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In Vitro and *In Vivo* Hepatoprotective Activity of *Madhuca Longifolia* L Leaves on Carbon Tetrachloride Induced Rats

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Abstract: *Administration of Madhuca longifolia Methanolic Extract (MLME) and derivative of Madhucic Acid (dMA) a bioactive from it leaves and standard drug silymarin in rats showed significant hepatoprotective action against CCl_4 induced hepatotoxicity. Elevated serum markers enzymes of SGOT, SGPT, ALP and serum bilirubin were found to be significantly reduced to near normal levels on treatment of rats with MLME and dMA treated rats. MLME (50 mg/kg) and dMA (5 mg/kg) decreased the cholesterol level and increased TG level. In vitro hepatoprotective activity of the MLME and dMA was evaluated at 50 and 5 µg/ml concentrations against CCl_4 (1 %) induced toxicity in freshly isolated rat hepatocytes. The combination of silymarin with MLME and dMA showed better hepatoprotective activity when compared with the standard.*

Keywords: *Madhuca longifolia L leaves, Carbon tetrachloride, SGOT, SGPT, Hepatoprotective activity.*

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I. Introduction

Indigenous plants have been the traditional source of raw materials for the manufacture of medicines. The trend of using natural products is increased and the active plant extracts are frequently screened for new drug discoveries [1].

The liver is the most important organ in the body. It plays a vital role in regulating various physiological processes. It is also involved in several vital functions, such as metabolism, secretion and storage. It has great capacity to detoxicate toxic substances and synthesize useful principles [2, 3]. It helps in the maintenance, performance and regulating homeostasis of the body. It is involved with almost all the biochemical pathways to growth, fight against disease, nutrient supply, energy provision and reproduction. In addition, it aids in metabolism of carbohydrate, protein and fat, detoxification, secretion of bile and storage of vitamins [4]. *Madhuca longifolia* L (Sapotaceae), commonly known as mahua in the indo-Pak subcontinent, is an important economic plant growing throughout the subtropical regions of the indo-Pak sub-continent [5]. The constituents reported from *Madhuca longifolia* L include fatty acids sapogenins, carbohydrates, triterpenoids, steroids, saponin, flavonoids [6, 7].There is scanty literature about the leaves and the objective of the present study was to isolate a bioactive from the leaves and investigate its hepatoprotective effect on carbon tetrachloride induced rats. Silymarin has also been reported to provide liver protection against CCl_4 and paracetamol-induced liver damage in rat models. In search of an effective, standardized and safe hepatoprotective combination therapy, we used the silymarin as standardized drug and methanolic extract against CCl_4-induced hepatotoxicity in rats.

II. Materials and Methods

Plant material

The leaves of *M.longifolia* L were collected in Nov 2009 from Konchavaram forest Gulbarga, Karnataka and authentication was done by Prof Y.N. Seetharam, Dept of Botany, Gulbarga University Gulbarga where a voucher specimen has been deposited in the herbarium (HGUG no: 723).

Extraction and isolation

Air dried leaves (500 g) of *M. longifolia* L were reduced to a fine powder, which was subjected to hot continuous extraction in a soxhlet extractor, successively with petroleum ether (40-60°C). Each time before extracting with the next solvent, the powdered material was dried in hot air oven below 50°C.Each extract was concentrated by distilling off the solvent followed by evaporation to dryness on a water bath. All extracts were kept in a descicator and stored in a refrigerator for phytochemical and pharmacological studies. The methanolic extract was subjected for column chromatography on silica gel (60-120 mesh) and eluted with following solvent systems: chloroform : methanol (8:2) and (1:9).The chloroform : methanol (1:9) fraction was repeatedly

chromatographed on column and the collected fraction was checked on TLC until it gave a single spot of bright red color (100 mg) (R_f value : 0.89). The isolated compound was subjected for spectral analysis and the compound was identified as

10-(carboxyoxy)-1,2,2,6a,9,9,hexamethyldocosahydropicene-4a-carboxylic acid which showed m.p at 310°C, λ_{max} 254nm, the IR (KBr) V_{max} cm^{-1} 3468.32 (OH) stretching, 2922-2808 (C-H) stretching, 1709(C=O),1612(COOH), 1213 C-O-C); ^{1}H-NMR (DMSO) suggesting the structural similarities with Madhucic Acid[8]which was identified and confirmed by LCMS,IR, ^{1}H-NMR in figure 5.1.

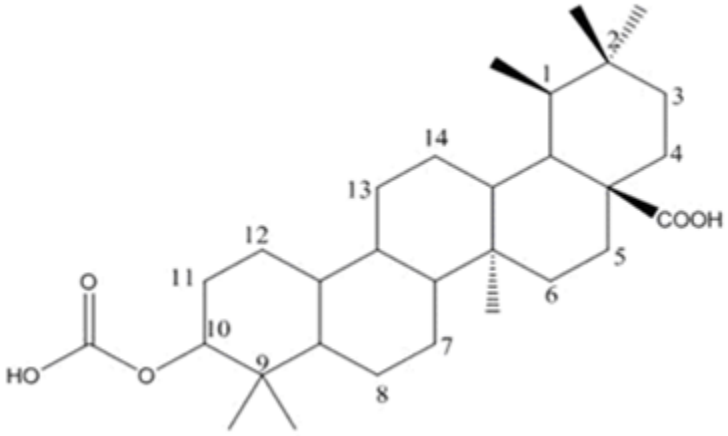


Figure 5.1: Chemical structure of compound isolated from *Madhuca longifolia* L leaves 10-(Carboxyoxy)-1, 2, 2, 6a, 9, 9-hexamethyldocosahydropicene-4a-carboxylic acid.

Animals

Swiss albino rats weighing 150-200 g were maintained under standard environmental conditions and had access to standard diet and water *ad libitum*. Animal were housed in polythene cages with 12:12 h light and dark cycle. All the experiments were performed in accordance with the guidelines for the care and use of laboratory, as adopted and promulgated by the institutional ethical animal committee, CPCSEA, India (Reg no 34800/2009/ CPCSEA).

Chemicals

All chemicals used were of analytical grade and procured from Sigma chemicals .Co. USA and Qualigens fine chemicals, Mumbai, India.

Assessment of hepatoprotective activity

Animals were divided into five groups of six rats each. Group I normal, group II received Carbon tetrachloride (1.0 ml/kg body weight) daily for 7 days. Group III received twice a day oral dose of 100 mg/kg bodyweight silymarin with CCl_4.Group IV received twice a day oral dose of MLME (50 mg/kg) and dMA (5 mg/kg) body weight along with CCl_4 respectively. In this study silymarin was used as positive control, as well as the hepatoprotective potential of both the doses of test samples were compared with the effect of silymarin. Rats were sacrificed 48h after the last dose of the drug. On 7th day, blood was collected by cardiac puncture for biochemical analysis and serum was separated and analyzed for various biochemical parameters.

Determination of serum biochemical markers

The collected blood was allotted to clot and centrifuged at 12000 rpm for 15 min to obtain the serum. The biochemical parameters like serum enzymes glutamate-pyruvate transaminase[9],glutamate- oxaloacetate transaminase[10],serum alkaline phosphatase [11],triglycerides[12],total cholesterol[13] and total bilirubin [14] were estimated using concerned assay kits according to the methods described by the manufactures.

Statistical analysis

The data of the current experiment is presented as mean ± SEM (standard error mean).The level of statistical significance was determined by analysis of variance (ANOVA) followed and the results were statistically significant at the value of probability less than 5% ($P < 0.05$).

III. Results

Figure 5.2 represents the effect of MLME and dMA on total bilirubin in CCl_4 induced hepatotoxicity in rats. The total bilirubin content was found to be increased (0.86 mg %) after CCl_4 administration when compared to control (0.17 mg %), which was further decreased after treatment with methanolic extract and dMA of *Madhuca longifolia* L. (0.65 and 0.57%) respectively. Administration of CCl_4 to rats produced significant ($P<0.001$***) levels of liver marker enzymes in plasma.

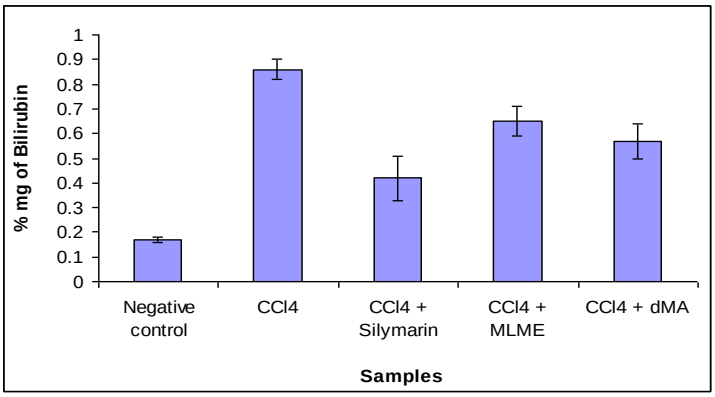


Figure 5.2: Mean ± SEM for total bilirubin estimation against carbon tetrachloride induced toxicity (1ml/kg) with silymarin (100mg/kg), MLME (50mg/kg) and derivative of Madhucic Acid (5mg/kg).

Figure 5.3 and Figure 5.4 depict levels of SGOT and SGPT in MLME and dMA administered rats respectively. Both SGOT and SGPT were found to be reduced and this reduction was almost equal to control. The level of ALP was found to be increased (224.01 U/L) in carbon tetrachloride induced rats. However, it was decrease (125.31 U/L and 100.88 U/L) when test samples (MLME and dMA respectively) were used. Reduction in ALP levels was almost equal to control in Figure 5.5.

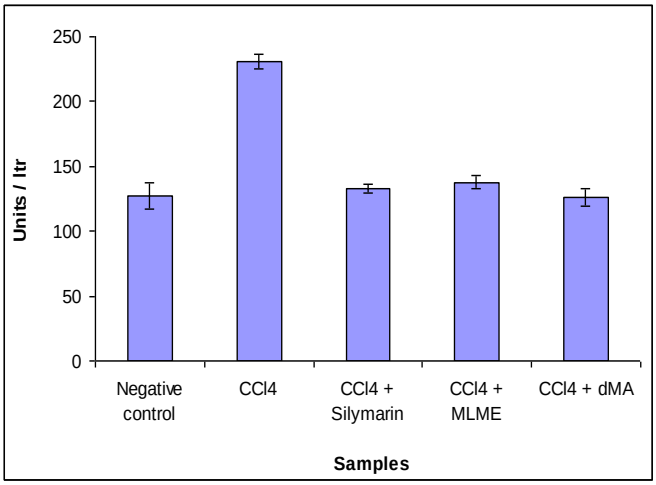


Figure 5.3: Mean ± SEM for SGOT estimation against carbon tetrachloride induced toxicity (1ml/kg) with silymarin (100mg/kg), MLME (50mg/kg) and derivative of Madhucic Acid (5mg/kg).

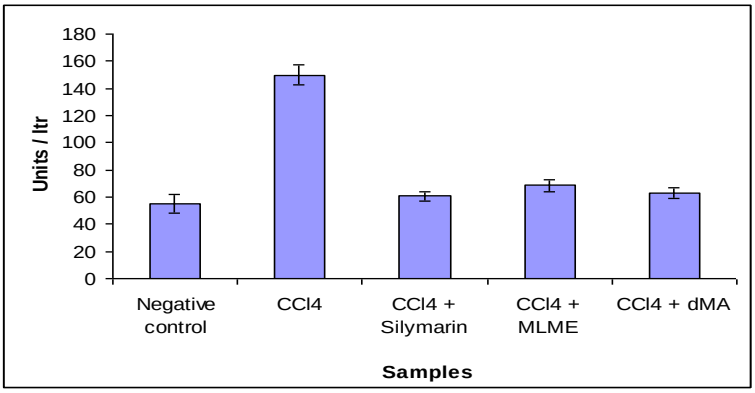


Figure 5.4: Mean ± SEM for SGPT estimation against carbon tetrachloride induced toxicity (1ml/kg) with silymarin (100mg/kg), MLME (50mg/kg) and derivative of Madhucic Acid (5mg/kg).

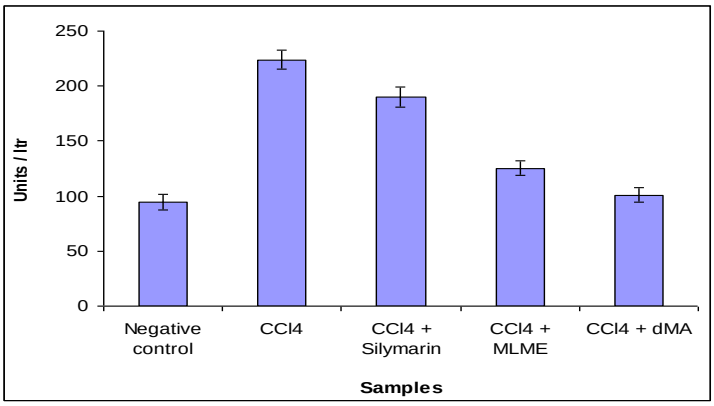


Figure 5.5: Mean ± SEM for ALP estimation against carbon tetrachloride induced toxicity (1ml/kg) with silymarin (100mg/kg), MLME (50mg/kg) and derivative of Madhucic Acid (5mg/kg).

SGOT, SGPT, ALP and total bilirubin in plasma have been reported to be sensitive indicators of liver injury [13].The disturbance in the transport function of the hepatocytes as a result of hepatic injury, causes the leakage of enzymes from cells due to altered permeability of membrane [14].

Figure 5.6 represents the effect of MLME and dMA on total protein content which was found to be decreased after CCl_4 administration when compared to control but increased after treatment with methanolic extract and dMA of *Madhuca longifolia* L. Figure 5.7 and Figure 5.8 depicts the effect of methanolic extract and dMA on cholesterol and trigycerides levels in CCl_4 induced hepatotoxicity in rats. Levels of both cholesterol and triglycerides were found to be reduced. Reduction in cholesterol and triglycerides were almost equal to control. The animals which were pretreated with MLME and dMA showed protection against the injurious effect of CCl_4 that may result from the interference with cyt P-450. These biochemical restorations may be due to the inhibitory effects on the cyt P-450/ promotion of its glucronidation [15].

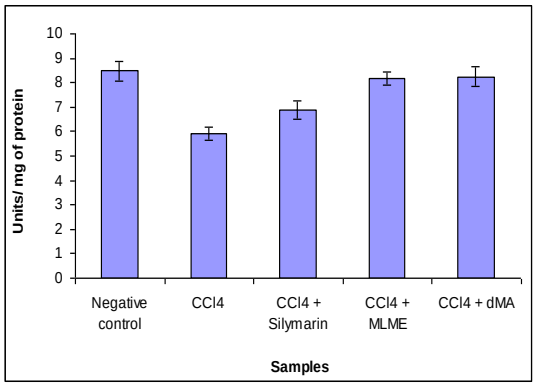


Figure 5.6: Mean ± SEM for total protein estimation against carbon tetrachloride induced toxicity (1ml/kg) with silymarin (100mg/kg), MLME (50mg/kg) and derivative of Madhucic Acid (5mg/kg).

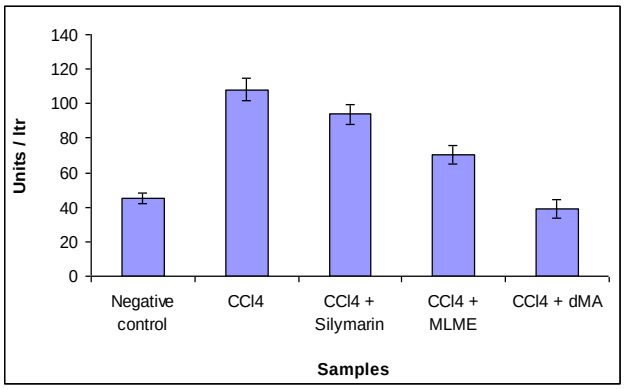


Figure 5.7: Mean ± SEM for cholesterol estimation against carbon tetrachloride induced toxicity (1ml/kg) with silymarin (100mg/kg), MLME (50mg/kg) and derivative of Madhucic Acid (5mg/kg).

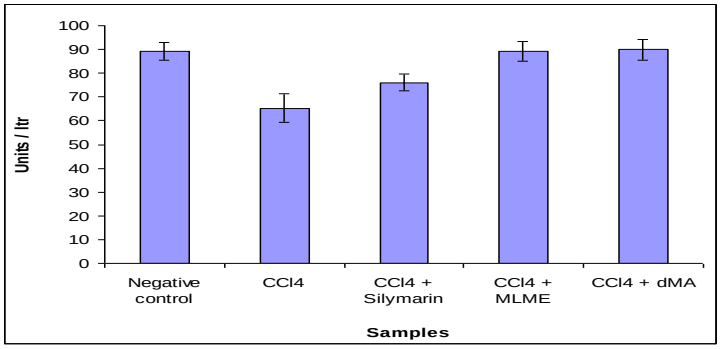
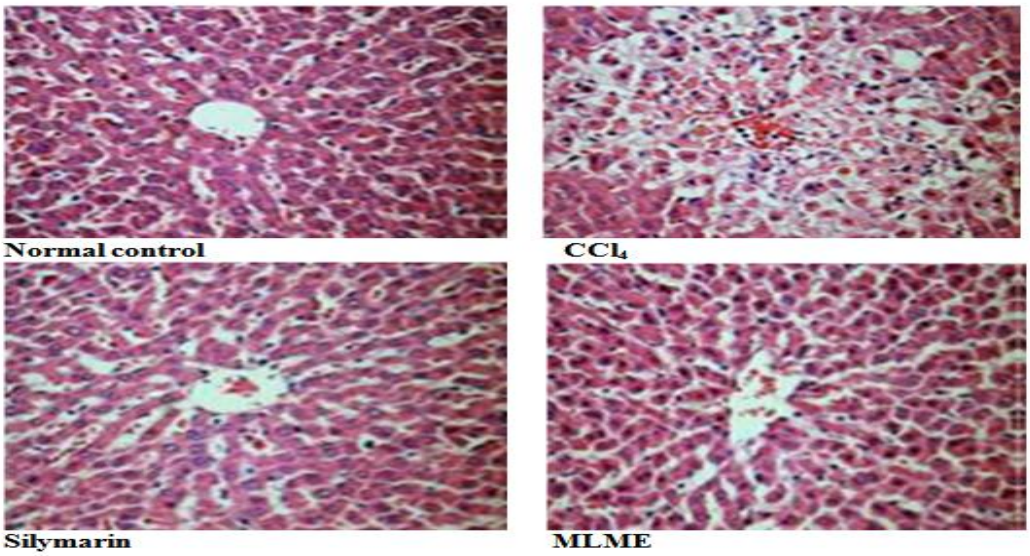
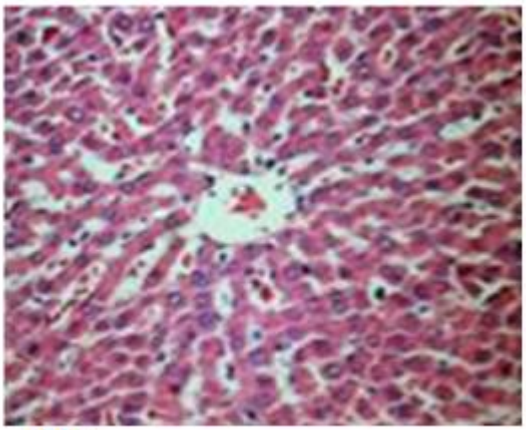


Figure 5.8: Mean±SEM for triglyceride estimation against carbon tetrachloride induced toxicity (1ml/kg) with silymarin (100mg/kg), MLME (50mg/kg) and derivative of Madhucic Acid (5mg/kg).

Histopathological examination – Liver of animals induced with CCl_4 on gross examination was presented with scattered white areas attributed to fatty acid and necrotic changes. Examination of liver tissues of animals treated with MLME, dMA and silymarin exhibited recovery towards normal in a dose related manner. Light microscopic examination of stained slides of liver sections of the animals induced with CCl_4 alone showed vacuolated hepatocytes, degenerated nuclei and focal necrosis and scattered lymphomononuclear infiltrate in hepatic parenchyma (Figure 5.9).





MLME (*Madhuca longifolia* Methanolic Extract) dMA (derivative of Madhucic Acid)

Figure 5.9: Effect of MLME on histopathological study of rats liver in CCl_4 induced hepatotoxicity (a) – Liver section of control rats showing normal liver architecture; (b)- liver section of CCl_4 alone (1 ml/kg) treated rats showing patches of liver cell necrosis with inflammatory collections and the loss of cellular boundaries; (c)- liver section of rats treated with CCl_4 and silymarin (100 mg/kg) showing well brought out central vein, hepatic cell with well- preserved cytoplasm, prominent nucleus. (d)-liver section of rats treated with CCl_4 and MLME (50 mg/kg) showing regeneration of hepatocytes with prominent nucleus, normal hepatic cells, no signs of necrosis and minimal inflammatory cellular infiltration; and (e) - liver section of rats treated with CCl_4 and dMA (5 mg/kg) showing well brought out central vein, hepatic cell with well-preserved cytoplasm, prominent nucleus like standard.

IV. Discussion

The ability of a hepatoprotective drug to reduce the injurious effects or to preserve the normal hepatic physiological mechanisms, which have been disturbed by a hepatotoxin, is the index of its protective effects. Protection of hepatic damage caused by carbon tetrachloride administration has been widely used as an indicator of liver protective activity of drugs in general [16]. CCl_4-mediated hepatotoxicity was chosen as the experimental model. It has been established that CCl_4 is accumulated in hepatic parenchyma cells and metabolically activated by cytochrome P450-dependent monooxygenases to form a trichloromethyl radical (CCl_3). The CCl_3 radical alkylates cellular proteins and other macromolecules with a simultaneous attack on polyunsaturated fatty acids, in the presence of oxygen, to produce lipidperoxides, leading to liver damage. Thus, antioxidant or free radical generation inhibition is important in protection against CCl_4-induced liver lesions [17].Serum glutamyl oxalacetic acid transferase, serum glutamyl pyruvate transferase, alkaline phosphatase and total bilirubin in plasma have been reported to be sensitive indicator of liver injury [13].The disturbance in the transport function of the hepatocytes as a result of hepatic injury, causes the leakage of enzymes from cells due to altered permeability of membrane. The present study reveals a significant increase in the activities of SGOT, SGPT, ALP and total bilirubin and total protein after exposure to CCl_4, indicating considerable hepatocellular injury. Administration of silymarin, silymarin MLME, silymarin dMA attenuated the increased levels of the serum enzymes produced by CCl_4, and caused a subsequent recovery towards normalization. These recoveries have been presented in Table 3. Serum parameters of different test sample, where it was found that the combination of silymarin, silymarin MLME and silymarin dMA exhibit higher recovery of serum parameters. Silymarin MLME and silymarin dMA showed highest recovery of SGOT, SGPT, total bilirubin and total protein. This suggests that MLME and dMA in combination with silymarin is able to condition the hepatocytes, so as to cause accelerated regeneration of parenchyma cells, thus protecting against membrane fragility and decrease of leakage of the marker enzymes into the circulation. Silymarin is a known hepatoprotective compound. It is reported to have a protective effect on the plasma membrane of hepatocytes [14]. In the present study, the level of SGOT, SGPT, ALP and serum bilirubin have been raised in CCl_4 induced group but decreased in silymarin MLME and silymarin dMA, silymarin group, which is an indication of hepatoprotection by these groups.

Histopathological findings revealed that the hepatic cells and the central veins were almost normal in MLME and dMA administered rats, compared with CCl_4 alone induced rats. This study confirms that both MLME and dMA have hepatoprotective effect on hepatic damage induced by CCl_4. Thus it can be concluded that the mechanism of hepatoprotective activity of MLME may be due its synergestic effect of the triterpene present in the leaves.

V. Conclusion

It can be concluded, that the *Madhuca longifolia L*. leaves possess significant hepatoprotective activity, this synergistic effect may be due to the presence of phytoconstituent in leaves and may prove to be effective for the treatment of liver disorders.

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