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EVALUATION OF HEPATOPROTECTIVE ACTIVITY OF *MORUS INDICA* LINN. AGAINST TOXICITY INDUCED BY CARBON TETRACHLORIDE IN RATS

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Keywords:

Morus indica, Carbon tetrachloride, Aqueous and dechlorophyllised extracts, hepatotoxicity.

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ABSTRACT: *Morus indica- MI-S36* is a potential source of phytochemicals and is well explored for its medicinal properties such as antidiabetic and antioxidant activity. In the present study, *Mous indica* aqueous (MAq) and dechlorophyllised (MDc) extracts efficacy to protect against the carbon tetrachloride (CCl_4) induced hepatotoxicity was studied in rats in comparison with standard drug Liv52. The experimental group rats were pre treated with MAq, MDc and Liv52 for 7days. Healthy control and positive control group animals were fed wth Olive oil and after 7days two doses of CCl_4 was given at 12 and 36 hrs intervals to all the animal groups. After 12 hrs of CCl_4 dosage animals were sacrificed and biochemical parameters analysed in the serum. The total protein, albumin, urea, creatinine, total bilirubin were within the normal level in all the groups whereas the hepatic enzymes AST, ALT and ALP activity was less in Liv52 and Morus treated groups. Total cholesterol, triglycerides, were determined. The total cholesterol and triglyceride levels in Morus treated groups were significantly ($p \leq 0.05$) lower than Liv52, CCl_4 and healthy control groups. Also pre treatment with Morus extracts restored the hepatic architecture near to the standard drug treatment which is showed in histopathologicl sections of liver. Hence the results indicate the liver protective property of *Morus indica*.

INTRODUCTION: Liver is the largest organ in the vertebrate body and the site for intense metabolism. It plays an astonishing array of vital functions in the maintenance and performance of the body. The most important functions include carbohydrate, protein and fat metabolism, detoxification and secretion of bile [1]. Liver is often damaged by environmental toxins, poor eating habits, alcohol and medicines and Over-the-counter drugs. Diverse homeostatic mechanisms are affected if liver function is impaired, with potentially serious consequences [2].

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Unfortunately, conventional or synthetic drugs used in the treatment of liver diseases are inadequate and sometimes can have serious side effects [3]. In the absence of a reliable liver protective drug in modern medicine there are a number of medicinal preparations in Ayurveda recommended for the treatment of liver disorders [4]. Conventional medicine is pursuing the use of natural products such as medicinal plants to provide support to the liver on a daily basis to revitalize the liver and treat hepatic dysfunction [5].

In this modern age it is very important to provide scientific proof by following systemic research methodology to justify the usage of various herbal drugs and providing scientific basis for the traditional herbal medicines that they have less side effects and possess the hepatoprotective activity [6]. However, examining the effectiveness of herbal medicines and looking for satisfactory remedies for

serious liver diseases in order to develop effective and safe drugs is an area of interest [7].

In view of severe undesirable side effects of synthetic agents, there is growing focus to follow systematic research methodology and to evaluate scientific basis for the traditional herbal medicines that are claimed to possess hepatoprotective activity [6]. The experimental intoxication induced by carbontetra chloride CCl_4 is widely used for modeling liver injury in rats [8]. The hepatotoxicity of CCl_4 is the result of reductive dehalogenaiton, which is catalyzed by P-450 and which forms highly reactive trichloromethyl free radical. That readily interacts with molecular oxygen to form the trichloromethyl peroxy radical. Both trichloromethyl and its peroxy radical are capable of binding to proteins or lipids, or of abstracting a hydrogen atom from an unsaturated lipid imitating lipid peroxidation leading to liver damage [9].

Numerous epidemiological surveys have shown an inverse relationship between the intake of herbs, fruits, vegetables and cereals and the incidence of coronary heart disease and certain cancers. Medicinal plants or parts thereof that are rlatively high in phytochemicals are known to exert hepatoprotective actions. *Morus indica* extracts Aqueous and dechlorophylised, with good antioxidant activity in *in vitro* and *ex vivo* levels were chosen to study their hepatoprotective activity.

MATERIALS AND METHODS:

Chemical and Reagents: Alanine transaminase (ALT), Aspartate transaminase (AST), Alkaline phosphatase (ALP), Total protein, albumin, urea, creatinine, total bilirubin, triglycerides, total cholesterol assay kits were purchased from Aggappe Diagnostics, Ernakulam, India. Reduced glutathione (GSH), 5,5-dithio (bis) nitro benzoic acid (DTNB) were purchased from Sigma-Aldrich, Bangalore, India. All the chemicals and reagents used in the study were of analytical grade.

Collection and Preparation of Samples: The leaves of *Morus indica* were collected from CSRTI, Mysore, Karnataka, India and subsequently identified by Dr. G. R. Shivamurthy, Department of Studies in Botany, University of Mysore, Mysore, India. The samples were washed, dried overnight (50^0C), powdered, passed through 60 mesh sieve (BS) and stored in airtight container at 4^0C till further use.

Aqueous (MAq) and dechlorophylised (MDc) extracts of *Morus indica* –S36 (MI-S36) leaf were used for studying their potential hepatoprotective effect against CCl_4 intoxication. The cold aqueous extracts of MI-S36 leaf were prepared by extracting powdered material with cold water (RT) in a mechanical shaker (24 h), filtered and freeze dried in freeze drier (Thermo Modulyo D, Hong Kong). 80% methanol extract was prepared by taking 15g sample, extracted with 50ml of 80% methanol (methanol : water - 8:2 ratio, v/v) in a mechanical shaker (6 h). The extracts were evaporated at 40º C under reduced pressure to dryness in a rotary evaporator (Superfit, India).

To avoid the interference of chlorophyll, it was separated as per the method of Rich A and Rich C1[0]. Briefly, hexane was added to the 80% methanol extract, shaken for 30 min and the chlorophyll - rich hexane top layer was separated. The remaining extract was further evaporated (Rotary evaporator) and oven dried (50 °C) and stored in air-tight container at 0°C until used.

Experimental Animals: Adult wistar strain albino rats weighing around 140-180g were acclimatized for 14 days under standard conditions. The rats were housed in the polyacrylic cages, maintained at 25±2º C, 45 to 60 % RH and 12h photo period. During acclimatization, the animals were observed for general conditions every-day. Standard pellet diet (Amrut feeds, Pune, India) and water *ad libitum* were provided. The experimental protocol of hepatoprotective studies was reviewed and approved by the Institutional Animal Ethical Committee (IAEC) for the purpose of control and supervision of experiments on animals (UOM/IAEC/29/2011).

Methodology: Adult Wistar strain albino rats weighing around 140-160g were divided into various groups (Table 1) consisting of 6 animals each. The rats were housed in the polyacrylic cages, maintained at 25±2º C, 45 to 60 % RH and 12h photo period. Standard pellet diet (Amrut feeds, Pune, India) and water *ad libitum* were provided.

CCl_4 Induced Hepatotoxicity: The sample extracts in the form of suspensions, a standard drug Liv 52 (polyherbal tonic used anti-hepatotoxin) and olive oil (Control & Positive Control) were administered orally for 7 days in the respective groups. A mixture of CCl_4 (0.5ml/kg BW i.p) in olive oil was injected two times, at 12 and 36 h after the final administration of sample extract, and the animals were euthanized and decapitated after 12 h of final administration of CCl_4.

Biochemical parameters and Histopathological Procedures. Blood was collected and serum was separated after centrifuge at 2500 ×g, for 20 min. Activities of AST, ALT and ALP were determined in serum along with estimation of total protein, albumin, urea, creatinine, total bilirubin, total cholesterol, triglycerides (TGL) using respective standard kits. The contents of Glutathione (GSH), TBARS as markers of lipid peroxidation were determined by the methods of Ellman (1959) [11] and Ohkawa *et al* (1979) [12] respectively in serum, liver and kidney homogenates.

Various organs like liver, kidney, heart, brain and spleen were excised immediately, washed with phosphate buffered saline and weighed. Small portions of liver and kidney were fixed in 10% formaldehyde, then dehydrated in graduate ethanol (50 – 100%), cleared in xylene and embedded in paraffin. The sections (4 - 5µm) were stained with haemotoxylin and eosin (H-E) dye and examined using a photomicroscope (40x) for any hisopathological changes.

Statistical Analysis: The values are expressed as mean ± SD. The data was subjected to one way ANOVA followed by Tukey's multiple comparisons test for significant difference ($p \leq 0.05$) using SPSS 11.5 software.

RESULTS AND DISCUSSION:

Effect on Biochemical Parameters and Hepatic Enzymes: The relative body and vital organs weights of all the animals were within the normal range (**Table 1**). The data on serum total protein, albumin, creatinine, total bilirubin and urea is shown in **Table 2**. No significant ($p \leq 0.05$) difference was observed in biochemical parameters of Liv52 and Morus treated groups (MAq and MDc). The protein and albumin content of the liver and kidney are given in **Table 3**. Apart from CCl_4 treated group the protein and albumin content were high in all the groups. This shows the protective effect of Morus on the protein metabolism against CCl_4 toxicity.

The protective effect of sample extracts against CCl_4 induced hepatic injury was assessed by analysing activity of hepatic enzymes (**Fig.1A**). A significant ($p \leq 0.05$) difference in serum biochemical markers were observed between normal and experimental groups. Pre-treatment with Liv52 and Morus extracts significantly reduced the ALP, AST and ALT activities compared to CCl_4. The activity of hepatic enzymes was higher than the control in all the groups, however, the Liv52, and other Morus treated MAq and MDc were less than CCl_4. The MAq showed better activity than MDc.

The total cholesterol and triglycerides levels in positive control group (CCl_4) were significantly high ($p \leq 0.05$) (**Fig.1B**). It was interesting to note that the total cholesterol and triglyceride levels in all the extract treated groups were significantly ($p \leq 0.05$) lower than Liv52 group.

The lipid peroxides (LPO) and Glutathione (GSH) of serum, liver homogenate and kidney homogenate are given **Fig. 1C** and **1D**. The control group with high glutathione levels showed low LPO than all the other groups. In CCl_4 treated group GSH levels were low and LPO were higher than Liv52 and Morus treated groups. These results indicate that the pre-treatment with Morus extracts had increased the antioxidant storage levels in animals which had inhibited the generation of lipid peroxides in serum, liver and kidney.

Histopathological Procedures: The histological sections of the liver of control and extract treated groups are represented in **Fig. 2I.** In CCl_4 group the architecture of liver in portal triad was destroyed along with necrosis of periportal hepatocytes and destruction of bile duct and blood vessels. There was also moderate to severe fatty change, plenty if chronic inflammatory infiltrate in portal triad. In Liv52 group the architecture of liver was normal, minimal chronic inflammatory infiltration in peripheral hepatocytes, and no fatty changes were observed.

The pretreatment with sample extracts helped in restoring the hepatic architecture near to the standard drug treatment, with significantly minimal damage when compared to the CCl_4 group. In all the experimental groups, necrosis in portal triad with chronic inflammatory infiltration and occasional foci was observed along with mild fatty change in peripheral hepatocytes, however in MDc group there was mild to moderate fatty change in peripheral hepatocytes.

TABLE 1: *MORUS INDICA* EFFECT ON THE BODY AND VITAL ORGANS WEIGHTS OF ANIMALS TREATED WITH CCl_4 (G).

Group	Body wt.	Liver	Kidney	Heart	Brain	Spleen
Ctrl	177±29.1	5.63±0.06	1.42±0.11	0.72. ±0.06	1.38±0.24	0.418 ±0.096
CCl_4	184 ±33.72	6.29±0.08	1.26±0.10	0.585±0.07	1.55±0.17	0.385±0.061
Liv52	175 ±34.01	6.40±0.01	1.4±0.22	0.665±0.01	1.56±.0.23	0.5415±0.13
MAq	171±21.8	5.36±0.35	1.44±0.23	0.615±0.061	1.298 ±0.176	0.522±0.0117
MDc	190±25.98	7.43±0.75	1.53±0.25	0.703± 0.116	1.44±0.0881	0.541±0.028

Ctrl-control; CCl_4-Carbontetrachloride; MAq- Morus Aqueous hot extract; MDc– Morus Dechlorophyllised extract; TC-total cholesterol;

TABLE 2: PROTECTIVE EFFECT OF *MORUS INDICA* L EXTRACTS ON SERUM BIOCHEMICAL PARAMETERS AGAINST CCl_4 TOXICITY

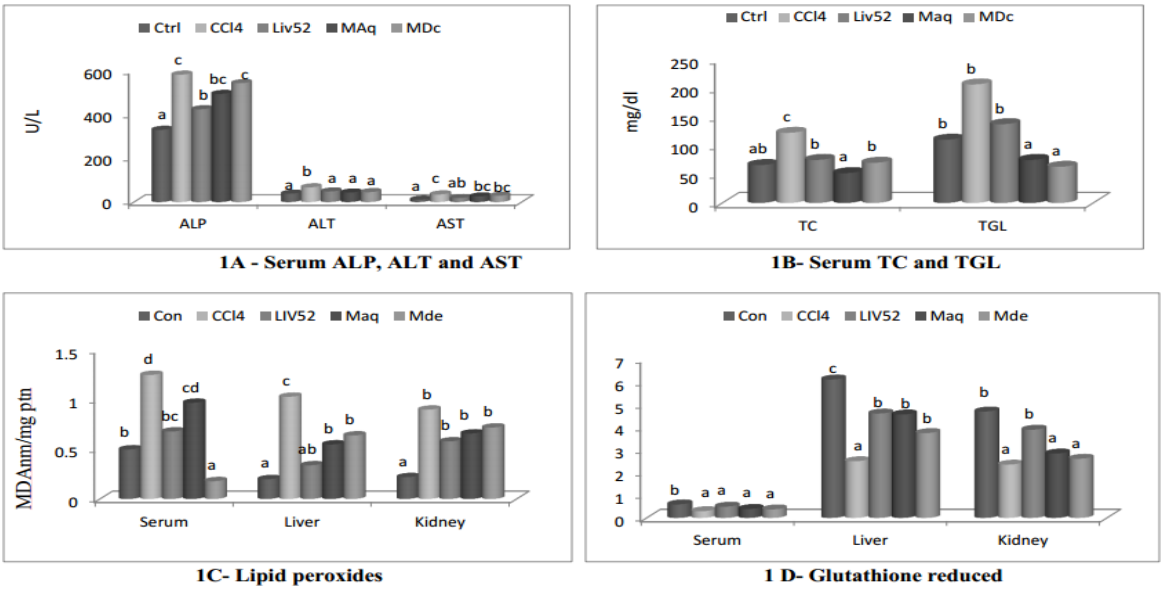
Group	TP@	Alb@	Creatinine@	T.Bil@	Urea@
Ctrl	$5.45±0.7^{b}$	$3.43±0.26^{b}$	$1.2±0.08^{a}$	$0.2±0.01^{a}$	$36±2.02^{a}$
CCl_4	$4.2±0.56^{a}$	$2.52±0.18^{a}$	$1.8±0.31^{b}$	2.1±0.06c	$37±±6.98^{a}$
Liv52	$5.23±0.18^{b}$	$2.92±0.11^{ab}$	$1.28±0.16^{a}$	$0.7±0.02^{ab}$	$41±11,23^{a}$
MAq	$4.32±0.12^{a}$	$3.79±0.18^{b}$	$0.84±0.11^{a}$	$0.6±0.11^{ab}$	$46±9.1^{ab}$
MDc	$4.53±0.63^{a}$	$2.9±0.14^{ab}$	$1.08±0.11^{a}$	$0.9±0.08^{b}$	$54±4.1.^{b}$

ctrl-control; CCl_4-Carbontetrachloride; MAq- Morus Aqueous hot extract; MDc – Morus Dechlorophyllised extract; TP-Total protein, Alb-albumin, T.Bil- total bilirubin,.Mean values carrying different superscripts a, b, c… differ significantly ($p \leq 0.05$). @- expressed as mg/dl

TABLE 3: PROTECTIVE EFFECT OF *MORUS INDICA* L EXTRACTS ON PROTEIN AND ALBUMIN CONTENT OF VITAL ORGANS AGAINST CCl_4 TOXICITY ON SERUM, LIVER AND KIDNEY

Group	Liver-TP	Kidney-TP.	Liver-Alb	Kidney-Alb
Ctrl	$1.71±0.7^{b}$	$0.52±0.06^{b}$	$0.47±0.04^{c}$	$0.23±0.05^{b}$
CCl4	0.81±0.04a	$0.38±0.04^{a}$	$0.35±0.04^{a}$	$0.13±0.01^{a}$
Liv52	$1.41±0.04^{b}$	$0.44±0.09^{a}$	$0.61±0.05^{d}$	$0.23±0.04^{b}$
MAq	$1.05±0.35^{a}$	$0.42±0.06^{a}$	$0.49±0.17^{b}$	$0.19±0.04^{b}$
MDc	$0.96±0.06^{a}$	$0.52±0.03^{b}$	$0.45±0.03^{b}$	$0.2±0.01^{b}$

ctrl-control; CCl_4- Carbontetrachloride, MAq- Morus Aqueous hot extract; MDc – Morus Dechlorophyllised extract; TP-Total protein, Alb-albumin; Values are mean ±SD ,(n=6) Values carrying different superscripts a, b, c.... differ significantly);*- mg/dl; ($p \geq 0.05$)



1A - Serum ALP, ALT and AST

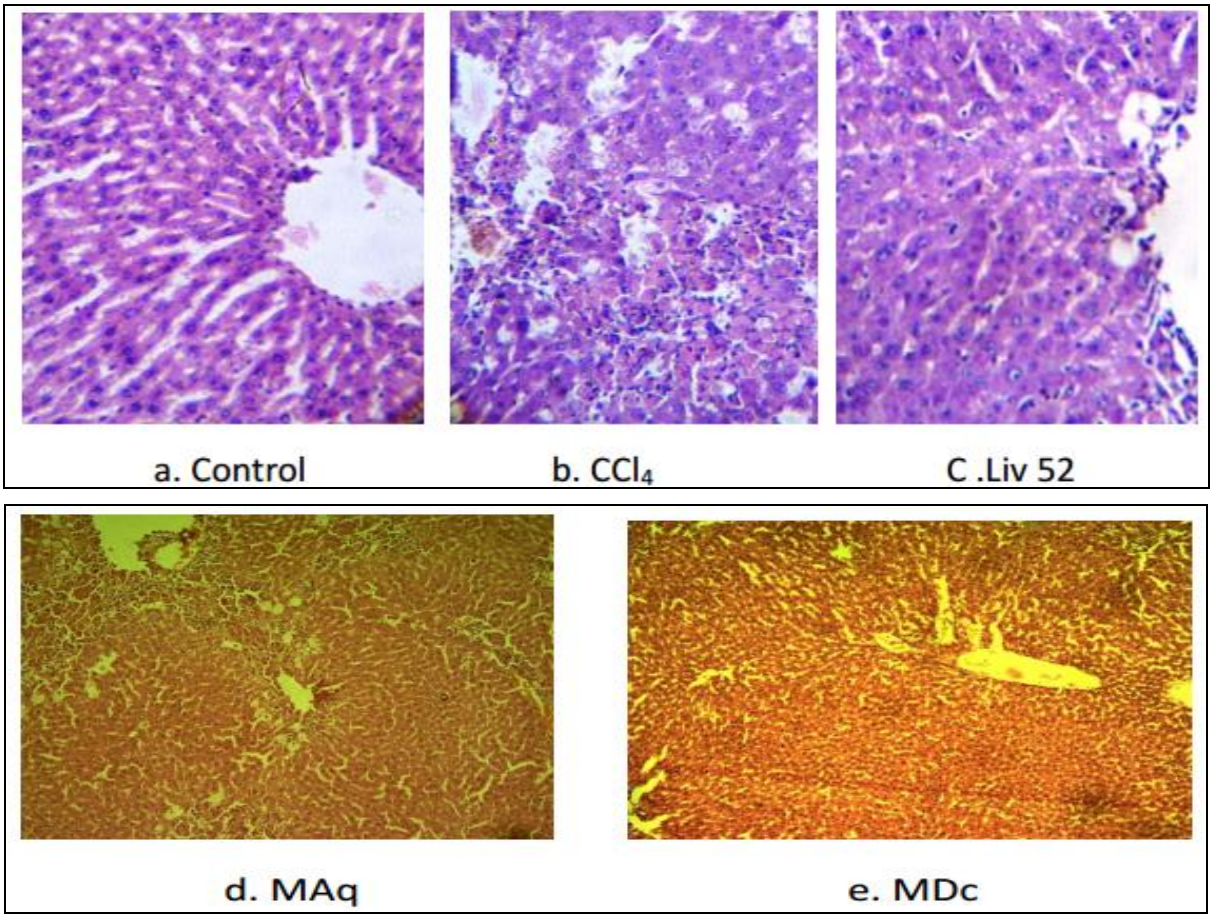
1B- Serum TC and TGL

1C- Lipid peroxides

1 D- Glutathione reduced

ctrl-control; CCl_4- Carbontetrachloride, MAq- Morus Aqueous hot extract; MDc – Morus Dechlorophyllised extract; ALP-alkaline phosphatase, ALT- Alanine transaminase; AST-Aspartate transaminase, TC-total cholesterol; Values are mean ±SD ,(n=6) Values carrying different superscripts a, b, c.... differ significantly); ($p \geq 0.05$)

FIG. 1: PROTECTIVE EFFECT OF MORUS INDICA L EXTRACTS ON ENZYME, LIPID METBOLISMA ND ANTIOXIDANTS AGAINST CCl_4 TOXICITY ON SERUM, LIVER AND KIDNEY



Ctrl-control; CCl_4- Carbontetrachloride, MAq- Morus Aqueous hot extract; MDc – Morus Dechlorophyllised extract;

FIG. 2: PROTECTIVE EFFECT OF *MORUS INDICA* EXTRACTS ON HEPATOCYTES AGAINST CCL_4 INDUCED TOXICITY

DISCUSSION: Oxidative stress and the metabolic stress contribute for hepatic injury and dysfunction. Medicinal plants are blessed with a wide array of phytochemicals most of which are potent anti-oxidants, and even some of the phytoconstituents from the medicinal plants have the capacity to potentiate the regeneration of the damaged hepatocytes [13].

Many hepatic injury models or protocols have been developed to assess the Hepato protective efficacy of the medicinal plants, of which CCl_4 induced hepatic damage in rats/mice is most widely accepted model system to study the protective effect of samples against oxidative damage in hepatocytes. CCL_4 is accumulated in hepatic parenchyma cells and metabolically activated by cytochrome P450-dependent monoxygenases to form a trichloromethyl radical (CCl_4). The CCl_4 radical alkylates cellular proteins and other macromolecules with simultaneous attack on polyunsaturated fatty acids, in the presence of oxygen to produce lipid peroxides leading to liver damage.

Thus, antioxidant or free radical generation inhibition is important in protection against CCl_4 – induced liver lesions. Hepatotoxic compounds such as CCl_4 are known to cause marked elevation in serum enzymes and bilirubin levels. It causes a marked decrease in total protein levels[14]. Various studies have demonstrated that the liver is not the only target organ of CCl_4; it causes free radical generation in other tissues also such as kidneys, heart, lung, testis, brain and blood [15]. The extent of toxic effect on normal liver functioning can be estimated by the activities of serum marker enzymes, like AST, ALT, ALP and by the

histopathological observation. The tendency of the aforementioned enzymes to return to near normal activities in extract administered group is a clear manifestation of hepatoprotective effect of the extracts.

Indeed we observed that pretreatment of rats with crude extract of Morus prevented the rise in the

serum level of AST and ALT towards the normal range was observed when compared to CCl_4 indicating hepatoprotective effect. Reduction in ALP levels with concurrent depletion of raised bilirubin levels suggests the stability of the biliary function in damaged hepatocytes due CCl_4 toxicity. Hence, all the extracts significantly ($p \leq 0.05$) blunted the increased activities of these enzymes and the level of bilirubin in the serum, showing the hepatoprotective effect [16]. Similar results were reported in *Morus alba*, where alcohol extract exerted a protective action against CCl_4 induced liver injury [6].

Research studies have shown that drugs having antioxidant activity are protective against CCl_4 induced hepatotoxicity [17]. The antioxidant activity or the inhibition of the generation of free radicals is important in the protection against CCl_4 induced hepatic injury [18]. The *Morus indica* is a good source of phytochemicals and also dechlorphylised and aqueous extracts contain polyphenols [19].

Literature indicates that 160 phyto-constituents from 101 plant families have anti-hepatotoxic activity [20]. Of these plant metabolites the phenolic components such as flavonoids, polyphenols, saponins, alkaloids and terpenoids [21-26] are known to reduce oxidative stress by virtue of their antioxidant and anti-inflammatory activities.

The examination of histopathological sections of negative group showed normal cellular architecture with distinct hepatic cells, sinusoidal spaces and central vein. Disarrangement of normal hepatic cells with centrilobular necrosis vacuolization of cytoplasm and fatty degeneration were observed in carbon tetrachloride intoxicated mice. The liver sections of the rats treated with the crude extract of *Morus indica* L. at 500mg/kg BW dosage, followed by carbon tetrachloride intoxication exhibited a significant protection as it was evident by the absence of necrosis, tissue damage and vacuoles.

Hence it can be inferred that the presence of antioxidant phytochemicals in the sample may act to reduce the CCl_4 induced oxidative stress, in turn reducing the generation of free radicals due ionizanti diabetic propertiesation of CCl_4. Since the plant demonstrated anti-hepatotoxic effect against CCl_4 induced oxidative damage, it can be utilized in any food formulation, as a nutraceutical or by individual administration to reduce or prevent the disorders involving oxidative stress.

CONCLUSION: *Morus indica* is a non toxic natural source of phytochemicals such as polyphenols, flavonoids, glutathione, anthocyanins, tannins, saponins etc. The plant is well investigated for its antioxidant, anti-microbial and anti-diabetic properties etc. In addition to these, the present work revealed the protective nature of the Morus against the CCl_4 induced toxicity. Hence, Morus with such therapeutic properties can discharge its activity as a nutraceutical in disease conditions associated with liver.

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