedii* extract have hepatoprotective effect. Therefore, this vegetable can further be exploited as a source of drug/supplement development in the prevention and treatment of liver damage.

Keywords: *Pterocarpus mildbraedi*, Carbon Tetrachloride, Intoxicated Rats, Sub-Fractions, Hepatoprotective.

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1. Introduction

The liver is the central organ of metabolism involved in bile production, storage of iron, vitamins, trace elements and detoxification of drugs [1]. These crucial roles make the liver susceptible to different injury leading to several diseases [2]. These diseases are mainly caused by toxic chemicals like carbon tetrachloride, acetaminophen, excess consumption of alcohol, infections and autoimmune/disorder in which excess reactive oxygen species is implicated [3]. Liver diseases are still serious health problems in both developed and developing world [4].

The effectiveness of orthodox medicines is inconsistent in the treatment of liver diseases and most of these drugs often accompanied by devastating adverse reactions such as renal dysfunction, poor tolerance and anemia. Most often, the treatment is worse than the disease [5]. These drugs only provide symptomatic relief, without significant changes to the progression of the disease [6]. They are laden with high relapse rate and are expensive.

Therefore, the search for natural antioxidants for the prevention and treatment of liver diseases arising from oxidative stress becomes very necessary in drug discovery and formulation of neutraceuticals. It has been established that some vegetables and fruits contain antioxidants that could be harnessed in the treatment and management of diseases arising from oxidative stress such as cancer, liver diseases, diabetes, ulcer, ageing process, arthritis, asthma, inflammation, ischemia, mongolism, neurodegeneration, Parkinson's disease [7,8] and fungal diseases [9].

P. mildbraedii belong to the family Papilionaceae and is found majorly in some West African countries like Nigeria, Ghana, Liberia, Cameroun, and Cot devoire. It is commonly called paduk and with different local names ("Oha" in Igbo, "Madobiyar rafi" in Hausa, "Urube" in Edo,"Geneghar" in Ijaw and "Kakupupu" in Urhobo [10]. The leaves of *P. mildbraedii* are used as cooked vegetable in southern Nigeria. In Ghana the trees have been planted in cocoa plantation to provide shade. *P. mildbraedii* is widely exploited for its timber in Tanzania and the wood which is whitish are widely used for carving cutlass handles, tie-rod and mortar making [11]. Apart from their food value, traditional vegetables are useful for other purposes, such as medicine, cash income and in cultural observance In Eastern and Southern Nigeria ,the leave extracts from *P. mildbraedii* are used in the treatment of headaches, pains, fever, convulsions, respiratory disorders and as antimicrobial agents [12]. Despite the extensive use of this plant as vegetable and herbal medicine there are very few validated scientific reports regarding the pharmacological activities, therefore, this study evaluates the hepatoprotective properties of P. *mildbraedii*.

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2.0 Materials and Methods

2.1 Collection of Samples and Identification of samples

P. mildbraedii vegetable was collected from a farm in Anambra State, Nigeria and identified by Dr. Ugbabe, G. of National Institute of Pharmaceutical Research, Abuja, Nigeria (NIPRD). The vegetable was assigned a Voucher/Specimen number of NIPRD/H6713 and deposited at NIPRD herbarium.

2.2 Plant Preparation and Extraction

The extraction was done according to the method of [13]. The debris were removed from the vegetable, washed and air-dried at room temperature with adequate ventilation. The dried sample was pulverized using a Binatone blender (Binatone model BLG- 555, China). Fifty grammes (50 g) of the powdered sample was weighed into 400ml of methanol in a reflux flask and refluxed for 2 hours. The extract was filtered with a muslin cloth and subsequently evaporated using a rotary evaporator (Bibby Sterling Ltd, Stone Staffordshire ST 15OSA, UK). The dried extract was weighed, placed in sterile sample bottles and stored in a refrigerator until required for use.

The crude extract of *P. mildbraedii* (100 g) was dissolved in 100ml of distilled water in a beaker and was poured into a liter of separatory funnel and partitioned using n-hexane, chloroform, ethyl acetate to give four fractions (n-hexane, chloroform, ethyl acetate and aqueous fractions). The aqueous fraction of *P. mildbraedii* with the highest *invitro* antioxidant properties was then subjected to vacuum liquid chromatography (VLC). The methanol fraction obtained from VLC that was observed to have highest Invitro-antioxidant activity were further subjected to column chromatography to give eight subfractions (Me1 - Me8). The partially purified active sub-fractions (Me6 - Me8) were chosen for this study.

2.3 Experimental Animals

Wistar albino rats weighing between 120-180 g were obtained from the Animal house, Department of Biochemistry, Ahmadu Bello University, Zaria, Kaduna. They were fed on standard rat pellet diet (Ladokun Feeds, Nigeria) and water was provided *ad libitum* and the animals were allowed to adapt to the laboratory environment for two weeks in order to acclimatize.

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2.4 Laboratory Animal Ethics

The principles governing the use of laboratory animals were followed as approved by the Ethical Committee on the use of laboratory animals of Federal University of Technology, Minna which is also in accordance to the Guidelines of National Research Council Guide for the Care and Use of Laboratory Animals [14] and principles of Good Laboratory Procedure [15].

2.5 Acute Toxicity Test

The acute toxicity study of sub-fractions Me6, Me7 and Me8 of *P. mildbraedii* was performed by the method of Organization of Economic Co-operation and Development (OECD) guideline 423 [16].

2.6 Experimental Design and Animal Groupings

The effect of purified sub-fractions; Me6, Me7 and Me8 on carbon tetrachloride induced hepatotoxicity in rats were determined as described by [17]. Forty five rats weighing between 120-180 g were divided randomly into nine groups of five rats each. The 2ml/kgbw of Carbon tetrachloride (CCl_4) in olive oil (1:1) was used to induce hepatotoxicity in rats at intraperitoneal region (i.p) every 72 hours followed by the treatments for 10 days daily.

Group I: received normal saline (2 ml/kgbw, i.p), Group II: CCl_4 only, Group III: 50 mg/kgbw of silymarin + CCl_4, Group IV: 50 mg/kgbw of Me6 sub-fraction of *P. mildbraedii* + CCl_4, Group V: 100 mg/kgbw of Me6 sub-fraction of *P. mildbraedii* + CCl_4 , Group VI: 50 mg/kgbw of Me7 sub-fraction of *P. mildbraedii* + CCl_4, Group VII: 100 mg/kgbw of Me7 sub-fraction of *P. mildbraedii* + CCl_4, Group VIII: 50 mg/kgbw of Me8 sub-fraction of *P. mildbraedii* + CCl_4, Group IX: 100 mg/kgbw of Me8 sub-fraction of *P. mildbraedii* + CCl_4

Twenty four hours after the last administration of CCl_4 which signify the end of the experiment, the rats were euthanized by cervical dislocation and blood samples were collected by cardiac puncture from the inferior vena cava of the heart into serum bottles [18]. The blood samples were allowed to clot at room temperature and sera were obtained by centrifuging at 3000 rpm for 10 min [20].

2.7 Assessment of Liver Function

Biochemical parameters such as AST, ALT [21], ALP [22], albumin [23], total bilirubin and total protein [24] were analyzed using Randox diagnostics kits (United Kingdom) according to individual kits manual.

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2.8. Preparation of Liver Homogenate and Assessments of oxidative stress

The preparation of liver homogenate was done as reported by [25]. A section of the rat's liver was rinsed in ice-cold 1.15% KCl, blotted and weighed. They were then homogenized in four volumes of ice-cold isotonic phosphate buffer (100mM, pH 7.4) in pre-cooled laboratory mortar and pestle placed in a bowl of ice chips. The homogenates were centrifuged at 12,500G for 15minutes at 4°C in a high speed refrigerated ultra-centrifuge (Model LR10-2.4A, Shendi, and Shangahai). The supernatants were used to assay for superoxide dismutase [26], catalase [27] and glutathione peroxides [28] enzyme activity. The homogenate was also used for the assay of determination of lipid peroxidation (LPO) by measuring the release of malondialdehyde (MDA) by the method of [29] and the estimation of reduced glutathione enzyme [30]

2.9. Histopathological studies

A section of the liver tissues were fixed in 10% formalin and embedded in paraffin wax. Sections of 4-5 microns thickness were made using rotary microtome and stained with hematoxylin-eosin. Histological observations were made under light according to the method described by [31].

2.10. Statistical Analysis

All values were expressed as Mean ± SEM. Statistical analysis was performed by one-way Analysis of Variance (ANOVA) and individual comparisons of the group mean values were performed using post hoc Duncan multiple range test. SPSS software was used in the data analysis.

3.0. Results

3.1. Acute Toxicity (LD_{50})

No lethality was observed in rats administered with single dose of subfractions Me6, Me7 and Me8 of *P. mildbraedi* extract at 2000 mg/kgbw. Animals did not show any significant changes in behavioral, physiological and physical activities. There were also no signs of late toxicity in rats when observed up to 14 days as shown in Table 1

3.2. Marker Enzymes of Liver Damage

The effect of Me6, Me7 and Me8 subfractions of *P. mildbraedii* leaf extract on the activities of marker enzymes of liver damage is presented in Table 2. Significant elevations ($p < 0.05$) were observed in the activities of serum ALT,

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AST and ALP in the group induced with CCl_4 when compared with control. However, simultaneous administration of standard drug (silymarin) and different sub-fractions of leaves of *P. mildbraedii* with CCl_4 significantly ($p < 0.05$) reduced the activities of these enzymes when compared with the toxic group.

3.3 Biochemical Parameters in Normal and CCl_4 Induced Hepatotoxic rat.

Treatment of rats with CCl_4 alone significantly ($p < 0.05$) reduced serum total protein when compared with control (Table 3). However, group treated with CCl_4 with a simultaneous administration of 50 mg/kgbw and 100 mg/kgbw of sub-fractions significantly ($p < 0.05$) increased the protein concentration. A significant decrease ($p < 0.05$) in albumin concentration was also observed in rats treated with CCl_4 when compared with control. The change effected on albumin by the treatment with CCl_4 was significantly ($p < 0.05$) reversed by the administration of 50 mg/kg and 100 mg/kgbw of sub-fractions of leaf of *P. mildbraedii*. The dose of 100 mg/kgbw however caused a more significant increase in the concentration of serum total protein and albumin than the 50 mg/kgbw for all the treated groups.

A significant ($p < 0.05$) increase in the levels of total bilirubin and direct bilirubin in the hepatotoxic group was observed when compared with control. However, all the groups treated with subfractions Me6, Me7 and Me8 significantly ($p < 0.05$) reduced the total bilirubin and direct bilirubin concentration when compared with group treated with CCl_4. The groups treated with subfractions Me6 (1.60 0.27 µmol/L) and Me8 (1.70 ± 0.32 µmol/L) at 100 mg/kg showed a better decrease in direct bilirubin concentration than the standard drug silymarin (2.10 ± 0.10 µmol/L).

3.4 Superoxide Dismutase (SOD) Activity

A significant reduction by 71% in SOD activity was observed in hepatotoxic group compared to normal control (Figure 1). An increase in SOD activity was observed in rats that received either silymarin or Me6, Me7 and Me8 subfractions of *P. mildbradii* at 50 and 100 mg/kgbw compared with the hepatotoxic group. The subfractions Me6 (24.00%) and Me8 (24.00%) at 50mg/kgbw showed the same effect as the standard drug (24.05%) while the groups treated with the sub-fractions at 100 mg/kgbw showed a significant ($p < 0.05$) increase in SOD activity of 34.67 - 38.02% compared to the standard drug (24.00%).

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3.5. Catalase Activity

All the treatment groups showed an increase in catalase activity significantly ($p < 0.05$) compared with the hepatotoxic group (Figure 2). Subfractions Me7 and Me8 of *P. mildbradii* at a dose of 100 mg/kgbw were more effective in enhancing the activity of this enzyme by 60 and 62.37% respectively in comparison with the CCl_4 hepatotoxic group. The standard drug, silymarin showed only 58.82% increments in enzyme activity compared to the toxic group.

3.6. Glutathione Activities

Figure 3 showed that Me6, Me7 and Me8 subfractions of *P. midbraedii* at a dose of 50 and 100 mg/kgbw were able to increase GSH concentration significantly ($p < 0.05$) when compared with the hepatotoxic group. The sub-fraction Me8 at both doses of 50 and 100 mg/kg body weight showed a more significant increase by 97 and 98% respectively when compared to hepatotoxic control group. This significant increase in GSH concentration by Me8 at 50 and 100 mg/kg body weight was observed to be better than that of Silymarin by 12 and 13%

3.7. Glutathione Peroxidase Activity

The effect of sub- fractions Me6, Me7 and Me8 of *P. midbraedi* on glutathione peroxidase activity is presented in (Figure 4). A significant ($p < 0.05$) decrease in the enzymatic activity of glutathione peroxidase in the hepatotoxic group (1.80 µmoles NADPH oxidized/mg protein) was observed when compared to the normal group (5.68 µmoles NADPH oxidized/mg protein). All the treatment groups offered some protection at 50 and 100 mg/kgbw against CCl_4- induced but not treated group. However, the sub-fraction Me7 at 100 mg/kg body weight showed the highest increment in glutathione peroxidase activity compared to other treatment groups.

3.8. Lipid peroxidation

The extent of lipid peroxidation was measured by assaying thiobarbituric acid reactive substances (MDA) for all groups (Figure 5). A significant ($p < 0.05$) increase in lipid peroxidation was observed in the CCl_4 hepatotoxic rats group. Treatment with sub-fractions of *P. midbraedii* (50 and 100 mg/kgbw) significantly ($p < 0.05$) decrease the MDA level to almost normal. Specifically, the effect of sub-fraction Me6 at both doses were comparable with that of standard

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3.9. Histopathology

Histopathology profile of Control showed normal hepatic architecture. However, CCl_4 induced non treated animals exhibited severe hepatotoxicity indicated by massive infiltration (fatty liver) caused by marked hepatocellular necrosis and vascular degeneration. All sub-fractions and silymarin treated groups showed reduction in hepatic damage in line with biochemical analysis results. Treatment with the subfractions of *P. mildbraedii* reduced the severity of hepatic damage in a dose dependent manner (Plates 1- VI).

4. DISCUSSIONS

Toxicity studies are useful parameters in the investigation of therapeutic index of drugs and xenobiotics [32]. The acute toxicity test showed that oral administration of Me6, Me7 and Me8 caused no death in the test animals. Therefore, it is inferred that oral LD_{50} of the sub-fractions was greater than 2000 mg/kgbw. The tested sub-fractions can be categorized as having high safety margins since substances possessing LD_{50} higher than a minimum allowable dose of 2000 mg/kgbw are considered acutely nontoxic [33]. According to guidelines of the Organization for Economic Cooperation and Development [16]; substances possessing LD_{50} dose of 2000 mg/kgbw or higher are categorized as unclassified nontoxic.

The significant increase ($p < 0.05$) in the activities of liver marker such as serum ALT, AST and ALP showed hepatotoxicity in the experimental animals. Increased levels of serum ALT, AST, ALP have been reported to be sensitive indicators of liver injury [34]. The leakage of these enzymes from the cells through peroxidative damage of the membrane into the bloodstreams usually caused increase in their activities. ALT is specifically produced in the hepatocytes to catalyze the transfer of amino group from L-alanine to α-ketoglutarate to form L-glutamate and oxaloacetate thus making it relatively a better determinant of hepatic injury than AST, which is observed in other tissues [35]. AST elevation indicate cellular leakage as well as loss of functional ability of cell membrane in liver [36]. Alkaline phosphatase increase gives an assessment of the integrity of the plasma membrane [37] and diagnosis of hepatobiliary effects for instance high serum ALP is associated with biliary disorder such jaundice and cirrhosis [38].

Sub-fractions Me6, Me7 and Me8 of the *P. mildbraedii* leaf extract significantly decreased the level of ALT, AST and ALP in a dose dependent manner. Sub-fraction Me6 at 100 mg/kg body weight reduced these enzyme activities

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better than all others including the standard drug, silymarin. The lowering of enzyme levels or activities is in agreement with results of a previous study in which n butanol fraction of *Gongronema latifolium* showed a similar effect [39]. The present finding may represent a pioneering effect of sub-fractions of *P. mildbraedii* leaf extract to offer protection against the toxicity of CCl_4. These protective effects may be due to the healing of hepatic parenchyma and regeneration of hepatocyte by the presence of phytochemicals such as flavonoids and phenols present in *P. mildbraedii* [40] these secondary metabolites may be acting alone or synergistically in quenching free radicals generated in the toxic groups.

The low serum levels of total proteins and albumin due to CCl_4-intoxication may be attributed to the initial damage of the endoplasmic reticulum resulting in the loss of Cyt P-450 leading to fatty livers [41]. This decrease in serum protein levels observed in CCl_4 intoxication correspond to extensive liver damage as a result of impaired protein turnover. Albumin is a useful indicator of hepatic synthetic function [42]. Liver injury is associated with a decrease in the concentration of serum albumin, and hypoalbuminemia has also been considered to be significant in the decreased rate of hepatic synthesis of essential protein in both humans and animals [43]. Concomitant treatment with sub-fractions of *P. mildbraedii* leaf extract remarkably increased the concentration of total protein and albumin in the serum. Values obtained in this study are higher than those obtained from a previous study [44]. The higher dose was generally more effective and similar to the standard drug, silymarin. Me7 and Me8 sub-fractions were much better than the standard drug in terms of total protein and albumin levels respectively. Therefore, sub-fractions from *P. mildbraedii* extract possess the ability to promote protein synthesis and this plays significant role in the overall hepato-protective effect.

The significant rise in the levels of total and direct bilirubin observed in CCl_4-intoxication may be attributed to hepatic dysfunction induced. Bilirubin is the major bile pigment that is formed from the breakdown of hem in red blood cells. It is transported to the liver and secreted into the bile [45]. Elevated bilirubin level may also be associated with reduced hepatocyte uptake of bilirubin, impaired conjugation of bilirubin and impaired hepatocyte secretion of bilirubin [46]. The significant reduction ($p < 0.05$) in the level of serum total and direct bilirubin following administration of sub-fractions of leaves of *P. mildbraedii* is indicative of the hepatoprotective potential of this vegetable. The reduction in bilirubin concentration, liver marker enzymes, elevation of protein and albumin levels in

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serum is in agreement with views that their levels revert to normal with the healing of hepatic parenchyma cells as well as the regeneration of hepatocytes [47, 48]

Oxidative stress resulting from enhanced free radical formation and/or defects in antioxidant defense cause severe tissue damage which have been implicated in a number of diseases, like liver diseases, coronary artery diseases, atherosclerosis, cancer and diabetes[49]. Antioxidant enzymes SOD, CAT and GPx are the three major scavenging enzymes that represent protection against oxidative tissue-injury [50].

SOD catalyzes the dismutation of superoxide anion to a hydrogen peroxide. CAT and GPx converts H_2O_2 to non-toxic products H_2O and O_2. The CCl_4 induction of rats lead to reduced antioxidant capacity as evident in the decreased activity of the antioxidant enzymes. These results are consistent with earlier reports [39, 41]. Treatment with sub-fractions of *P. mildbraedii* leaf restored the antioxidant enzyme profiles and reversed the oxidative hepatic damage. The higher dose was generally more effective than the standard drug. These positive effects could be associated with the phytoconstituents in these fractions as it has earlier been reported that some phytochemicals like tannins, terpenoids, phenols steroids and flavonoids enhance antioxidant enzyme activities *in vivo* [51]. Enhanced antioxidant activity as enumerated by these enzymes in the sub-fractions of *P. mildbraedii* may be attributed to their phytochemical constituents.

This study also showed an evidence of oxidative stress by the reduced level of glutathione in the CCl_4 induced rats. This is in agreement with reports from hepatotoxicity studies which show that decreased levels of GSH, SOD and CAT in the liver suggests hepatic damage [52]. Glutathione is a major non- enzymatic antioxidant capable of regulating intracellular redox homeostasis in all cell types [53]. It scavenges free radical directly or acts as a substrate for GPx, glutathione-S-transferase (GST) during detoxification of H_2O_2, lipid hydroperoxides and maintains membrane protein thiol. The present results show that all sub-fractions at 100 mg/kgbw treatment restored the GSH level better than the standard drug, silymarin. Therefore, the ability of this extract to restore the GSH level will help in maintaining the body's antioxidant defense mechanism thus protecting cell membrane integrity. The exact mechanism by which the sub-fractions enhanced GSH level is not clear but could be attributed to the presence of phytochemicals. Marked increase in MDA level, an index of lipid peroxidation observed in the liver of CCl_4 induced rats is indicative of membrane damage. Treatment with subfractions Me6, Me7 and Me8 of *P. mildbraedii*

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significantly reversed these changes. Stigmasterol present in bark of *Butea monosperma* exhibited decrease in hepatic lipid peroxidation and increase in the activities of catalase, superoxide dismutase and glutathione thereby suggesting its antioxidant property [54]. Therefore, inhibition of lipid peroxidation by *P. mildbraedii* sub-fractions may be attributed to their various phytochemicals.

Histopathological examination of the liver sections of control animals showed normal hepatic cells with well-preserved lobular pattern and visible central vein. Liver sections of CCl_4 -treated rats showed severe distortion in liver histoarchitecture with massive fatty change, necrosis, congested central vein, severe infiltration of inflammatory cells and vacuolar degeneration. These are signs of toxicity of CCl_4 reported by other researchers [52, 55]. Necrosis is pathological death that occurs after abnormal stress by chemical injury or toxins. Necrotic cells are unable to maintain membrane integrity thus leak out their contents and thus eliciting inflammation in surrounding tissues[56]. Inflammation is fundamentally a protective response, the ultimate goal of which to rid of the organism of both the initial cause of the cell injury (e.g., toxins) and the consequences of such injury [56].

Histological architecture of liver sections of the rats treated sub-fractions of *P. mildbraedii* leaf extract as well as silymarin showed mild hepatotoxic change with a mild degree of fatty material, necrosis and lymphocyte infiltration comparable to the control and silymarin treated groups. Histoarchitectural preservation or protection of the hepatocytes was more effective with the 100 mg/kgbw doses. This result further validates the result of the biochemical studies showing the hepatoprotective effect of the *P. mildbraedii* vegetable.

The clinical impact of this study is the possession of hepatoprotective effect by P. *mildbraedii* leaf extract which is attributed to its ability to: reduce the rate of lipid peroxidation, enhance the antioxidant defense status and guard against the pathological changes of the liver induced by CCl_4 intoxication. The strong in vivo antioxidant activity also suggests that a dietary supplement of *P. mildbraedii* may confer a beneficial effect against oxidative stress.

As far as we observed, the hepatoprotective of the partially purified *P. mildbraedii* is being reported in this research for the first time. As such, the most promising sub-fraction (Me8) as well as Me6 and Me7 that also active at 100 mg/kgbw can be purified further to obtain non-toxic, readily available and less expensive hepatoprotective compound(s) that can be used for drugs production in future.

5. Conclusion

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Conclusively, *P. mildbraedii* vegetable can be exploited as a naturally available alternative for drug/supplement development in the prevention and treatment of liver damage.

Conflicts of interest: All authors have no conflict of interest to report.

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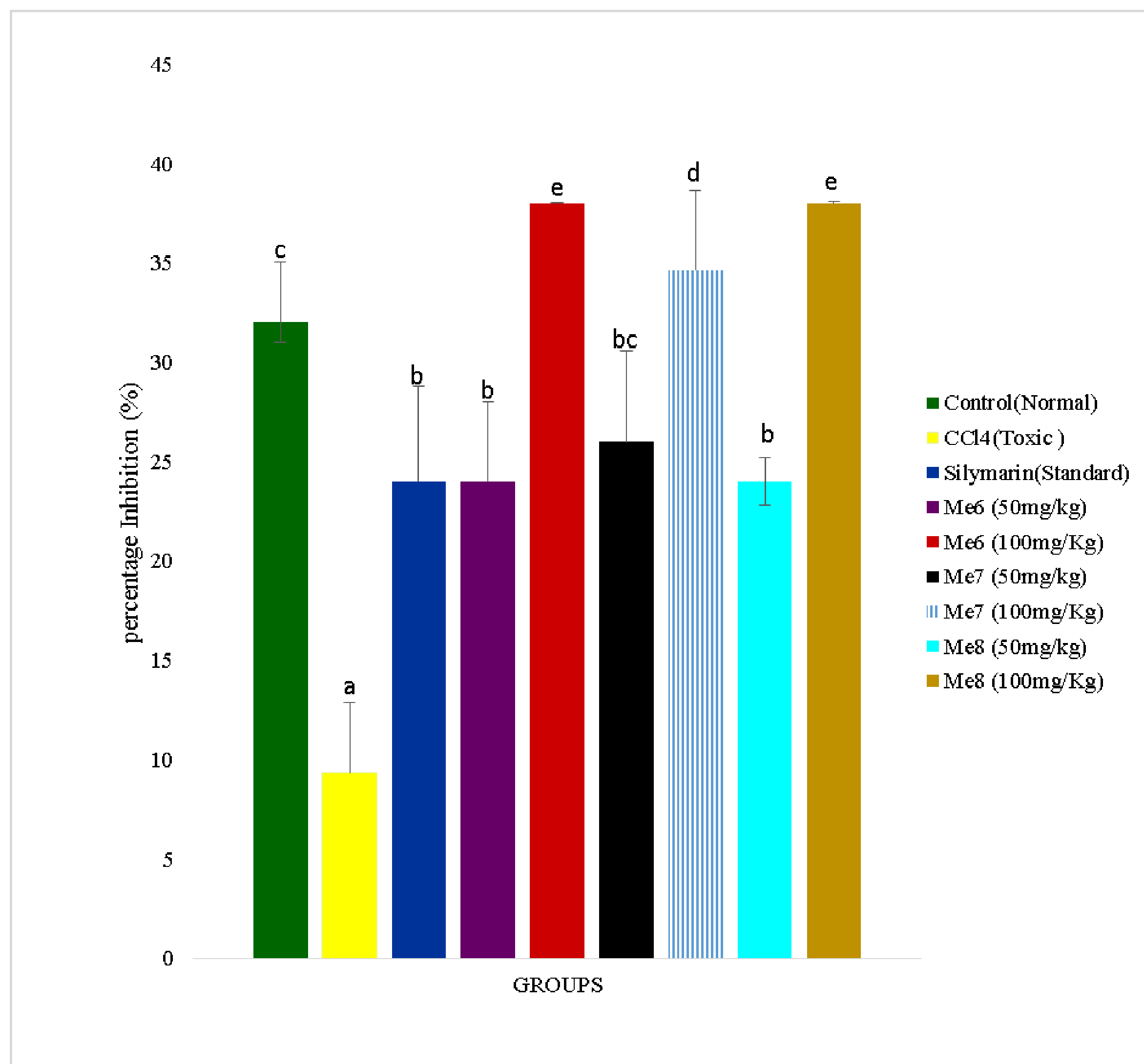

Figure 1: Effect of Me6, Me7 and Me8 Subfractions of *P. milbraedii* on Superoxide Dismutase Activity in Normal and CCl_4 Induced Rats.

Results are mean of five determinations ± SEM. Bar with different superscripts are significantly different ($p<0.05$).

Key: Normal=Control, CCl_4= Toxic and not treated, Silymarin= Treated with standard drug, $Me6_{50}$= Treated with sub-fraction Me6 at 50mg/kgbw, $Me6_{100}$=Treated with sub-fraction Me6 at 100mg/kgbw, $Me7_{50}$=Treated with sub-fraction Me7 at 50mg/kgbw, $Me7_{100}$=Treated with sub-fraction Me7 at 100mg/kgbw, $Me8_{50}$= Treated with sub-fraction Me8 at 50mg/kgbw, $Me8_{100}$= Treated with sub-fraction Me8 at 100mg/kgbw.

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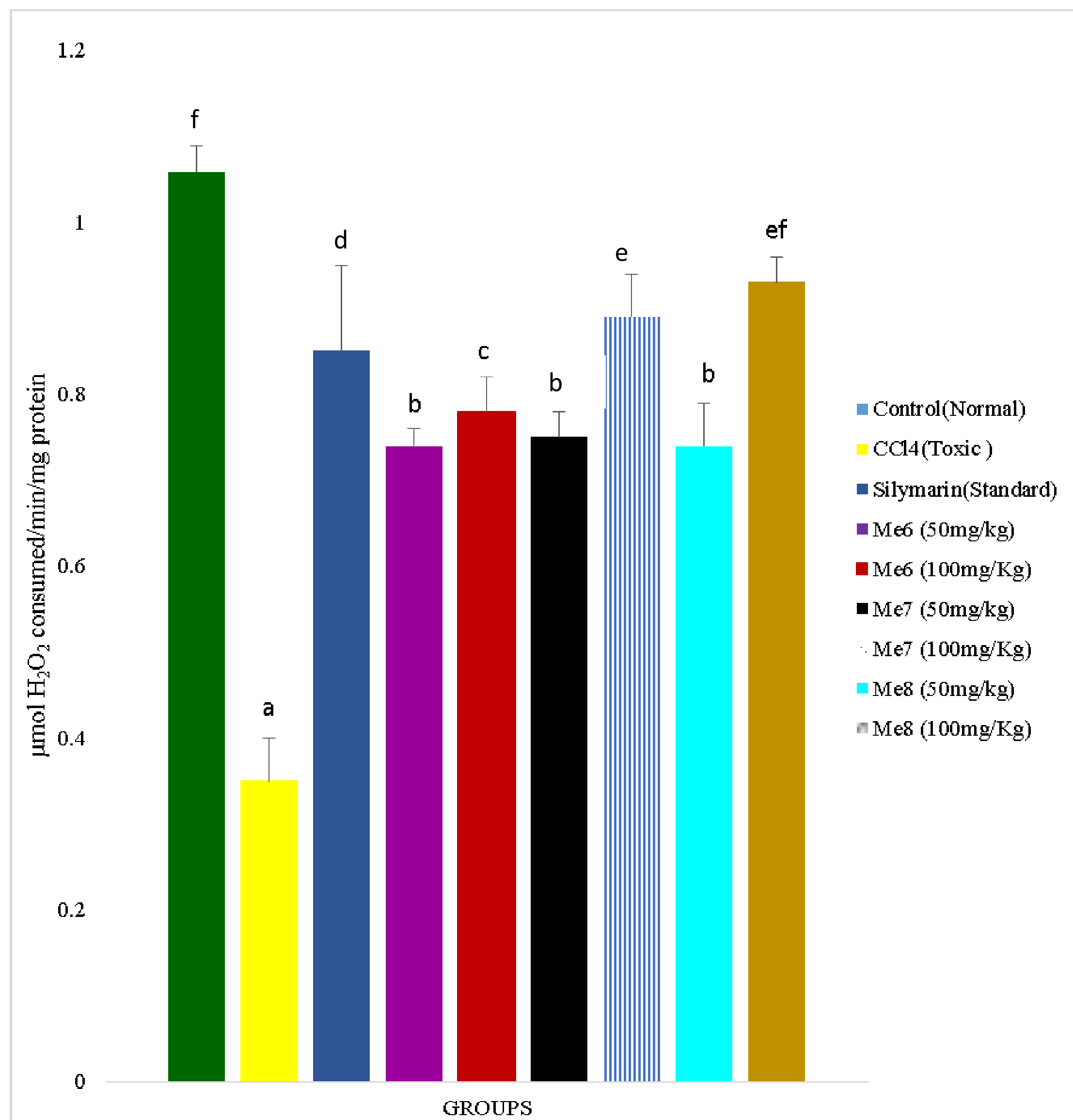

Figure 2: Effect of Me6, Me7 and Me8 Subfractions of *P. milbraedii* on Catalase Activity in Normal and CCl_4 Induced Rats.

Results are mean of five determinations ± SEM. Bar with different superscripts are significantly different ($p<0.05$).

Key: Normal=Control, CCl_4= Toxic and not treated, Silymarin= Treated with standard drug, $Me6_{50}$= Treated with sub-fraction Me6 at 50mg/kgbw, $Me6_{100}$=Treated with sub-fraction Me6 at 100mg/kgbw, $Me7_{50}$=Treated with sub-

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fraction Me7 at 50mg/kgbw, $Me7_{100}$=Treated with sub-fraction Me7 at 100mg/kgbw, $Me8_{50}$= Treated with sub-fraction Me8 at 50mg/kgbw, $Me8_{100}$= Treated with sub-fraction Me8 at 100mg/kgbw.

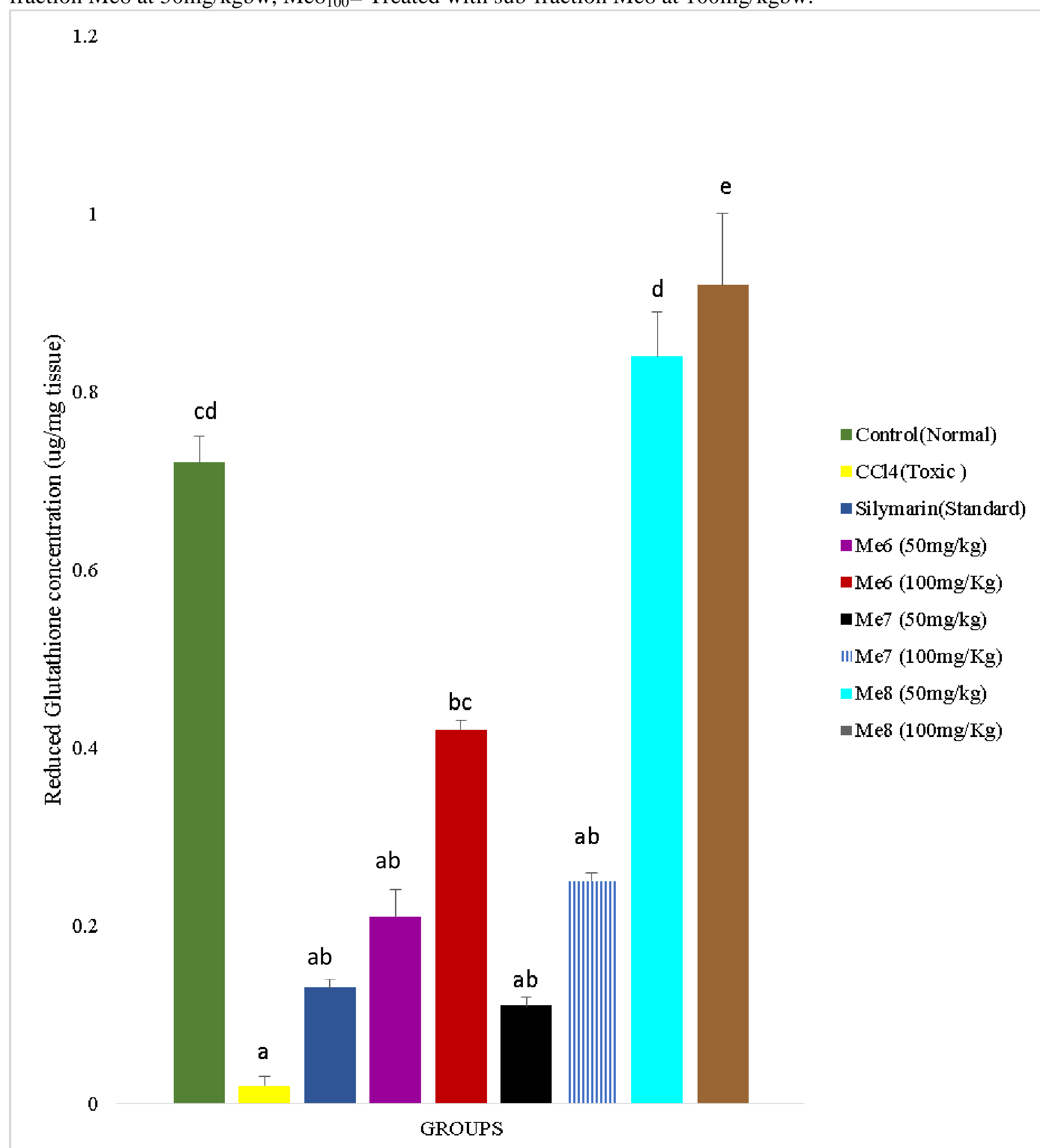

Figure 3: Effect of Me6, Me7 and Me8 Subfractions of *P. milbraedii* on Glutathione Concentration in Normal and CCl_4 Induced Rats.

Results are mean of five determinations ± SEM. Bar with different superscripts are significantly different ($p<0.05$).

Key: Normal=Control, CCl_4= Toxic and not treated, Silymarin= Treated with standard drug, $Me6_{50}$= Treated with sub-fraction Me6 at 50mg/kgbw, $Me6_{100}$=Treated with sub-fraction Me6 at 100mg/kgbw, $Me7_{50}$=Treated with sub-fraction Me7 at 50mg/kgbw, $Me7_{100}$=Treated with sub-fraction Me7 at 100mg/kgbw, $Me8_{50}$= Treated with sub-fraction Me8 at 50mg/kgbw, $Me8_{100}$= Treated with sub-fraction Me8 at 100mg/kgbw.

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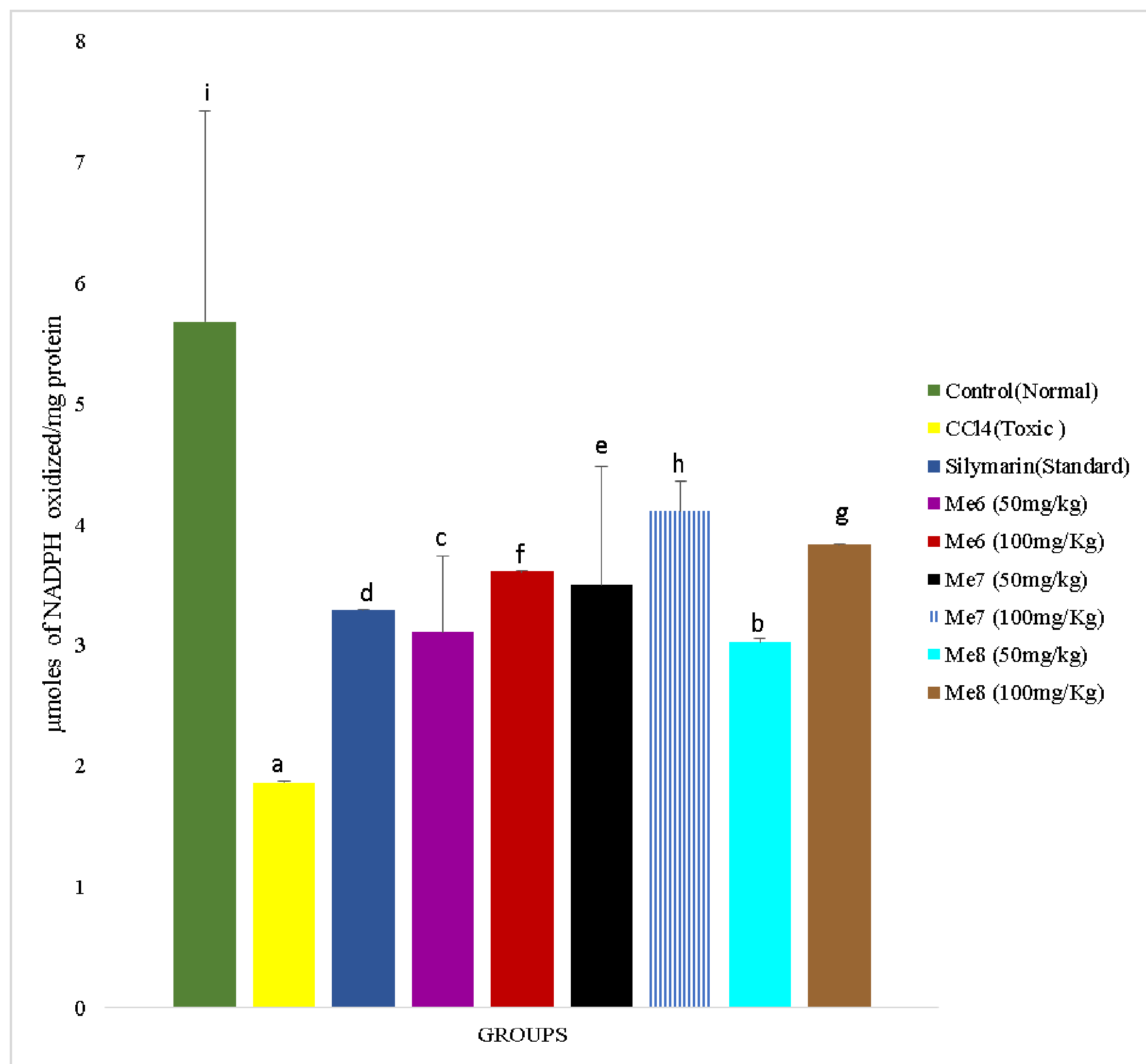

Figure 4: Effect of Me6, Me7 and Me8 Subfractions of *P. milbraedii* on Glutathione Peroxidase Activity in Normal and CCl_4 Induced Rats.

Results are mean of five determinations ± SEM. Bar with different superscripts are significantly different ($p<0.05$).

Key: Normal=Control, CCl_4= Toxic and not treated, Silymarin= Treated with standard drug, $Me6_{50}$= Treated with sub-fraction Me6 at 50mg/kgbw, $Me6_{100}$=Treated with sub-fraction Me6 at 100mg/kgbw, $Me7_{50}$=Treated with sub-fraction Me7 at 50mg/kgbw, $Me7_{100}$=Treated with sub-fraction Me7 at 100mg/kgbw, $Me8_{50}$= Treated with sub-fraction Me8 at 50mg/kgbw, $Me8_{100}$= Treated with sub-fraction Me8 at 100mg/kgbw.

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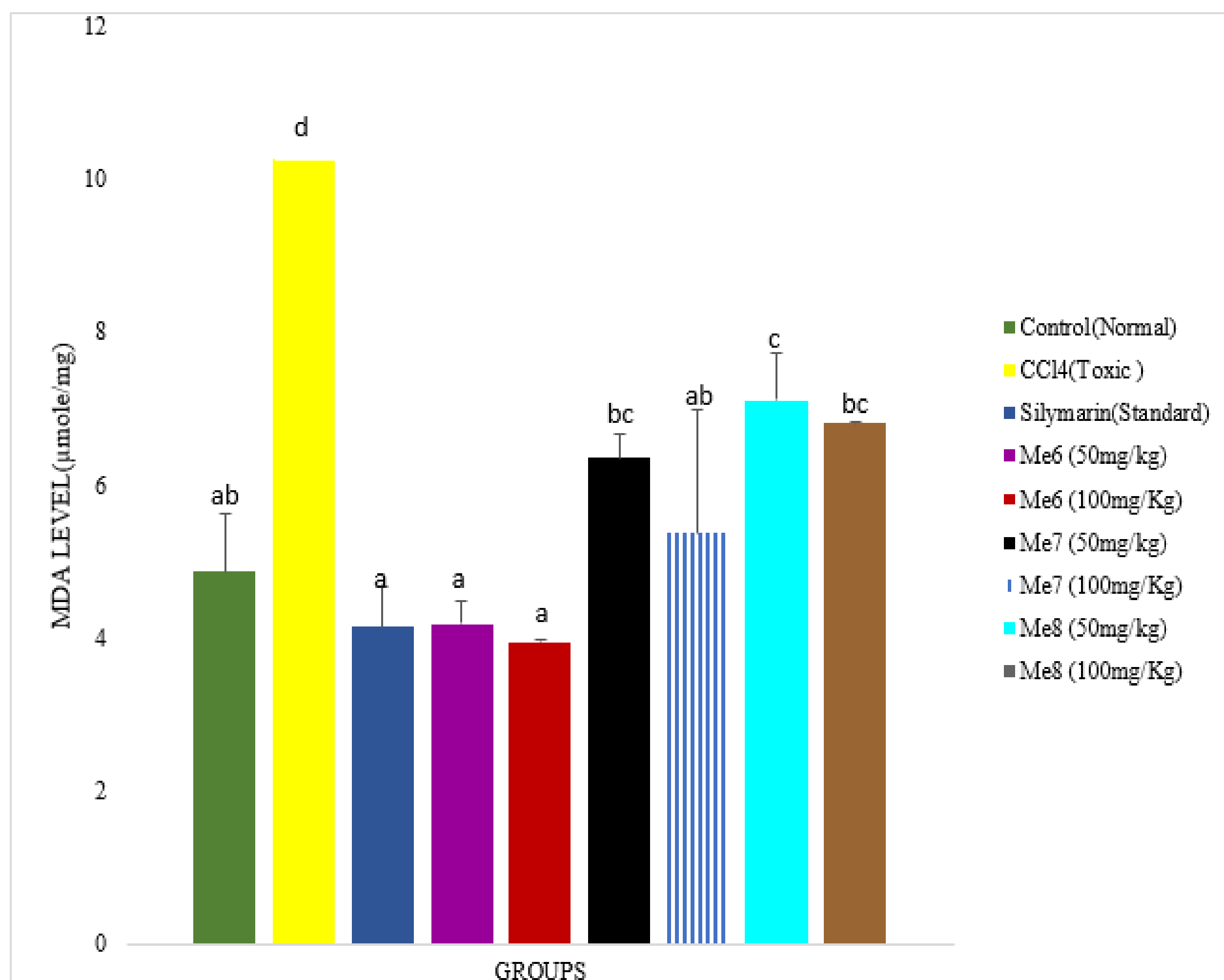

Figure 5: Effect of Me6, Me7 and Me8 Subfractions of *P. milbraedii* on MDA Level in Normal and CCl_4 Induced Rats.

Key: Normal=Control, CCl_4= Toxic and not treated, Silymarin= Treated with standard drug, $Me6_{50}$= Treated with sub-fraction Me6 at 50mg/kgbw, $Me6_{100}$=Treated with sub-fraction Me6 at 100mg/kgbw, $Me7_{50}$=Treated with sub-fraction Me7 at 50mg/kgbw, $Me7_{100}$=Treated with sub-fraction Me7 at 100mg/kgbw, $Me8_{50}$= Treated with sub-fraction Me8 at 50mg/kgbw, $Me8_{100}$= Treated with sub-fraction Me8 at 100mg/kgbw.

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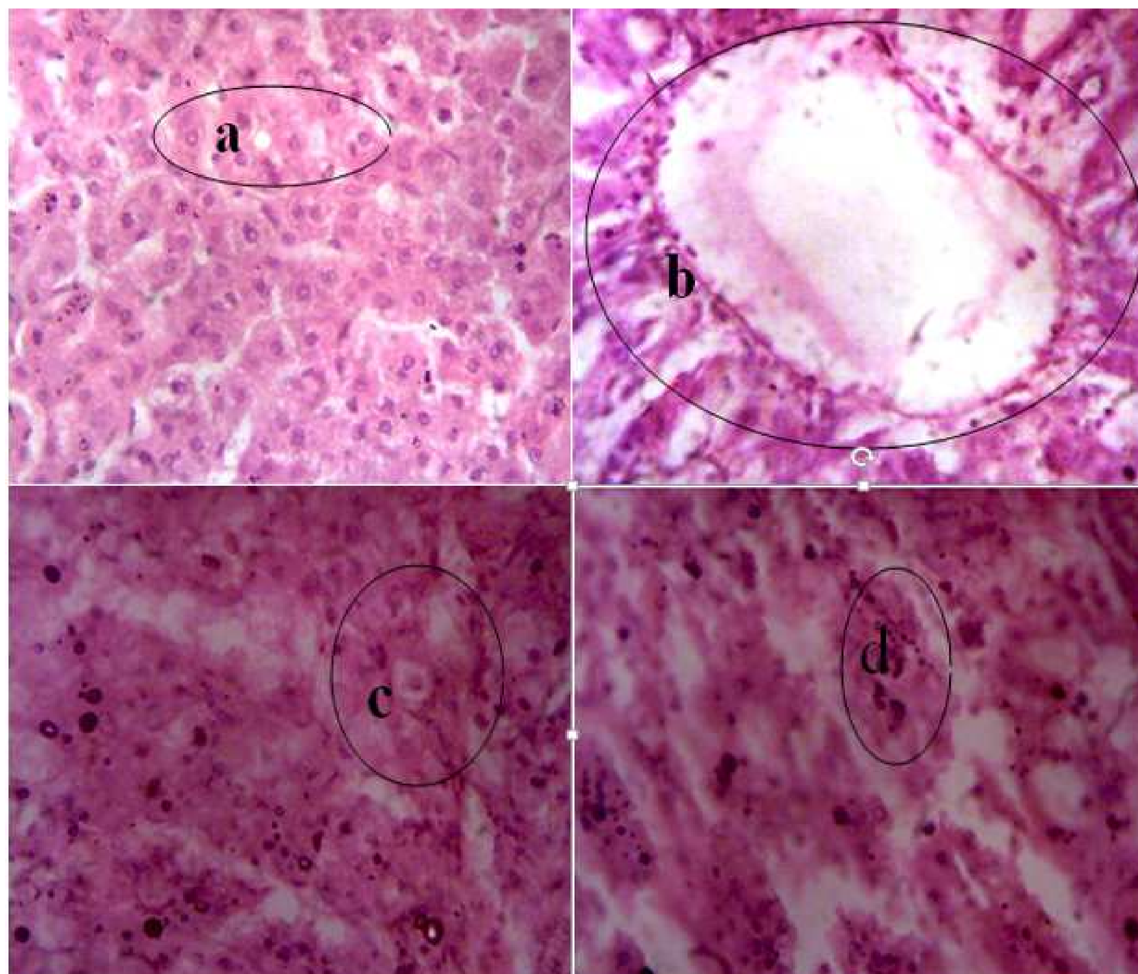

Plate 1: Liver section of a. Control group (Normal), b. untreated group, c. Standard drug (Silymarin) group, d. Subfraction Me6 (50mg/kg)

Liver section of control group I (Normal) showing normal liver histoarchitecture. (b) Liver section of CCl_4 induced untreated group (group II) showing distorted liver histoarchitecture and severe inflammation; fatty change, congested central vein; infiltration of inflammatory cells, necrosis, vascular degeneration. (c) Liver section of silymarin treated (group III) showing mild distortion of the liver histoarchitecture; moderate fatty change, macro and micro vesicles, hepatocytes, Central vein, and sinusoids with few necrotic cells (d) Liver section of Me6 (50mg/kg) treated group showing mild distortion of the liver histoarchitecture; Micro vascular fatty change, Infiltration of inflammatory cells.

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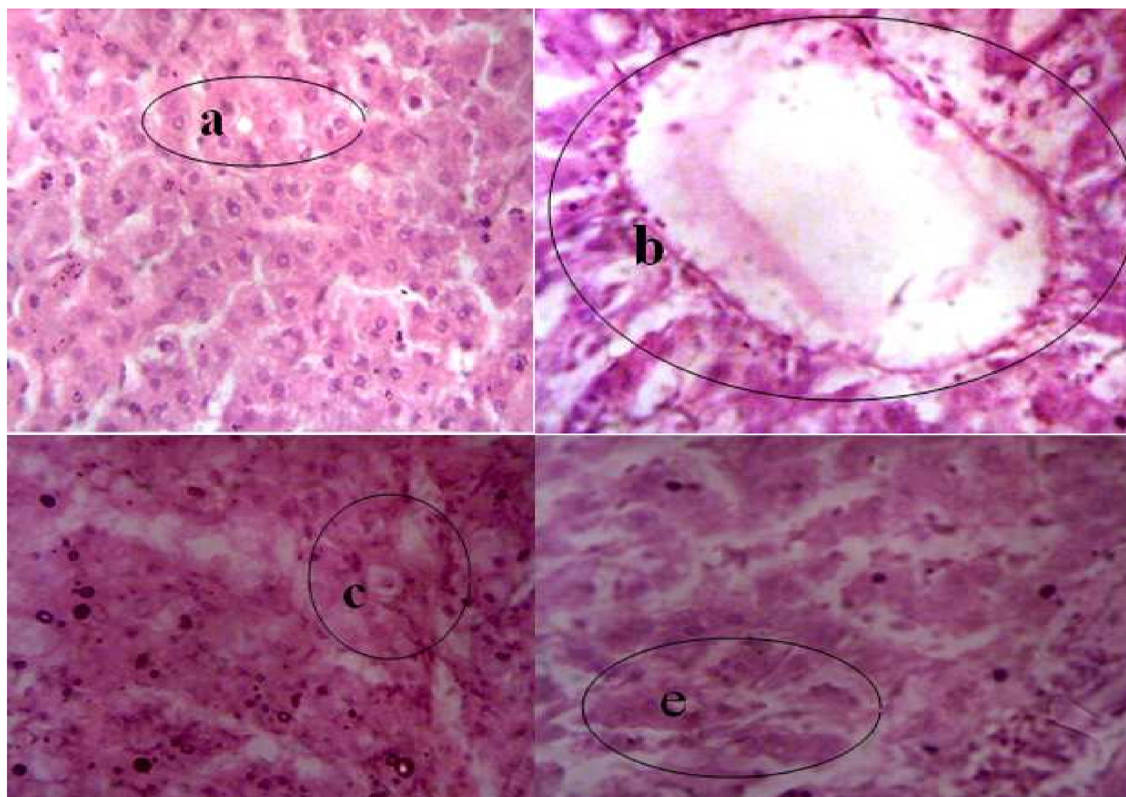

Plate 1I: Liver section of a. Control group (Normal), b. Untreated group, c. Standard drug (Silymarin) group, e. Subfraction Me6 (100mg/kg)

(a) Liver section of control group I (Normal) showing normal liver histoarchitecture. (b) Liver section of CCl_4 induced untreated group (group II) showing distorted liver histoarchitecture and severe inflammation; fatty change, congested central vein; infiltration of inflammatory cells, necrosis, vascular degeneration. (c) Liver section of silymarin treated (group III) showing mild distortion of the liver histoarchitecture; moderate fatty change, macro and micro vesicles, hepatocytes, Central vein, and sinusoids with few necrotic cells. (e) Liver section of Me6 (100mg/kg) treated group showing mild distortion of the liver histoarchitecture, mild to moderate bridged inflammation.

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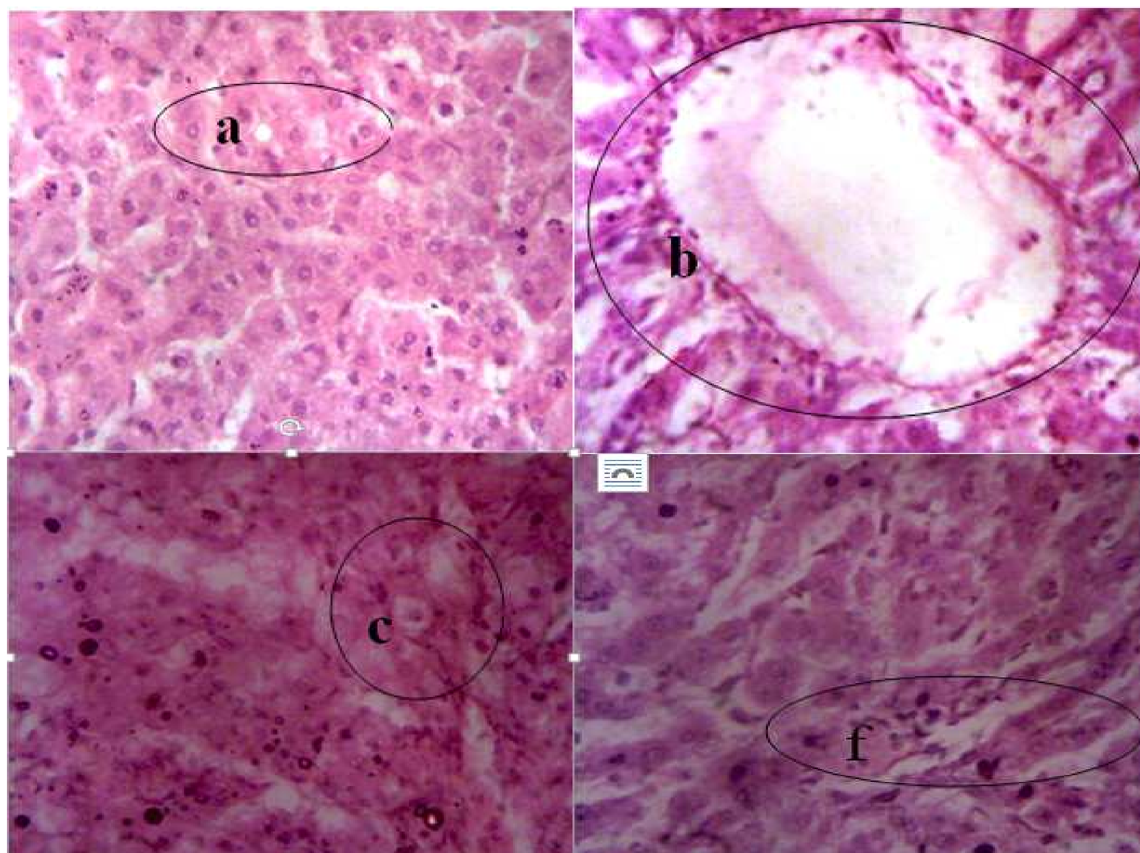

Plate 1II: Liver section of a. Control group (Normal), b. Untreated group, c. Standard drug (Silymarin) group, f. Subfraction Me7 (50mg/kg)

(a) Liver section of control group I (Normal) showing normal liver histoarchitecture. (b) Liver section of CCl_4 induced untreated group (group II) showing distorted liver histoarchitecture and severe inflammation; fatty change, congested central vein; infiltration of inflammatory cells, necrosis, vascular degeneration. (c) Liver section of silymarin treated (group III) showing mild distortion of the liver histoarchitecture; moderate fatty change, macro and micro vesicles, hepatocytes, Central vein, and sinusoids with few necrotic cells. (f) Liver section of Me7 (50mg/kg) treated group showing mild distortion of the liver histoarchitecture, moderate inflammation and bridging with bile ductular reaction, Infiltration of inflammatory cells.

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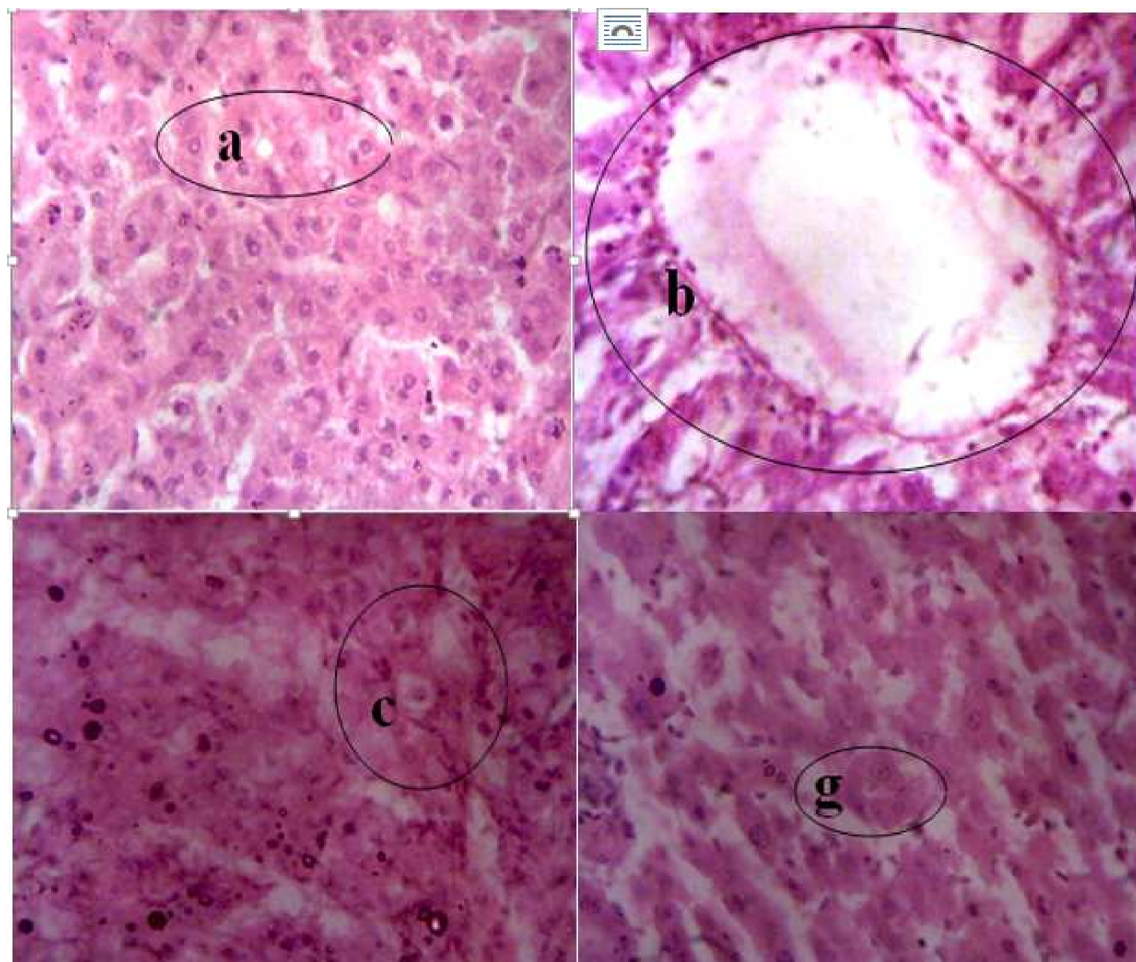

Plate IV: Liver section of a. Control group (Normal), b. Untreated group, c. Standard drug (Silymarin) group, g. Me7 (100mg/kg).

(a) Liver section of control group I (Normal) showing normal liver histoarchitecture. (b) Liver section of CCl_4 induced untreated group (group II) showing distorted liver histoarchitecture and severe inflammation; fatty change, congested central vein; infiltration of inflammatory cells, necrosis, vascular degeneration. (c) Liver section of silymarin treated (group III) showing mild distortion of the liver histoarchitecture; moderate fatty change, macro and micro vesicles, hepatocytes, Central vein, and sinusoids with few necrotic cells. (g). Liver section of Me7 (100mg/kg) treated group showing mild distortion of the liver histoarchitecture, subtle fatty change, and mild bridging inflammation with ductular reaction

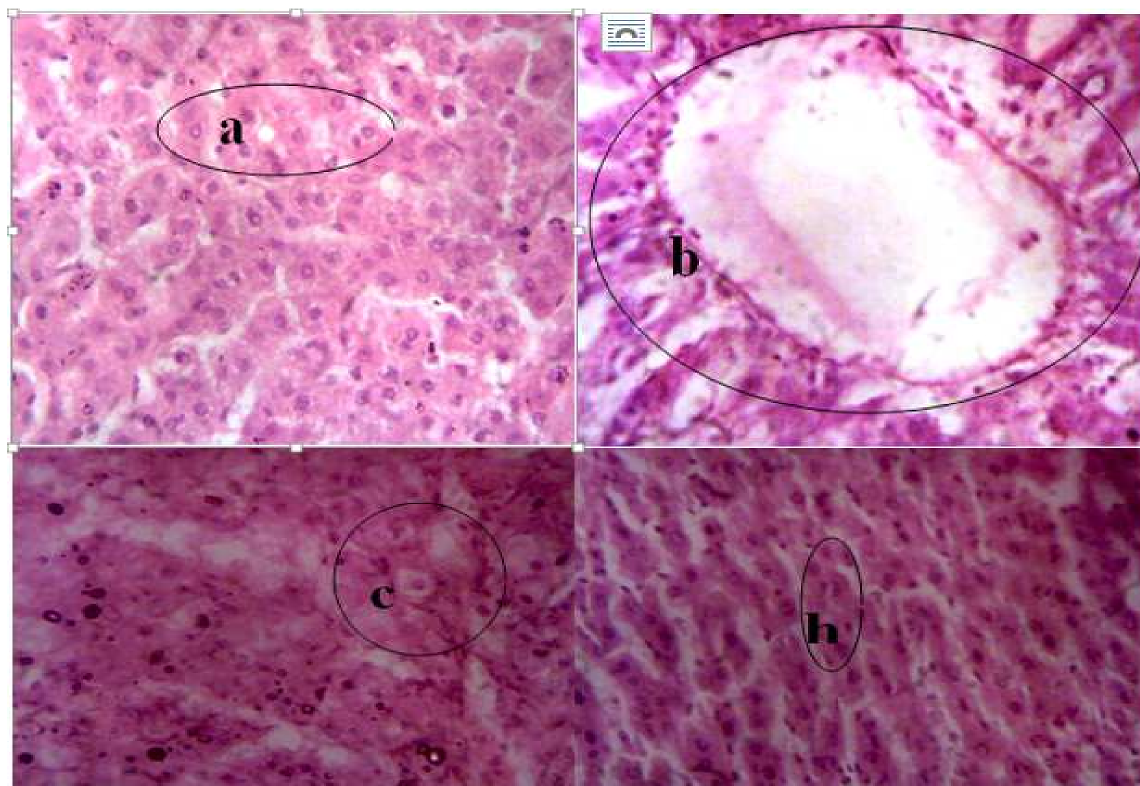

Plate V : Liver section of a. Control group (Normal), b. Untreated group, c. Standard drug (Silymarin) group, h. Subfraction Me8 (50mg/kg).

(a) Liver section of control group I (Normal) showing normal liver histoarchitecture. (b) Liver section of CCl_4 induced untreated group (group II) showing distorted liver histoarchitecture and severe inflammation; fatty change, congested central vein; infiltration of inflammatory cells, necrosis, vascular degeneration. (c) Liver section of silymarin treated (group III) showing mild distortion of the liver histoarchitecture; moderate fatty change, macro and micro vesicles, hepatocytes, Central vein, and sinusoids with few necrotic cells. (h) Liver section of Me8 (50mg/kg) treated group showing very mild distortion of the liver histoarchitecture almost normal; subtle bile ductular reacti

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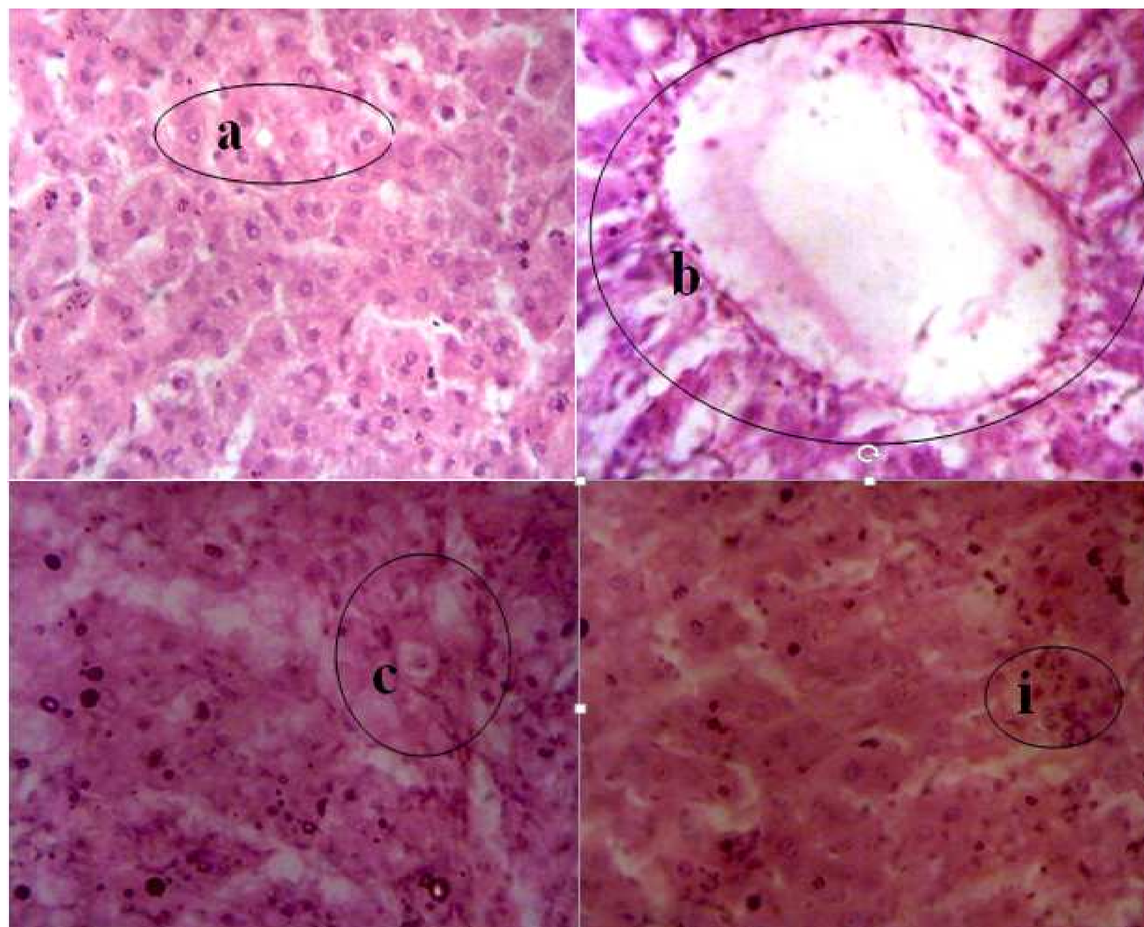

Plate VI: Liver section of a. Control group (Normal), b. Untreated group, c. Standard drug (Silymarin) group, i. Subfraction Me8 (100mg/kg)

(a) Liver section of control group I (Normal) showing normal liver histoarchitecture. (b) Liver section of CCl_4 induced untreated group (group II) showing distorted liver histoarchitecture and severe inflammation; fatty change, congested central vein; infiltration of inflammatory cells, necrosis, vascular degeneration. (c) Liver section of silymarin treated (group III) showing mild distortion of the liver histoarchitecture; moderate fatty change, macro and micro vesicles, hepatocytes, Central vein, and sinusoids with few necrotic cells. (i) Liver section of Me8 (100mg/kg) treated group showing moderate fatty change, few macro vesicles, numerous micro vesicles and no inflammation.

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Table 1: LD_{50} of Me6, Me7 and Me8 Subfractions of *P. mildbraedii*

Samples	No of Animal Used	Mortality	Dose	Toxicity signs
Me6	5	0	2000mg/kgbw	No observable sign of toxicity
Me7	5	0	2000mg/kgbw	Heavy breathing and inactive between 1-30mins
Me8	5	0	2000mg/kgbw	Inactive within 1st - 30mins

Key: Normal=Control, CCl_4= Toxic and not treated, Silymarin= Treated with standard drug, $Me6_{50}$= Treated with sub-fraction Me6 at 50mg/kgbw, $Me6_{100}$=Treated with sub-fraction Me6 at 100mg/kgbw, $Me7_{50}$=Treated with sub-fraction Me7 at 50mg/kgbw, $Me7_{100}$=Treated with sub-fraction Me7 at 100mg/kgbw, $Me8_{50}$= Treated with sub-fraction Me8 at 50mg/kgbw, $Me8_{100}$= Treated with sub-fraction Me8 at 100mg/kgbw.

LD_{50}>2000mg/kgbw if 3 animals survived. Therefore LD_{50} of Me6, Me7 and Me8 is greater than 2000mg/kgbw.

Treatment	AST(U/L)	ALT(U/L)	ALP(U/L)
Normal	19.89 ± 0.15^{ab}	20.33 ± 0.89^{a}	117.87 ± 1.15^{b}
CCl_4	82.06 ± 2.08^{e}	51.26 ± 1.77^{e}	206.28 ± 1.47^{d}
Silymarin	24.31 ± 2.21^{ab}	40.66 ± 176^{cd}	122.87 ± 8.49^{b}
$Me6_{50}$	35.99 ± 2.11^{bc}	25.64 ± 0.88^{b}	162.67 ± 1.94^{c}
$Me6_{100}$	17.69 ± 2.21^{a}	20.20 ± 0.12^{a}	61.91 ± 0.044^{a}
$Me7_{50}$	55.46 ± 1.56^{d}	44.99 ± 0.86^{d}	129.06 ± 1.15^{b}
$Me7_{100}$	41.99 ± 2.21^{cd}	23.54 ± 0.72^{ab}	117.28 ± 3.33^{b}
$Me8_{50}$	26.52 ± 1.00^{abc}	36.75 ± 3.70^{c}	121.41 ± 5.01^{b}
$Me8_{100}$	19.02 ± 0.01^{ab}	21.28 ± 0.45^{ab}	76.02 ± 3.54^{a}

Table 2: Effect of Me6, Me7 and Me8 Subfractions of *P. milbraedii* on Liver Enzymes

Results are presented as mean of five determinations ± SEM. Values with different superscripts down the same column are significantly different ($p<0.05$).

Key: Normal=Control, CCl_4= Toxic and not treated, Silymarin= Treated with standard drug, $Me6_{50}$= Treated with sub-fraction Me6 at 50mg/kgbw, $Me6_{100}$=Treated with sub-fraction Me6 at 100mg/kgbw, $Me7_{50}$=Treated with sub-fraction Me7 at 50mg/kgbw, $Me7_{100}$=Treated with sub-fraction Me7 at 100mg/kgbw, $Me8_{50}$= Treated with sub-fraction Me8 at 50mg/kgbw, $Me8_{100}$= Treated with sub-fraction Me8 at 100mg/kgbw.

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Table 3: Effect of Me6, Me7 and Me8 Subfractions of *P. milbraedii* on Biochemical Parameters in Normal and CCl_4 induced Hepatotoxic rats.

Treatment	Total Protein(mg/dl)	Albumin(g/dl)	Total Bilirubin(μmol/L)	Direct bilirubin (μmol/L)
Normal Control	$17.45±0.13^{b}$	$4.03±0.19^{e}$	$4.93±0.72^{ab}$	$2.10± 0.58^{ab}$
CCl_4	$13.87±0.21^{a}$	$2.73±0.12^{a}$	$16.87±1.02^{d}$	$5.03± 0.47^{c}$
Silymarin	$15.75±0.23^{ab}$	$3.67±0.12^{d}$	$4.93±0.27^{ab}$	$2.10±0.10^{ab}$
$Me6_{50}$	$15.28±0.17^{ab}$	$3.70±0.02^{d}$	$9.63±1.61^{c}$	$2.45±0.25^{ab}$
$Me6_{100}$	$15.14±0.13^{ab}$	$3.50±0.03^{c}$	$4.00±0.20^{a}$	$1.60±0.27^{a}$
$Me7_{50}$	$16.44±0.29^{ab}$	$3.13±0.44^{b}$	$5.30±0.50^{ab}$	$2.70±0.10^{b}$
$Me7_{100}$	$17.16±0.31^{b}$	$3.50±0.01^{c}$	$4.70±0.65^{ab}$	$2.30±0.81^{ab}$
$Me8_{50}$	$14.60±0.21^{ab}$	$3.70±0.29^{d}$	$7.40±2.30^{bc}$	$2.00±0.11^{ab}$
$Me8_{100}$	$15.01±0.19^{ab}$	$4.30±0.50^{e}$	$4.60±0.51^{ab}$	$1.70±0.32^{ab}$

Results are presented as mean of five determinations ± SEM. Values with different superscripts down the same column are significantly different ($p<0.05$).

Key: Normal=Control, CCl_4= Toxic and not treated, Silymarin= Treated with standard drug, $Me6_{50}$= Treated with sub-fraction Me6 at 50mg/kgbw, $Me6_{100}$=Treated with sub-fraction Me6 at 100mg/kgbw, $Me7_{50}$=Treated with sub-fraction Me7 at 50mg/kgbw, $Me7_{100}$=Treated with sub-fraction Me7 at 100mg/kgbw, $Me8_{50}$= Treated with sub-fraction Me8 at 50mg/kgbw, $Me8_{100}$= Treated with sub-fraction Me8 at 100mg/kgbw.

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