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Assessment of hepatoprotective activity of coded plant (222) leaf ethanolic extract against carbon tetrachloride-induced hepatotoxicity in Wistar rats – Part IV

N M Krishnakumar*, P G Latha, S Rajasekharan, S R Suja, Mathew Dan and M Navas
Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI),
Thiruvananthapuram – 695 562, Kerala, India
*krishnakumarnmohandas@gmail.com

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Abstract

The present study was designed to assess the possible hepatoprotective activity of the leaf ethanolic extract of coded plant (Code No. 222**) against carbon tetrachloride (CCl_4)-induced hepatic injury in Wistar albino rats. The animals were divided into different groups and treated with 222 leaf ethanolic extract at different concentrations for five days. Silymarin, the known hepatoprotective standard compound (100 mg/kg) was administered for five days. Hepatotoxicity was induced by the subcutaneous administration of a single dose of CCl_4: Olive oil (2 mL/kg) on days 2 and 3. The administration of CCl_4 resulted in marked increase in serum hepatic enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST) and serum bilirubin levels. CCl_4 intoxication also resulted in a significant ($P \leq 0.05$) increase in malondialdehyde (MDA), which is a common marker of lipid peroxidation. The other biochemical parameters such as cholesterol, triglycerides, creatinine, urea and uric acid levels were also increased significantly ($P \leq 0.05$) compared to normal control group. Changes in serum hepatic enzymes, biochemical parameters and MDA levels induced by CCl_4 were reversed by the leaf ethanolic extract of 222 (125 mg/kg) dose. The standard drug silymarin treated group also reversed CCl_4-induced changes in biomarkers of liver function and MDA levels. Histopathological studies of the liver samples confirmed the hepatoprotective property of the coded drug 222. It was seen that histopathological damage induced by CCl_4 were improved in rat liver, treated with 222 extract. The results of the present study suggested that coded plant (222) leaf ethanolic extract may be used as a hepatoprotective agent against toxic effects caused carbon tetrachloride in the liver.

Keywords: Hepatoprotective, carbon tetrachloride, lipid peroxidation, malondialdehyde

1. Introduction

In continuation of our earlier communication on evaluation of the protective effect of coded plant leaf ethanolic extract (222) against paracetamol-induced hepatotoxicity and oxidative stress in Wistar albino rats-Part III (Krishnakumar *et al*., 2017), in this paper, the hepatoprotective effect of the leaf ethanolic extract of the same coded plant (Code No. 222) has been evaluated against carbon tetrachloride (CCl_4)-induced hepatotoxicity *in vivo* model.

Liver is an important organ that plays a key role in the conjugation and detoxification of many drugs and its function is generally impaired by xenobiotics or infections (Karakus *et al*., 2011). The excessive exposure of xenobiotics leads to

* Corresponding author
** Name of the medicinal plant species will be disclosed only after obtaining the Patent

cirrhosis or malignant lesions in untreated cases and at present, millions of people suffer from hepatic injury induced by alcohol, chemicals and infections. Thus, acute and chronic liver diseases continue to be serious health problems in the world (Cemek *et al*., 2010). The chemicals such as paracetamol, carbon tetrachloride, polycyclic aromatic hydrocarbons and nitrosamines induce significant liver damage (Al-Harbi *et al*., 2014). Carbon tetrachloride (CCl_4) has been one of the most intensively studied hepato-toxicants and provides a relevant model for other halogenated hydrocarbons that are widely used (Clawson, 1989). It is the best-characterized *in vivo* model of xenobiotic induced free radical mediated hepatotoxicity and a single exposure to CCl_4 leads to severe centrizonal necrosis and steatosis in liver (Recknagel *et al*., 1974). The changes associated with CCl_4 – induced liver damage are similar to that of acute viral hepatitis/cirrhosis (Rubinstein, 1962; Mi *et al*., 2000).

There are no specific allopathic medicines used as hepatoprotective agents, although different research studies are going on some drugs. There is a need for the development of an efficient alternative to manage the liver diseases with efficacy and safety. The hepatoprotective agent should have the ability to restore the normal hepatic architecture and preserve the normal physiological functions distorted by the hepatotoxins (Yadav and Dixit, 2003). Herbal drugs are more widely used than allopathic drugs as hepatoprotective agents because they are inexpensive, have better cultural acceptability, better compatibility with the human body and minimal side effects (Soni *et al*., 2014).

The present scientific study was carried out based on traditional knowledge related to a coded medicinal plant (Code No. 222) disclosed by a traditional healer and the claim of the traditional healer was that he was using the particular medicinal plant species for treating diabetes and related complications. According to him, the coded plant also provides protection to the liver and its therapeutic usage was kept as trade secret by the healer. We have reported the hepatoprotective effect of the leaf ethanolic extract of coded plant 222 against paracetamol-induced hepatotoxicity *in vivo* model (Krishnakumar *et al*., 2017). On verification, no pharmacological studies related to the hepatoprotective potential of the coded plant 222 against carbon tetrachloride induced hepatotoxicity had been so far conducted. The present ethnopharmacological study revealed the hepatoprotective potential of the coded plant 222 leaf and planned to evaluate hepatoprotective activity of ethanolic extract on carbon tetrachloride-induced hepatotoxicity in Wistar rats.

2. Materials and Methods

2. 1. Preparation of the coded plant leaf extract

The coded plant 222 leaves were collected from the Traditional Healer at Karunagappally, Kollam District, Kerala. The leaves were washed thoroughly under tap water, shade dried, powdered and 100 g of the leaf powder was extracted with 1000 mL of ethanol overnight, at room temperature with constant stirring (200 rpm). The extract was filtered, concentrated and the solvent evaporated completely on a rotary evaporator at 40°C temperature under reduced pressure, dried in a desiccator and it was reconstituted in 0.5% Tween-80 to required concentrations and used for the experiments.

2. 2. Chemicals and commercial kits

Sodium dodecyl sulphate (SDS) and thiobarbituric acid (TBA) were purchased from Sigma Aldrich, USA. Commercial kits for the estimation of Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Serum alkaline phosphatase (ALP), Serum bilirubin (SB), Glucose, Serum cholesterol, Triglycerides, Total protein, Creatinine, Urea, Uric acid and Albumin were purchased from Coral Clinical system, Goa, India.

2. 3. Animals

Wistar albino rats, males (200–250 g) obtained from the Institute's Animal House were used for the present study. They were housed in poly-acrylic cages with three animals per cage and maintained under standard laboratory conditions (temperature 24-28 ± 1°C, relative humidity 60 ± 5% and 12 h light/dark cycles), fed commercial rat feed (Lipton India Ltd., Mumbai, India) and boiled water *ad libitum*. All experiments involving animals were carried out according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, after getting the approval of the Institute's Animal Ethics Committee (B-form No. B-01/12/2011/EM & EP - 08).

2. 4. Carbon tetrachloride-induced hepatotoxicity study

The hepatoprotective potential of the coded plant leaf ethanolic extract (222) was evaluated on carbon tetrachloride (CCl_4)-induced hepatotoxicity in Wistar rats. Rats were divided into different groups of six animals in each group. Group I, the Normal control group animals were administered (p.o.) a single daily dose of 0.5% Tween-80 (1 mL) on all five days and olive oil (1 mL/kg) (s.c.) on days 2 and 3. Group II, the CCl_4 control group animals were administered with a single daily dose of 0.5% Tween-80 (1 mL), (p.o.) on all 5 days and on the second and third day, they were administered CCl_4: Olive oil (1:1), (s.c.). Group III animals were administered silymarin, the known hepatoprotective compound, at a dose of 100 mg/kg, (p. o.) on all five days and a single dose of CCl_4: Olive oil (2 mL/kg) (s.c.) on days 2 and 3, 30 min after silymarin administration. Other treated groups were administered the extracts at different doses (p.o.) on all five days and a single dose of CCl_4: Olive oil (2 mL/kg) (s.c.) on days 2 and 3, 30 min after the extract administration. On the fifth day, all the animals were sacrificed by carbon dioxide inhalation. Blood samples were collected and serum biochemical parameters were evaluated. Liver tissue samples were collected for the estimation of MDA and histopathological studies (Suja *et al*., 2004).

2. 4. 1. Biochemical Estimation

The collected blood was allowed to coagulate for 1h at room temperature and centrifuged at 1500 rpm for 15 min at 37°C to separate the serum. . The serum was then used for the assay of marker enzymes, namely Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Serum Alkaline phosphatase (ALP) and Serum Bilirubin (SB) according to standard methods. Other serum biochemical parameters such as serum cholesterol, triglyceride, creatinine, urea, uric acid, total protein and albumin levels were estimated and compared to the standard drug, silymarin.

2. 4. 2. Lipid peroxidation study (*in vivo*) in CCl_4-intoxicated rats

Lipid peroxidation was estimated by the method of Ohkawa *et al*. (1979). Liver homogenate was mixed with 100 µL of 8.1% sodium dodecyl sulphate and 600 µL of 20% acetic acid solution, kept for 2 min at room temperature, then 600 µL of 0.8% solution of thiobarbituric acid (TBA) was added, heated at 95°C for 60 min in water bath and cooled with ice cold water at 4°C. Then n-butanol was added, shaken vigorously and centrifuged at 10,000 rpm for 5 min. The absorbance of the organic layer was measured at 532 nm. Lipid peroxidation was expressed as n moles of MDA/g of wet liver tissue.

2. 4. 3. Histopathological studies

Liver specimens obtained from the control and the treated groups of the experiment were trimmed to small pieces and preserved in formalin (10% solution) for 24 h. They were subjected to dehydration with acetone of strength 70, 80 and 100% respectively, each for 1 h. The infiltration and impregnation was done by treatment with paraffin wax, twice each time for 1 h. Specimens were cut into sections of 3-7 µm thickness and stained with haematoxylin and eosin and mounting of the specimens was done using Distrene Phthalate Xylene (DPX). The specimens were observed under high resolution microscope (magnification × 400) with camera and attachment (Carl Zeiss, Germany).

2. 5. Statistical analysis

All the analyses were carried out in triplicate. The statistical significance between the samples were determined by the Analysis of Variance (ANOVA) and the data were recorded as mean ± Standard Deviation (SD). $P \leq 0.05$ was considered to be statistically significant. Significant differences between means were determined by Duncan's Multiple Range tests (Raghava, 1987). The computer software employed for the statistical analysis was IBM SPSS Statistics, version 20 (USA).

3. Results and Discussion

Carbon tetrachloride (CCl_4) induced hepatotoxicity is a well known *in vivo* model for producing chemical hepatic injury and it also causes acute and chronic hepatic damage due to the generation of free radicals (Jain *et al*., 2008). Marked increase in the release of hepatic enzymes into the blood stream is often associated with massive necrosis of the liver caused by CCl_4. CCl_4 is transformed by Cytochrome P-450 (Cyt. P_{450}) system to produce trichloromethyl free radicals. These free radicals may again react with oxygen to form trichloromethyl peroxyl radicals which may attack lipids on the membrane of endoplasmic reticulum to elicit lipid peroxidation, finally resulting in cell necrosis and consequent cell death (Recknagel *et al*., 1989). CCl_4 is believed to produce lipid peroxidation in the form of conjugated dienes, lipid hydro-peroxides, malondialdehyde like substances and other short-chain hydrocarbons which eventually leads to hepatic damage (Amin and Hamza, 2005).

In the present study, CCl_4 caused severe hepatic damage which was demonstrated by the marked increase in hepatic marker enzyme levels, biochemical parameters and histopathological architecture of CCl_4 control group animals. Results showed that the treatment with leaf ethanolic extract of coded plant 222 at 125 mg/kg dose ameliorated CCl_4 –induced hepatotoxicity

and showed normalization of hepatic marker enzymes like ALT and AST, serum bilirubin and other biochemical parameters such as cholesterol, triglycerides, creatinine, urea and uric acid. Hepatic damage causes leakage of the liver enzymes, causing increased levels of these enzymes in the serum and it is the indication of cellular damage and loss of functional integrity of hepatocyte membrane (Cherubini *et al*., 2005). According to Zimmerman *et al*. (1965), CCl_4 induced elevated levels of ALT and AST due to cell membrane and mitochondrial damage of hepatocytes. The significant ($P \leq 0.05$) reduction of hepatic enzyme levels in the ethanolic extract 222 (125 mg/kg) and silymarin (100 mg/kg), treated groups after CCl_4 intoxication is an indication of stabilization of plasma membrane as well as repair of hepatic tissue damage caused by CCl_4 (Table 1). Silymarin is a known hepatoprotective compound isolated from *Silybum marianum* and it is reported to exhibit protective effect on plasma membrane of hepatocytes (Ramellini and Meldolesi, 1976). Serum bilirubin is a dominant marker of hepatic damage, which indicates the secretory mechanism of liver cells (Al-Harbi *et al*., 2014). Serum bilirubin level increased after the administration of CCl_4 compared to the normal control group. The leaf ethanolic extract of coded plant 222 (125 mg/kg) treated group exhibited a significant ($P \leq 0.05$) decrease in serum bilirubin levels compared to standard control silymarin treated group (Table 1), which suggests the possibility of the extract to stabilize the biliary dysfunction in rat liver after the hepatic injury with CCl_4.

The other biochemical parameters such as serum cholesterol, triglycerides, creatinine, urea and uric acid levels were also increased after CCl_4 intoxication. The activity of serum lipid profile like total cholesterol and triglyceride levels was elevated in toxin control indicating deterioration in hepatic function due to damage caused by CCl_4 administration (Essawy *et al*., 2012). The increased serum total cholesterol and triglyceride levels in CCl_4-treated rats may have resulted from the increase in oxidative stress, which enhances deterioration in hepatic function and the accumulation of lipids due to the failure of secretory mechanisms (Oyinloye *et al*., 2017). Treatment with 222 leaf ethanolic extract (125 mg/kg) and silymarin (100 mg/kg) reduced the elevated cholesterol and triglyceride levels significantly ($P \leq 0.05$) compared to silymarin control (Table 2). Administration of CCl_4 significantly ($P \leq 0.05$) increased serum creatinine, urea and uric acid levels compared to normal control. But, the ethanolic extract of coded plant 222 leaves (125 mg/kg) significantly lowered the elevated levels of creatinine, urea and uric acid (Table 3) compared to silymarin control. Total protein and albumin levels were significantly ($P \leq 0.05$) decreased in CCl_4 control compared to normal control. Significant decrease in serum albumin had been associated with active cirrhosis and biliary liver damage (Whicher and Spence, 1987). According to Navarro and Senior (2006), diminution of total protein and albumin induced by CCl_4 is a further indication of liver damage. 222 ethanolic extract treatments increased the levels of serum total protein (Table 2) and albumin (Table 3) towards normal compared to silymarin control. Stimulation of protein synthesis has been advanced as a contributory hepatoprotective mechanism which accelerates the regeneration process and the production of liver cells (Rip *et al*., 1985).

The administration of CCl_4 resulted in a significant ($P \leq 0.05$) increase in hepatic MDA

content compared to normal control. Treatment with ethanolic extract of coded plant 222 leaves exhibited a significant ($P \leq 0.05$) reversal in CCl_4 induced elevation in liver MDA levels compared to silymarin control. The lipid peroxidative degeneration of biomembranes is one of the major causes of hepatotoxicity of CCl_4. The coded drug 222 leaf ethanolic extract at 125 mg/kg exhibited 34.02% of MDA inhibition in lipid peroxidation *in vivo* study (Table 4).

Previous studies reported that one of the main causes of CCl_4-induced hepatic damage is the generation of lipid peroxides by free radical derivatives of CCl_4. Thus, the anti-oxidant effect or the inhibition of the generation of free radicals could be one of the mechanisms in the protection against CCl_4-induced hepato-toxicity (Brent and Rumack, 1993). The increased serum levels of hepatic biomarkers could be due to lipid peroxidation caused by free radical derivatives of CCl_4 leading to the leakage of these enzymes from liver cells (Klaunig and Kamendulis, 2004). CCl_4-administration resulted in a significant ($P \leq 0.05$) increase in liver MDA contents compared to the control group. Treatment with ethanolic extract of coded plant 222 leaves showed a significant ($P \leq 0.05$) reversal in CCl_4-induced increase in liver MDA levels. The reduction in MDA caused by ethanolic extract of coded plant 222 leaves shows the free radical scavenging property of the extract.

In histopathological studies, the liver slices of normal control rats showed normal hepatic architecture and no fatty changes. CCl_4 intoxication resulted in histopathological changes such as centrilobular necrosis, marked fatty changes, infiltration of inflammatory cells like macrophages and hepatocyte ballooning. The coded drug 222 leaf ethanoilc extract (125 mg/kg) produced a marked degree of protection against CCl_4 induced alterations and the liver sections were almost comparable to that of silymarin control (Fig. 1). The results of histopathological studies further supported the findings of the estimation of serum biochemical parameters and hepatic biomarker enzyme levels of coded drug 222 leaf ethanoilc extract (125 mg/kg) treated group.

Table 1: The effect of the coded plant 222 leaf ethanolic extract on rat serum parameters after carbon tetrachloride intoxication

Groups	ALT (IU/L)	AST (IU/L)	ALP (KA units/100 mL)	Bilirubin (mg/dL)
Normal control	167.90 ± 1.10	65.18 ± 6.50	102.53 ± 1.05	0.19 ± 0.07
CCl_4 control	411.85 ± 2.33	372.77 ± 3.52	166.52 ± 2.61	2.59 ± 0.03
222 leaf ethanolic extract (50 mg/kg)	289.05 ± 0.49	158.89 ± 3.83	177.17 ± 0.89	0.22 ± 0.02**
222 leaf ethanolic extract (125 mg/kg)	253.70 ± 1.88	111.16 ± 4.77	147.34 ± 0.89	0.13 ± 0.01**
222 leaf ethanolic extract (250 mg/kg)	327.65 ± 0.34	141.31 ± 0.20	123.78 ± 0.01	0.27 ± 0.06
Silymarin (100 mg/kg)	213.47 ± 2.41	94.28 ± 4.13	118.42 ± 0.89	0.22 ± 0.02**

Values are the mean ± SD, n=6 in each group, Analysis of Variance (ANOVA) followed by Duncan's multiple range test. **Significance $P \leq 0.05$, compared to CCl_4 control.

Table 2: The effect of the coded plant 222 leaf ethanolic extract on rat serum parameters after carbon tetrachloride intoxication

Groups	Glucose (mg/dL)	Cholesterol (mg/dL)	Triglycerides (mg/dL)	Total protein (mg/dL)
Normal control	47.39 ± 0.89	41.67 ± 0.89	14.82 ± 4.67	65.60 ± 0.27
CCl_4 control	129.69 ± 0.89	105.50 ± 0.89	80.34 ± 1.87	40.05 ± 0.09
222 leaf ethanolic extract (50 mg/kg)	96.23 ± 0.89	95.50 ± 0.89	53.85 ± 4.68	54.86 ± 0.01
222 leaf ethanolic extract (125 mg/kg)	60.35 ± 0.89	64.67 ± 0.89**	39.89 ± 5.37	58.78 ± 0.63**
222 leaf ethanolic extract (250 mg/kg)	101.04 ± 0.89	87.00 ± 0.89	61.54 ± 0.01	60.97 ± 0.01**
Silymarin (100 mg/kg)	65.20 ± 0.89	62.33 ± 0.89**	30.77 ± 1.53	65.44 ± 0.19**

Values are the mean ± SD, n=6 in each group, Analysis of Variance (ANOVA) followed by Duncan's multiple range test. **Significance $P \leq 0.05$, compared to CCl_4 control.

Table 3: The effect of the coded plant 222 leaf ethanolic extract on rat serum parameters after carbon tetrachloride intoxication

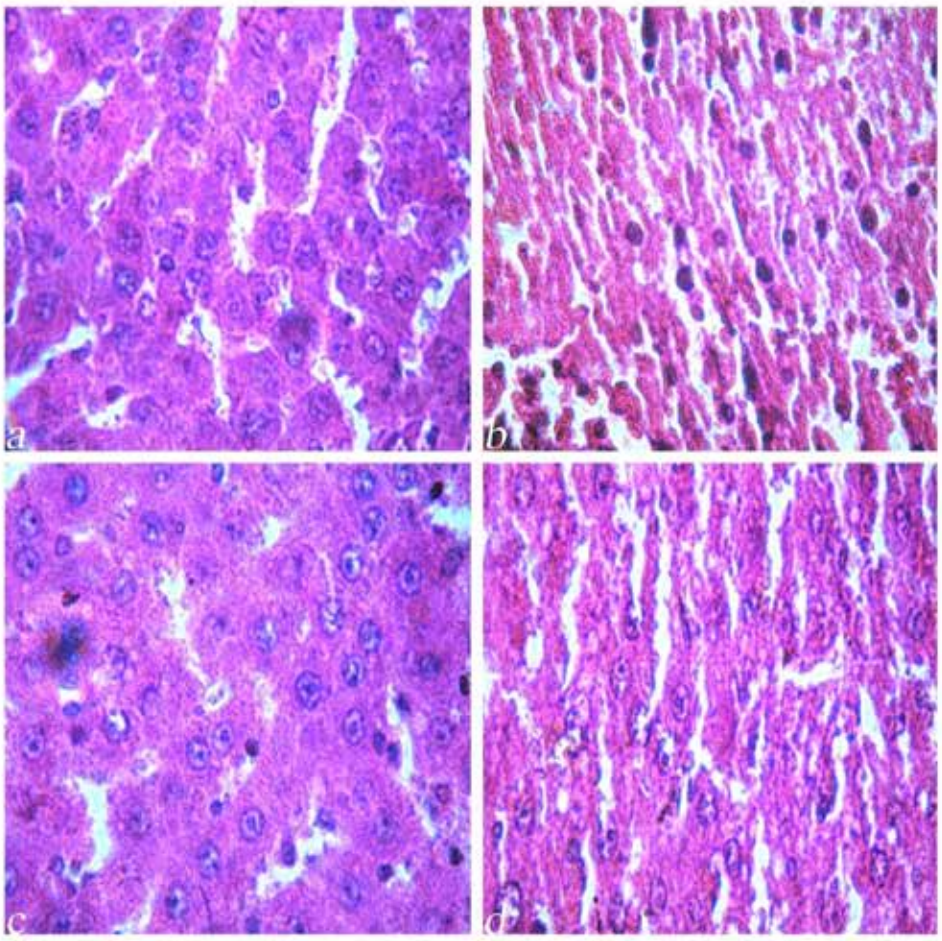
Groups	Creatinine (mg/dL)	Urea (mg/dL)	Uric acid (mg/dL)	Albumin (g/dL)
Normal control	0.63 ± 0.14	19.48 ± 1.27	2.62 ± 0.02	11.28 ± 0.18
CCl_4 control	2.85 ± 0.03	23.50 ± 0.73	5.76 ± 0.03	4.26 ± 0.01
222 leaf ethanolic extract (50 mg/kg)	0.85 ± 0.05	19.47 ± 0.05**	2.63 ± 0.01**	8.47 ± 0.13
222 leaf ethanolic extract (125 mg/kg)	0.63 ± 0.14**	19.84 ± 1.61**	2.81 ± 0.09**	9.33 ± 1.05**
222 leaf ethanolic extract (250 mg/kg)	0.70 ± 0.09**	20.04 ± 3.89	3.06 ± 0.01	10.70 ± 0.01
Silymarin (100 mg/kg)	0.60 ± 0.09**	19.30 ± 2.24**	2.70 ± 0.45**	10.24 ± 0.52**

Values are the mean ± SD, n=6 in each group, Analysis of Variance (ANOVA) followed by Duncan's multiple range test. **Significance $P \leq 0.05$, compared to CCl_4 control.

Table 4: The effect of the coded plant 222 leaf ethanolic extract on lipid peroxidation after carbon tetrachloride intoxication *in vivo*

Groups	MDA (n mol/g wet liver)	MDA inhibition (%)
Normal control	3.71 ± 0.01	----------
CCl_4 control	13.69 ± 0.03	----------
222 leaf ethanolic extract (50 mg/kg)	11.29 ± 0.04	17.53 %
222 leaf ethanolic extract (125 mg/kg)	9.03 ± 0.11**	34.02 %
222 leaf ethanolic extract (250 mg/kg)	10.02 ± 0.01	26.30 %
Silymarin (100 mg/kg)	7.62 ± 0.12**	44.33 %

Values are the mean ± SD, n=6 in each group, Analysis of Variance (ANOVA) followed by Duncan's multiple range test. **Significance $P \leq 0.05$, compared to CCl_4 control.



Effect of coded drug 222 ethanolic extract on CCl_4 –induced liver damage in Wistar rats. a) Normal Control liver with well defined nuclei and cytoplasm (x 400). b) CCl_4 -treated liver showing fatty changes, gross necrosis, broad infiltration of lymphocytes and Kupffer cells (x 400). c) Liver treated with 222 ethanolic extract (125 mg/kg) prior to CCl_4 administration showing lesser necrosis compared to Toxin control group (x 400). d) Liver pretreated with Silymarin prior to CCl_4 administration, showing normalcy of hepatic cells with mild fatty changes and necrosis (x 400).

4. Conclusion

In conclusion, the coded plant 222 leaf ethanolic extract exhibited hepatoprotective activity by significantly reducing the elevated serum enzymes levels and biochemical parameters in carbon tetrachloride (CCl_4)-induced hepatotoxicity. The extract also showed protection against CCl_4-induced lipid peroxidation by significantly reducing the formation of malondialdehyde (MDA) or by scavenging the free radicals by antioxidant activity. The histopathological studies along with hepatic enzyme levels, lipid peroxidation *in vivo* and biochemical evaluation suggests the protective effect of coded plant 222 leaf ethanolic extract against carbon tetrachlorde induced hepatotoxicity.

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