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Research Article

Protective Potential of *Canthium dicoccum* Methanolic Extract Against Hepatic Injury in Rats

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ABSTRACT

The methanolic extract of leaves of *C.dicoccum* was evaluated for its hepatoprotective activity against paracetamol induced hepatotoxicity since it is reported to cause oxidative stress in the animal thereby altering the enzymatic levels. Fresh leaves were collected, shade dried and extract was prepared by cold maceration followed by drying in a rota-vapour using methanol to obtain MECD as a sticky semi-solid mass. Thirty rats were taken and divided into 5 equal groups where, Group I, II, and III served as Normal Control, Negative control (PCM 3g/kg), and Positive control (Silymarin 200mg/kg) respectively; whereas, Group IV and V served as test groups where the rats were pre-treated orally with MECD 200mg/kg and 400mg/kg respectively for six days before administering PCM. On the 8th day all groups except Group I was administered with PCM (3g/kg). 48 hours post PCM induction, the animals were anesthetized, blood samples were obtained via retro-orbital sinus plexus and then the rats were sacrificed. The serum was assessed for the evaluation parameters like AST, ALT, ALP, and bilirubin levels. Apart from these; SOD, CAT, and MDA levels were also evaluated and it was concluded that treatment with MECD restored the levels to normal thereby exhibiting hepatoprotective activity. Moreover, histopathological evaluation was carried out to assess the liver for inflammation, infiltration, or necrosis where the MECD treated rats showed promising results.

Key words *Canthium dicoccum*, Hepatoprotective activity, Antioxidant enzymes, Liver markers, Paracetamol.

INTRODUCTION

Human existence on this planet has been become possible just due to the important functions carried out through the plant kingdom. Nature all the time keeps as golden standard to maintain the unexceptional symbiosis phenomenon[1]. Medicinal herbal plants are present even before humans kept their possibility of an appearance on the earth. The medicinal practice of traditional utilization of herbal medications is profound in each part of the globe. The most possible regions are traditions of Indian Chinese and European regions. The procedure for the progress of herbal medications for global needs has to vary from that of synthetic medicines[2].

Hepatic disorders possess higher consequences, following the critical relying of other organs on the liver metabolic functions. Injury of the liver and its consequences seem to follow specific patterns. In few cases, the diseased process is basically to the liver. Liver damage is always linked with necrosis of cells, rise in lipid peroxidation of tissue, and decrease in the GSH levels of tissues[3]. Along this levels of various biochemical markers in serum like AST, ALT ALP, triglycerides, cholesterol, bilirubin, are elevated.[4] Also a marked oxidative stress is involved thereby altering the antioxidant markers like SOD, CAT GPX-Px due to Lipid peroxidation[5].

Paracetamol is one among the several drugs reported to be associated with liver injury[6]. It is known as one of the commonly used analgesic and antipyretic and available without a prescription. Paracetamol induced hepatic injury is reported as one of the most common cause of acute liver failure. An enormous research to understand the mechanism of paracetamol lead to the conclusion that oxidative stress in involved in the various paracetamol associated toxicity induced liver damage[7]. Paracetamol is known to get metabolized by cytochrome P450 enzymes into reactive metabolite known as N-acetyl-p-benzoquinoneimine (NAPQI)[8] known to be a extremely reactive compound which depletes cellular GSH, and damage to the bio membranes causing oxidative stress and leakage of enzymes such as ALT, AST, ALP, and molecules such as bilirubin into the blood stream[9]. Cytochrome P 450 mediated metabolism of paracetamol is also assumed to generate ROS that contribute to the liver damage thereby altering the liver marker enzymes SOD, CAT and GSH-Px levels and non enzymatic compounds such as tocopherol, ascorbate and GSH[10].

C. dicoccum was selected to study its effective potential in treating hepatic injury since the genus has reported evidences of having phytoconstituents; however, the same species and its beneficial activities are not yet reported. The leaves of *C. dicoccum* were reported to have few pharmacological activities and attempts were made to study the hepatoprotective activity against the paracetamol-induced hepatotoxicity. Since methanol has the ability to extract polar, non polar and intermediately polar bioactive compounds it was chosen for the study.

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MATERIALS AND METHODS

Chemicals and Reagents

All the chemicals involved in the study were of analytical grade and obtained from UV Scientifics Pvt. Ltd, and the diagnostic kits involved for estimations wherever involved were obtained from Sigma Aldrich.

Experimental animals

Adult Swiss Albino rats were collected from Sainath Agencies, Hyderabad. The rats were categorized into five groups randomly consisting of six rats each for every group. Every rat that weighed in between 150-200 grams was selected (6 rats per each cage) and were left to acclimatize to the conditions of animal room for period of seven days. They were managed in prescribed laboratory conditions of temperature 22±2°C, humidity, cycles of 12 hours light and dark and fed with standard pellet diet (Hindustan lever Ltd., Bangalore) and sufficient tap water.

Collection of plant material

The leaves of the plant *C.dicoccum* utilized for the current investigation was collected from Sri Venkateshwara University, Tirupati, India. Dr.Madhava Chetty, Department of Botany, authenticated the plant and a specimen of voucher of the plant was conserved at the institute herbarium library.

Preparation of Methanolic Extract

Leaves of fresh plants were collected, to remove adhered dirt; leaves were washed and cleaned with distilled water, blotted and dried by shade instead of sunlight. The shade-dried leaves were powdered using a commercial mixer. This powder was further sieved to get fine powder and utilized for solvent extraction. Around 100 gms of the powdered plant leaves were kept for maceration using 1500 ml solvent of methanol. This particular cycle was repeated for a period of five days with occasional stirring. On the fifth day, the supernatant was filtered by using a filter paper, collected and concentrated with a rotary evaporator at 64°C. The extracts were then placed in desiccators to get rid of remaining moisture, if any present, and lastly preserved in air-tight containers at 4°C in the refrigerator before commencing for further use.

Determination of total flavonoid Content

About 200 mgs of *C.dicoccum* extract was added with 1ml of 0.5% (w/v) hexamethylene-tetramine, 20ml acetone and 2ml of 25% HCl, hydrolyzed by refluxing the extract within 30 minutes. The mixture was filtered and liquid portion collected. The residue was refluxed with 20ml acetone for 30 minutes, filtered and collected. Acetone was added to make the volume up to 100ml. About 20ml of this filtrate was transferred to a separating funnel and added with 20ml of water, and extracted thrice using 15ml ethyl acetate and collected. For reference solution quercetin was taken and solution prepared using 10ml of stock solution with glacial acetic acid to reach the volume to 25ml[11]. The sample solution was prepared by taking 10mL of stock solution, added with 1ml of $AlCl_3$ and glacial acetic acid solution to reach the volume to 25ml[12]. Quercetin and sample measurements were done 30 minutes after the addition of $AlCl_3$ using a spectrophotometer at α=425 nm. Total flavonoid content was expressed as mgs quercetin equivalent (QE) per 100 gms dried sample.

Determination of total phenolic content

The total phenolic content was determined by reported standard procedures with slight modification. The extract of *C.dicoccum* of 100 mg was diluted with 10mm distilled water to 10mg/ml. About 1mL of diluted *C.dicoccum* extract was diluted into 1mg/ml. 0.2mL of extract was added to 15.8 ml of distilled water and 1 ml of Folin-Ciocalteu reagent was added and vortexed, incubated for 10 minutes and added with 3 ml of 10% Na_2CO_3 solution was settled for 2 hours at room temperature. The UV-Vis absorbance was measured at α=765 nm.

Determination of antioxidant activity with DPPH

The assay is considered the best method to evaluate the antioxidant activity of any test compound. The assay is based on the principle where the sample must have the ability to reduce DPPH (1,1-diphenyl-2-picryhydrazine) into a stable free radical. Plant extracts of six different concentrations viz. 5, 10, 20, 40, 80, 160µg/ml in ethanol were prepared for the study. 0.1 mM DPPH ethanolic solution was prepared and added to 3 ml of sample solutions of different concentrations. These were shaken vigorously and allowed to react at room temperature for 30 minutes and absorbance was measured at 517nm using a UV-Visible Spectrophotometer. Ascorbic acid was used as standard compound for reference and its serial dilutions were prepared in the same manner.

$$\%\ \text{DPPH Radical Inhibition} = \frac{[\text{Abs}_{\text{Control}} - \text{Abs}_{\text{sample}}]}{\text{Abs}_{\text{control}}} * 100$$

Qualitative phytochemical screening[13]

The phytochemical tests of the herbal extract were performed according to the standard procedures to detect various phytoconstituents like Flavonoids, phenolic compounds, terpenoids alkaloids, carbohydrates, saponins, etc present in them.

Acute toxicity studies

Acute toxicity studies were carried out based on guidelines provided by OECD-423 category IV substance also referred as acute toxic class method[14]. Albino rats (n=3) of both sex chose by random technique of sampling was utilized in this study. For four hrs, animals were kept under fasting condition by providing free access to only distilled water. The methanolic extract of leaves of *C. dicoccum* orally with maximum dose of 2000 mg/kg body weight was administered. Mortality cases were observed for following three days after administration of extract. If any mortality was seen in 2/3 or 3/3 of animals, the conclusion, administered dose was a toxic dose. However, in case the mortality was seen only one rat out of 3 animals then the same procedure was repeated for confirming the toxic effect. If no mortality was seen, the same cycle was then repeated with higher dose[15] (Organization for economic Co-operation and development, 2001).

Experimental design[16]

Thirty (30) healthy male albino rats (Wistar strain) were randomly divided into five groups of 6 rats in each group as per the Table 1.

Biological estimations

The animals were anesthetized by utilizing anesthetic ether, blood collected through puncture of retro orbital and biochemical parameters such as ALT, AST, ALP, direct bilirubin, and total bilirubin

Table 1: Experimental design on hepatoprotective activity of MECD against PCM induced rats.

Groups	Type of Treatment	Pre treatment[a]	Dose (mg/kg)	Hepatotoxic Inducer
I	Normal Control	10% DMSO	-	10% DMSO
II	DMSO + PCM	10% DMSO	-	3 gms/kg PCM
III	Silymarin	Silymarin	200mg/kg	3 gms/kg PCM
IV	MECD + PCM	MECD	200mg/kg	3 gms/kg PCM
V	MECD + PCM		400mg/kg	3 gms/kg PCM

were estimated[16]. The animals scarification was carried out through overdosing of ether and dissected.

Histopathology studies

Hematoxylin and Eosin (H&E) staining is the most common staining technique in histopathology. This uses a combination of two dyes, Hematoxylin and Eosin used for demonstration of the nucleus and cytoplasmic inclusions in clinical specimens. Livers from each animal were isolated, rinsed by using ice-cold saline, the wet liver volume was weighed and measured. A piece of liver tissue was separated and stored in a 10% formalin solution for histopathological investigations.

RESULTS

Determination of total flavonoids and total phenolics and antioxidant power of *C. dicoccum* extract

The quantitative results of total flavonoids, and total phenolics, of *C.dicoccum* extract, were 13.5 and 26.27%, respectively which is mentioned in Table 2. The IC_{50} value corresponding to the antioxidant power was found to be 41.23 µg/ml which was weaker than that of the standard ascorbic acid (27.06 µg/ml) taken as standard.

Phytochemical screening results

MECD was subjected to preliminary photochemical screening and it was found to have various phytochemicals which are mentioned in the Table 3. The strong availability of needed phytochemical groups in methanolic extracts were observed. Thus, for further investigations MECD was chosen to evaluate the hepatoprotective activity of the same.

Acute Toxicity studies

In the current investigation, the methanolic extract of *C. dicoccum* was evaluated for its toxicity. For the determination of LD_{50}, methanolic extract was given up to dose 2000 mg/kg body weight and extract did not cause any type of mortality, hence 1/5th (400mg), 1/10th (200mg) of the maximum dose given were chosen for the current investigation.

Effect of MECD pre treatment on PCM intoxicated rat body weight (BW), rat liver weight (LW) and their ratio

Administration of paracetamol in rats leads to liver enlargement which was proved via a rise in the weight and volume of rat body weight and also wet liver weight. The groups were administered by Silymarin and MECD exhibited marked restoration of rat body weight and wet liver weight near to normal. The MECD at 200mg/kg b.w. and 400mg/kg b.w. exhibited a decrease of wet body weight and liver weight markedly at $p<0.05$. The results are shown Table 4 and respective graphs in Figure 1 and Figure 2.

Effect of MECD pre treatment on serum biochemical parameters biochemical parameters (ALT, AST and ALP) in PCM intoxicated rats

Treatment with Paracetamol progressed into a marked hepatic injury of the liver showed increased levels of serum of hepatospecific enzymes such as ALT, AST and ALP as compared to the normal control group. Prior administration of Silymarin and methanolic extract had exhibited the best protection on Paracetamol-induced toxicity to hepatic cells. The test showed a marked decrease in increased serum levels of the enzyme with extract administered animals as compared to animals of toxic control which is shown in Figure 3.

Table 2: Phytochemical substituent value of *C. dicoccum* extract.

Constituents	IC_{50} (ppm)	Percentage (w/w)
Total flavonoids		13.5%
Total phenolics		26.27%
Antioxidant power	41.23	

Table 3: Results of qualitative phytochemical tests.

S.No.	Test	Methanolic Extract
1	**Carbohydrates**	
	Mohlish's test	+
	Fehling's test	+
	Barfoed's test	+
2	**Starch**	-
3	**Amino Acids and Proteins**	
	Million's test	+
	Ninhydrin test	+
	Biuret test	+
4	**Phenolics and tannins**	
	Ferric chloride test	+
	Gelatin test	+
	Lead acetate test	+
5	**Phytosterols**	
	Salkowski test	-
	Libermann – Burchards test	
6	**Fixed oils and fats**	
	Spot test	+
	Saponification	-
7	**Alkaloids**	
	Mayer's test	+
	Dragendroff's test	+
	Wagner's test	+
	Hager's test	-
8	**Glycosides**	
	Borntrager's test	+
	Legals test	+
9	**Flavonoids**	
	Ferric chloride test	+
	Shinoda test	+
	Fluorescence test	+
	Reaction with alkali and acid	+
	Zinc, HCl reduction test	+
	Lead acetate solution	+
10	**Saponins**	
	Foam test	+
	Hemolysis test	+

Table 4: Results of MECD on Liver and Body weights.

Group	Treatment	Dose	Rat Body weight (gms)	Wet Liver weight (gms)	LW/BW (%)
I	Normal Control	-	158 ± 3.26	2.43 ± 0.53	1.54 ± 0.1
II	DMSO + PCM	-	162 ± 4.23	4.24 ± 0.09	2.61 ± 0.3a
III	Silymarin	200mg/kg	145 ± 4.89	2.49 ± 0.11*	1.71 ± 0.2b
IV	MECD + PCM	200mg/kg	156 ± 3.67	2.68 ± 0.12*	1.72 ± 0.4b
V	MECD + PCM	400mg/kg	164 ± 3.89	2.36 ± 0.27*	1.43 ± 0.2b

Values are expressed as means ± S.E.M. of six replicates.
PCM Dose was 3g/kg b.w.
[a] Significant difference as compared to normal control, $p<0.05$
[b] Significant difference as compared to negative control, $p<0.05$

Effect of MECD pre treatment on direct bilirubin and total bilirubin in PCM intoxicated rats

An increase in levels of total and direct bilirubin after treatment of paracetamol exhibited its hepatotoxicity. Prior administration with Silymarin, methanolic extract markedly decreased levels of direct and total bilirubin as compared to the group of toxic control indicating the

Table 5: Histopathological scoring of the liver sections of PCM intoxicated rats with or without MECD pre treatment.

Treatment	Dose (mg/kg)	Steatosis	Haemorrhage	Necrosis	Inflammation
Normal Control	-	-	-	-	-
DMSO + PCM	-	+	+	+++	++
Silymarin	200mg/kg	-	-	++	-
MECD + PCM	200mg/kg	+	-	+	+
MECD + PCM	400mg/kg	+	-	-	-

(+ - Indicates Presence, - Indicates Absence)

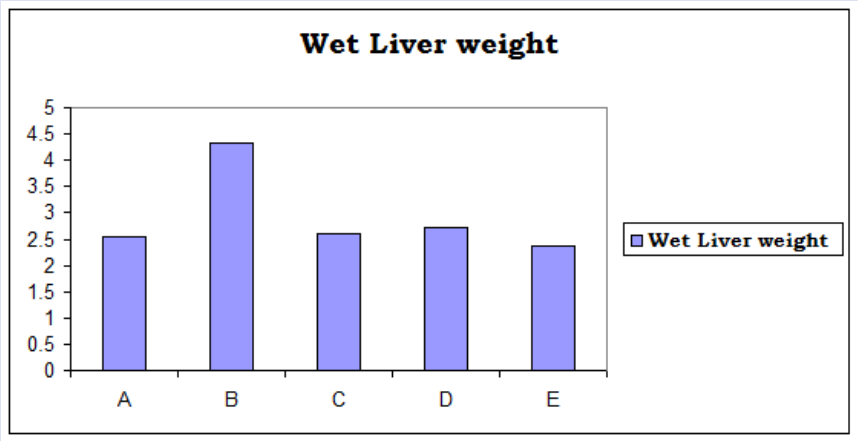

Figure 1: Effect of MECD on rat body weights in paracetamol induced hepatotoxic rats.

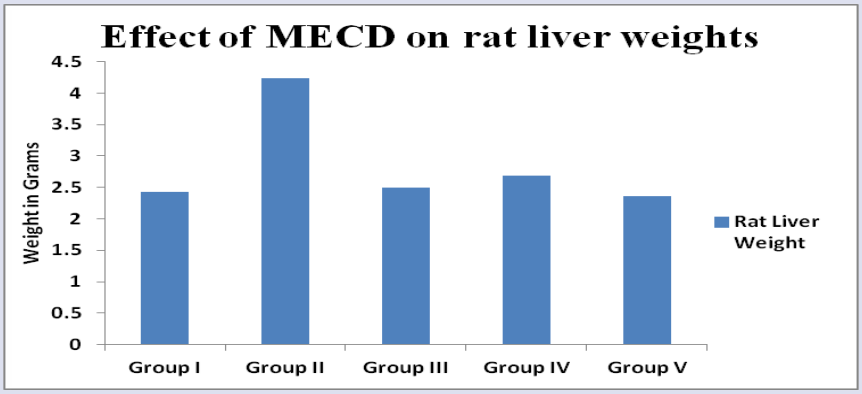

Figure 2: Effect of MECD on wet liver weights in Paracetamol-induced hepatotoxic rats.

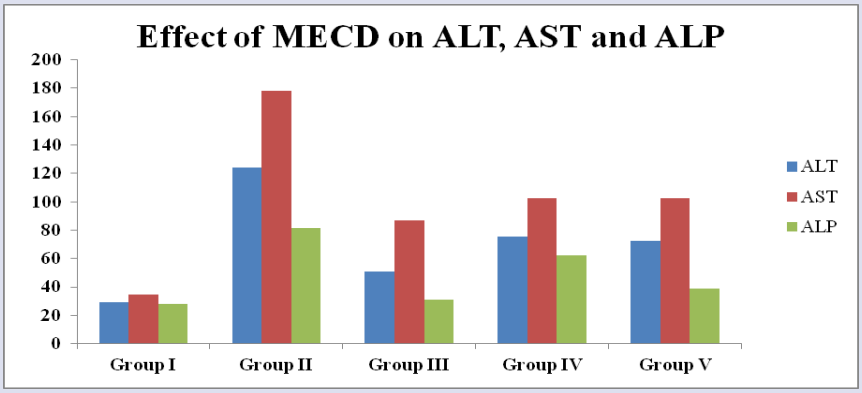

Figure 3: Effect of MECD on ALT, AST and ALP levels in paracetamol induced hepatotoxic rats.

hepatoprotective effect of methanolic extract of *C. dicoccum* it may be observed in Figure 4.

Effects of MECD pre-treatment on the activities of endogenous antioxidant enzymes defence (i.e., CAT and SOD) and the level of end product of lipid peroxidation (i.e., MDA)

Effect of MECD on the activities of CAT and SOD is shown in . In the PCM induced hepatotoxic rats (Group II) a significant ($p<0.05$) decrease is observed in liver CAT and SOD activities in comparison to the normal control group. However, pre treatment with Silmarin or MECD at doses of 200mg/kg and 400 mg/kg, it was found to reverse the toxic effect of PCM by causing a significant ($p<0.05$) increase in the SOD and CAT levels as compared to the PCM intoxicated groups (Group II) indicating that MECD has triggers the activation of endogenous antioxidant enzymes thereby proving its hepatoprotective potential. The table also shows the effect of pre treatment with MECD on MDA, the end product of lipid peroxidation. Silymarin and MECD at 200 and 400mg/kg doses reduces the toxic effect of PCM by reversing and restoring the levels of MDA towards its normal value when compared to PCM intoxicated groups, (Group II) indicating the MECD hepatoprotective activity partly via the attenuation of Lipid peroxidation.

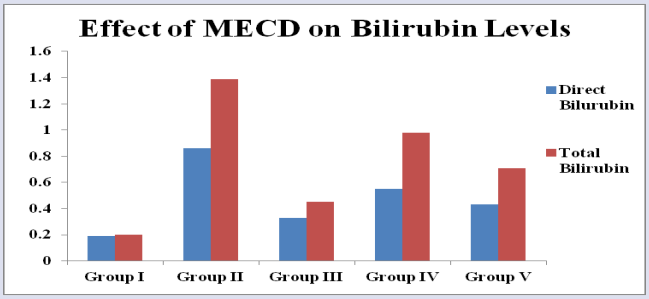

Figure 4: Effect of MECD on Direct Bilirubin & Total bilirubin levels in Paracetamol induced hepatotoxic rats.

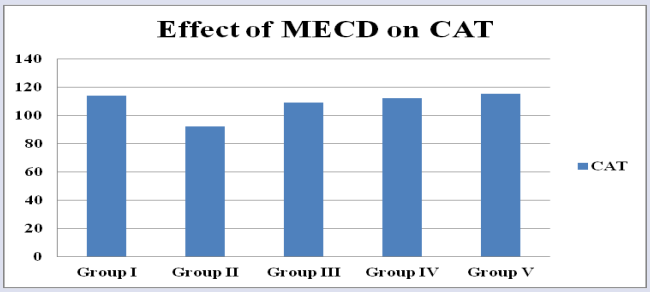

Figure 5: Effect of MECD on CAT in paracetamol induced hepatotoxic rats.

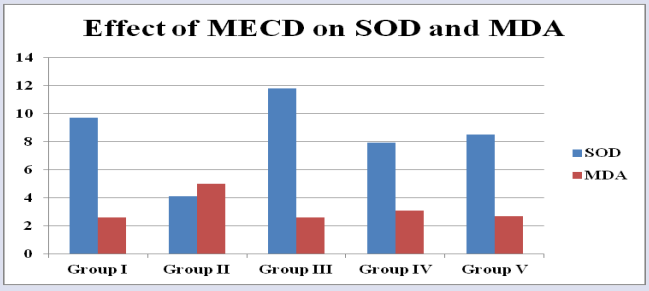

Figure 6: Effect of MECD on SOD and MDA in Paracetamol induced hepatotoxic rats.

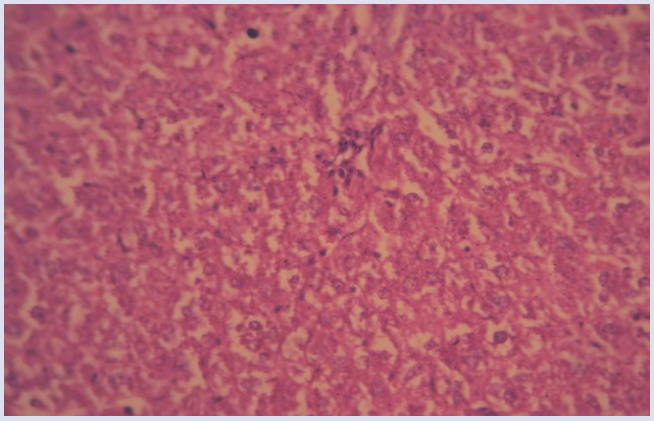

Figure 7: Group I (Control 10% DMSO).

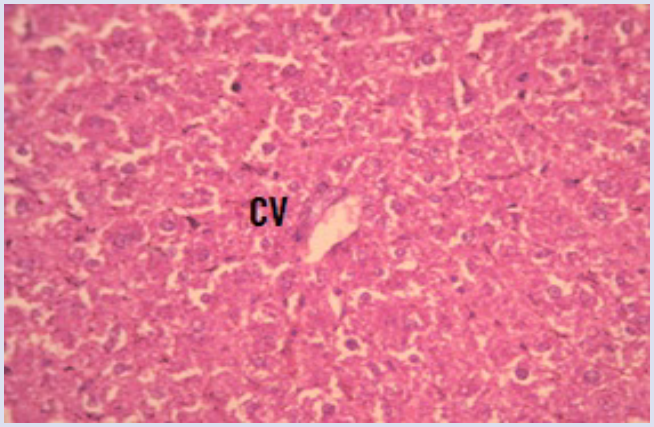

Figure 8: Group III Standard (Silymarin 200 mg/kg).

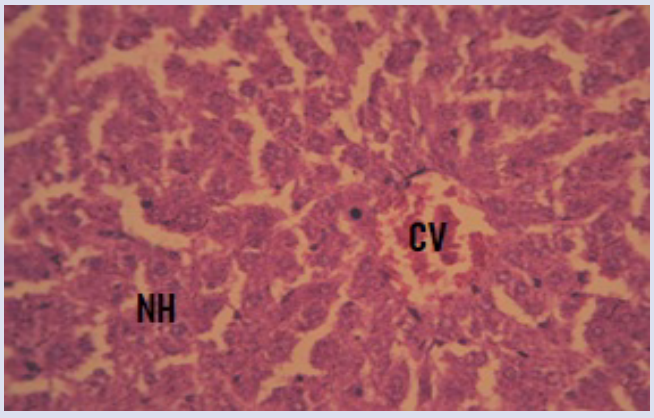

Figure 9: Group IV (MECD 200mg/kg).

DISCUSSION

The liver involved in different metabolic functions may by virtue of the availability of numerous enzymes and hence expose self different toxicants also, chemicals and medications which might damage it[17]. In our hepatoprotective investigation, paracetamol was utilized as hepatotoxicants to cause injury of the liver, because it is applied by human beings for either non-medical or medical needs.

Liver injury due to Paracetamol in rats was first documented in 2006 and has been broadly and successfully utilized by various investigators[18]. In the instance of liver toxicity, the weight of Wet liver and volumes of Wet liver are raised. In this instance, water is accumulated in the

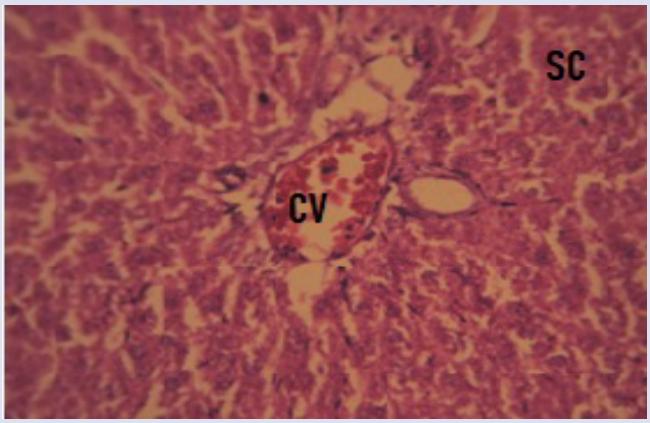

Figure 10: Group V (MECD 400mg/kg).

cytoplasm[19] of hepatocytes resulting in liver cells enlargement, which leads to raised total liver volume and mass[20]. It is demonstrated that the mass and volume of the liver are vital parameters in ascertaining the hepatoprotective activity of the medications, hence in this investigation therapy with methanolic extract of the leaves of *C. dicoccum* markedly decreased the weight of wet liver and volumes of wet liver of animals and thus it exhibits markedly significant ($p<0.05$) hepatoprotective effect.

Hepatotoxin gets transformed into radicals in hepatic cells through enzymes action & these attacks the fatty acids of unsaturated membranes in the availability of oxygen to produce peroxides of lipid consequently[21]. The functional unity of mitochondria of hepatic cells is changed, progress to damage of liver. Damage of hepatic cells cellular enzymes like ALT, AST, and ALP exist in the cells of liver release into the serum, leading in raised levels of ALT, AST, and ALP after treatment with seven days markedly[22]. ALT Serum levels may rise because of injury of the tissues causing hepatic necrosis acutely, like acute cholestasis and viral hepatitis[23]. Paracetamol-induced damage of the liver and alcoholic cirrhosis may link with mild to the moderate rise of transaminases too.

In the present investigation administration with MECD in rats markedly ($p<0.05$ in 200mg/kg b.w. and $p<0.01$ in 400 mg/kg b.w.), reduced the serum levels of ALT, an indication of hepatoprotective effect. AST is a mitochondrial enzyme secreted from the liver, heart, kidney, and skeletal muscle[24]. Liver toxicity raised the levels of AST in serum because of the injury to the tissues causing acute necrosis, like chronic acute cholestasis & viral hepatitis[20]. Alcoholic liver injury and cirrhosis can also link with mild to moderate rise of transaminase[25]. In the present investigation administration of animals with MECD markedly ($p<0.05$) reduced the AST levels in serum which is an indication of hepatoprotective effect.

In an instance of liver toxicity, levels of alkaline phosphatase are very high might be because of defective hepatic excretion or through the raised release of ALP by hepatic duct or parenchymal cells[26]. In the present investigation administration of animals with MECD markedly ($p<0.05$) reduced the ALP levels in serum which is indicative of hepatoprotective effect. In the condition of liver toxicity, levels of bilirubin are increased. Hyperbilirubinemia may outcome of altered hepatic uptake of unconjugated bilirubin. An instance may happen in the injury of generalized liver cells, few medications (like probenecid and rifampin) affect the bilirubin net uptake through the cells of the liver and might cause a mild unconjugated hyperbilirubinemia. Bilirubin level increases in the disorder of hepatocytes, blockage to biliary release into the duodenum, haemolysis, and alteration of hepatic uptake and bilirubin pigment conjugation like in Gilbert's disease[27]. In the present investigation administration of animals with MECD markedly ($p<0.05$) reduces the levels of bilirubin (total and direct) in serum which is indicative of hepatoprotective effect.

CONCLUSION

It was concluded from our study that *C.dicoccum* exhibited a significant protective effect on paracetamol induced hepatotoxicity. MECD exerted this protection by ameliorating the lipid peroxidation due to its scavenging activity of free radicals thereby improving the antioxidant defence system.

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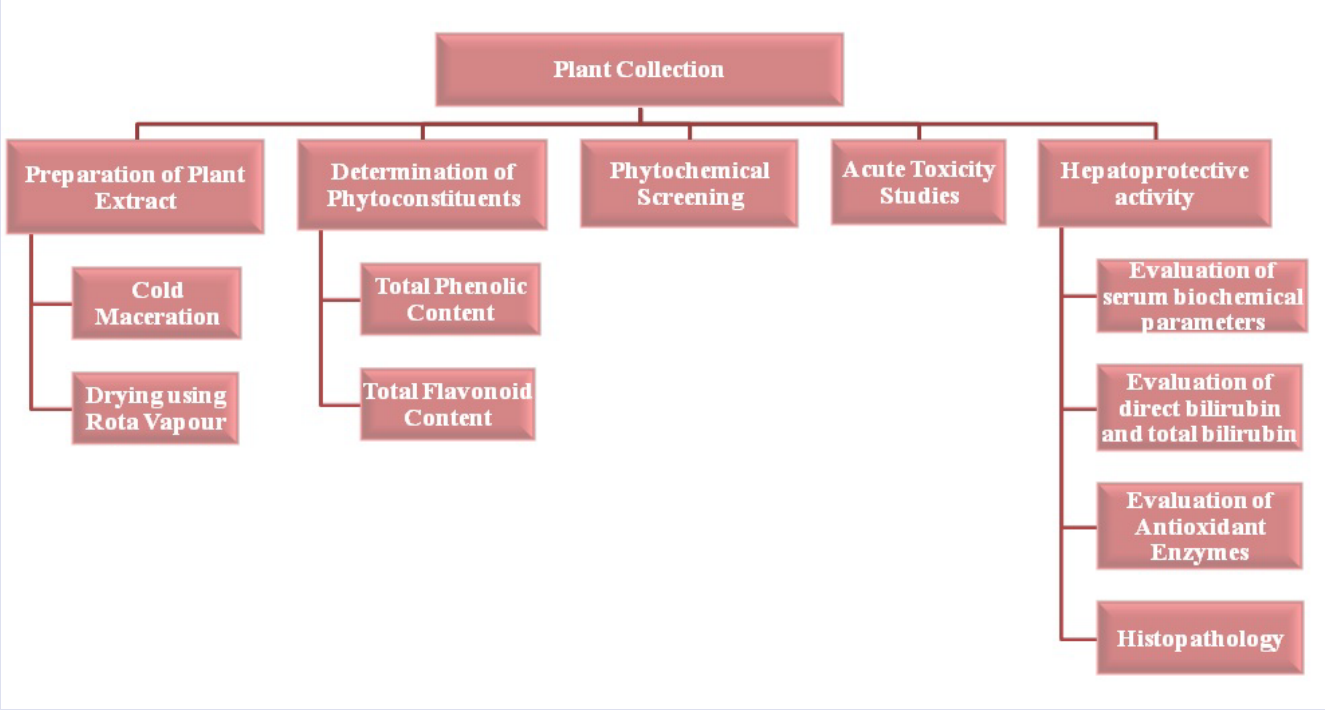
GRAPHICAL ABSTRACT



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