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Hepatoprotective and Nephroprotective Activity of *Cardiospermum halicacabum* Linn. against Paracetamol Induced Toxicity in Wistar Rats

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Abstract: ***Introduction: Many indigenous plants claim to have marked hepatoprotective and nephroprotective activity, the plant under study which is rich in polyphenols and flavonoids is still unexplored and being investigated for its hepatoprotective and nephroprotective properties. Objectives: To investigate the hepatoprotective and nephroprotective activity of* Cardiospermum *halicacabum Linn. (CH) against Paracetamol (PCM) induced toxicity in Wistar rats. Materials and Methods: Normal group received only saline, and the control group received paracetamol at a dose of 750 mg/kg p.o. for 7 days, except for the normal group all the other groups received the same above said dose of PCM, the standard group received silymarin and drug-treated groups received three doses; 200 mg/kg, 400 mg/kg and 800 mg/kg of petroleum ether, chloroform, and ethanol extract for 14 days. Normal group, standard group and all the drug-treated groups are compared with negative control and analyzed by one-way ANOVA followed by Dunnet's multiple comparison tests. The study assesses liver function by estimating the following biochemical parameters ALT, AST, ALP, total protein, and total bilirubin and kidney function by serum creatinine and blood urea nitrogen. Liver and kidney tissue homogenates are used to determine endogenous antioxidants such as GSH, SOD, and catalase and the extent of lipid peroxidation by measuring MDA levels. Results: It was observed that medium and high dose of ethanol extract, chloroform extract, and high dose of petroleum extracts showed hepatoprotective and nephroprotective effects by restoring biochemical parameters and increasing antioxidant levels which is statistically significant. It is quite relevant to correlate the presence of a high concentration of flavonoids & phenolic compounds to its antioxidant property. Conclusion: Among all the extracts the hepatoprotective and nephroprotective activity of ethanol was found to be more pronounced and promising. Further investigation is required to know which flavonoids and phenolic compounds in the ethanol and chloroform extract of* Cardiospermum halicacabum *are responsible for the hepatoprotective and nephroprotective activity.***

Keywords: hepatoprotective, nephroprotective, antioxidant, *Cardiospremum halicacabum*, paracetamol

1. Introduction

In normal physiological conditions, most of the oxygen is reduced by the electron transport chain in the mitochondria to form water, and a little amount of oxygen is reduced to form reactive oxygen species namely superoxide ($O2^{*-}$), hydrogen peroxide (H_2O_2) & hydroxyl ion (OH^-), these formed radicals are scavenged by the antioxidants defense in the body [1]. Our body is equipped with "redox homeostasis", one cannot rule out the beneficial effects of free radicals at low concentrations but higher concentrations of these reactive species cause a deleterious effect in the body. Continuous use of many drugs and chemicals at normal dose or the higher dose may induce oxidative stress and generate free radicals in the body, these reactive species in higher concentration depletes the antioxidant levels and may be the reason for the etiology of several degenerative diseases such as cirrhosis, rheumatoid arthritis, atherosclerosis, cancer, diabetes, and alzehmeir [2], [3].

An increase in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) is a clear indication of hepatic injury and elevated serum creatinine and blood urea nitrogen indicates kidney injury [4]. Paracetamol (PCM), a very common drug used as antipyretic and analgesic. Injudicious use of paracetamol cause hepatic injury as well as kidney dysfunction in humans and experimental animals [5]. A hepatorenal injury induced by Paracetamol is due to oxidative stress which depletes the antioxidants level. Paracetamol induced hepatorenal toxicity due to oxidative stress can be a quite appropriate model that can be used to study the hepatoprotective, nephroprotective & antioxidant property of plant extract [6].

As synthetic antioxidants are carcinogenic their use is limited and there is always a search for antioxidants, of natural origin [7]. The plant *Cardiospermum halicacabum (CH)* is widely used in traditional practices. The plant is used in the treatment of hardened tumors, rheumatism, diuretic, stiffness of limbs, snake bites, diaphoretic, & stomachic [8]. The plant contains a good amount of flavonoids, steroids, tannins, triterpenoids, carbohydrates, glycosides, β-sitosterol, and its D-glucoside, an alkaloid, oxalic acid, saponin, quebrachitol, and amino acid [8], [9]. The study is undertaken to investigate the *hepatoprotective and nephroprotective* property of the plant *Cardiospermum halicacabum.*

2. Materials and Methods

Animals: Wistar rats of either sex weighing 160 g to 200 g are selected. The animals were acclimatized for one week under laboratory conditions, housed in polypropylene cages maintained at ambient room temperature, with 12 hours dark/light cycle fed with standard laboratory diet and free assess of water, *ad libitium*. The animals are maintained as

per CPSCEA guidelines and approved by Institutional Animal Ethical Committee (IAEC), with an IAEC approval no: 878/ac/05/CPCSEA/01/2010.

Chemicals & Reagents: All the chemicals & reagents procured are of analytical grade.

Paracetamol (Pharmed, Bengaluru, India), Silymarin (Micro Labs-Bengaluru, India), Petroleum ether, Chloroform ($CHCl_3$), Ethanol (S D Fine chemicals), the assay kits are procured from ERBA diagonstics Mannheim GmbH (Germany). Trichloroacetic acid (Fisher), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB)(Sigma-Aldrich, USA), epinephrine (Sigma-Aldrich, USA), H_2O_2 (Fisher), Thiobarbituric acid (Loba Chemicals).

Plant collection: The whole plant of *Cardiospermum halicacabum* Linn. has been collected in the month of august-september, and the matured well grown plant bearing fruits is selected and authenticated by botanist, Govt College for Girls, Bagalkot. The herbarium specimen no. is; HSKCOP.PH.COL HERB. 09/2010-11**.** The whole plant is washed, shade dried, and powdered.

Extraction: 70-80 g per batch of shade-dried whole plant of *Cardiospermum halicacabum* powder is taken and extracted with petroleum ether (Pet ether), chloroform ($CHCl_3$), and ethanol (EtOH) using the soxhlet apparatus. The extracts were concentrated under reduced pressure using a rotary flash evaporator and the residues were dried in a desiccator. The extracts were subjected to preliminary phytochemical investigations by qualitative chemical tests before pharmacological evaluation.

Pharmacognostic investigations
Morphological features [10], [11] of the plant is studied, extractive values [11], [12] and ash values [12], [13], [14], [15] is determined.

Phytochemical investigations
Calculation of % yield and qualitative chemical identification tests [11] of the plant extract is done and reported.

Dose selection: No mortality is found up to 2000 mg/kg [16] based upon this, the plant *Cardiospermum halicacabum* is administered in three doses 200 mg/kg, 400 mg/kg & 800 mg/kg.

Paracetamol (PCM) induced hepatotoxicity & nephrotoxicity & treatment schedule [17]
Paracetamol is given 750 mg/kg/day, p.o. for 7 days; except Group I all the groups received Paracetamol in the above mentioned dose. Animals were divided into 12 groups of six animals each. Group I: Normal received the vehicle as normal saline (vehicle control), Group II: Toxic Control, received PCM, Group III: Standard, received silymarin 100 mg/kg p.o., Group IV: Low dose, 200 mg/kg p.o. of pet ether extract, Group V: Medium dose, 400 mg/kg p.o. of pet ether extract, Group VI: High dos, 800 mg/kg p.o. of pet ether extract, Group VII: Low dose, 200 mg/kg p.o. of $CHCl_3$ extract, Group VIII: Medium dose, 400 mg/kg p.o. of $CHCl_3$ extract, Group IX: High dose, 800 mg/kg p.o. of $CHCl_3$ extract, Group X: Low dose, 200 mg/kg p.o. of EtOH extract, Group XI: Medium dose, 400 mg/kg p.o. of EtOH extract, Group XII: High dose, 800 mg/kg p.o. of EtOH extract. All the extracts of above mentioned dose are administered p.o. for 14 days from the first day. Silymarin and the extracts were administered 1 hour before the PCM administration to the respective group of animals.

Biochemical Analysis

Assessment of hepatoprotective & nephroprotective Activity
After the experimental period, the blood is collected by the puncture of retro-orbital plexus under light anesthesia into sterilized dry centrifuge tubes which are coagulated for 30 min at 37^0C. The clear serum is separated at 2500 rpm for 10 min. The serum is subjected to standard biochemical estimations and different hepatic biochemical markers like serum alanine aminotransferase (ALT) [18], aspartate aminotransferase (AST) [18], alkaline phosphatase (ALP) [19], total bilirubin (TBIL) [20] total protein (TP) [21] and renal biochemical markers serum creatinine and blood urea nitrogen (BUN) are measured by following the kit procedure.

Estimation of endogenous antioxidants
Tissue (liver & kidney) homogenate (10%w/v) is prepared in ice-cold 10 mM Tris-buffer (pH 7.4) and centrifuged at 3000 rpm for 10 min at 4º C and the supernatant is used for estimation of endogenous antioxidants; reduced glutathione (GSH) [22], superoxide dismutase (SOD) [23], catalase (CAT) [24], lipid peroxidation (LPO) [25].

Statistical Analysis
The results were expressed as mean ± SEM, where all the groups are compared with the PCM treated (control) group. The data were analyzed using one way analysis of variance (ANOVA) followed by the Dunnet multiple comparison test. Where P values; *P < 0.05, **P < 0.01, ***P < 0.001 are considered as statistically significant.

3. Results

Observation is made on the morphological features of fresh leaf, stem and root of the plant *Cardiospermum halicacabum* L. is done and reported in table no:1 and proximate values such as extractive values, moisture content and ash values of whole plant of CH is reported in table no:2. Percentage (%) of dry weight in g, color, odor and consistence of the extract is reported in the table no:3 and results of qualitative phytochemical tests of the plant extract is reported in the table no:4.

Table 1: Morphological features of the plant CH

S. No.	Features	Observation
A]	Leaf	
1	Color	Greenish-pale yellow
2	Odor	Characteristic
3	Taste	Slightly bitter
4	Size	13-16 by 3-6mm long
5	Leaf	Compound leaf
6	Shape	Elliptic- oblong base rounded
7	Petioles	Very short
8	Leaf-let	Two rows with a rachis, alternate and opposite

B]	Flowers	
1	Color	white in umbellate cyme
C]	Stem	
1	Color	Light brown
2	Odor	Characteristic
3	Taste	Bitter
5	Size	20-75 cm long
6	Shape	Cylindrical
7	Base	Branching at the base
D]	Root	
1	Color	Brown
2	Odor	Characteristic
3	Taste	Bitter
4	Size	2.5-11.0 cm long
5	Shape	Nearly straight
6	Base	Gradually tapering
7	Fibrous	Number of fibrous
8	Roots	Small, secondary and tertiary

Table 2: Proximate values of the plant CH

S. No.	Parameter	Determined Value % w/w
A.	Extractive values for I: Cold maceration	
1	Alcohol soluble extractive value	7.00
2	Water soluble extractive value	15.00
3	Ether soluble extractive value	2.00
II: Hot extraction method		
1	Alcohol soluble extractive value	18.00
2	Water soluble extractive value	32.00
3	Ether soluble extractive value	12.00
B.	Moisture content by Loss on drying	10.00
C.	Ash Values	
1	Total ash	8.00
2	Acid insoluble ash	4.00

Table 3: Percentage yield and physical characteristics of various extracts of CH

Extract	% Dry weight in g	Color	Odor	Consistency
Successive extraction				
Pet ether	2.08	Brownish yellow	Characteristic	Sticky wax
CHCl3	2.66	Dark Green	Characteristic	Sticky
EtOH	8.24	Reddish Brown	Characteristic	Sticky

Pet ether- Petroleum ether, $CHCl_3$ – Chloroform, EtOH - Ethanol

Effect of different extracts of *Cardiospermum halicacabum* Linn.on serum biochemical parameters in Paracetamol induced hepatotoxicity and nephrotoxicity

Results has been summarized, the level of significance of the extracts of different dose as hepatoprotective and nephroprotective is same. Significant (***P < 0.001) increase in ALT, AST, ALP, TBIL, serum creatinine, BUN levels and decrease in total protein levels is observed in PCM treated (control) group whereas standard group significantly (***P < 0.001) restores all the serum biochemical parameters near to the normal group. Low dose of pet ether extract is not significant (ns) & medium dose of pet ether extract is less significant (*P < 0.05) and high dose of pet ether extract is significant (***P < 0.001) in restoring serum biochemical markers when analyzed and compared with the negative control group. Low dose of $CHCl_3$ extract is not significant (ns), medium dose and high dose of $CHCl_3$ extract is significant (**P < 0.01) and (***P < 0.001) respectively in restoring serum biochemical markers when analyzed and compared with the negative control group. Low dose of EtOH extract is not significant (ns), medium dose and high dose of EtOH extract is significant (**P < 0.01) and (***P < 0.001) respectively in restoring serum biochemical markers when analyzed and compared with the negative control group.

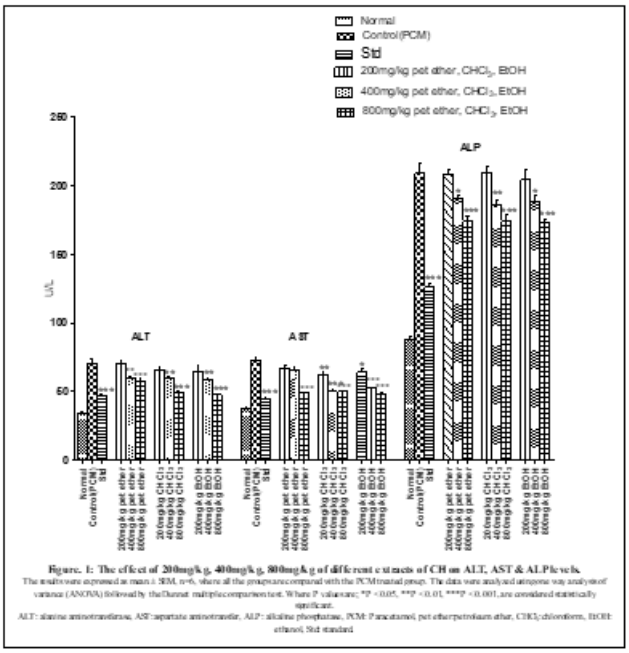

Figure. 1: The effect of 200mg/k g, 400mg/k g, 800mg/k g of different extracts of CH on ALT, AST & ALP levels.

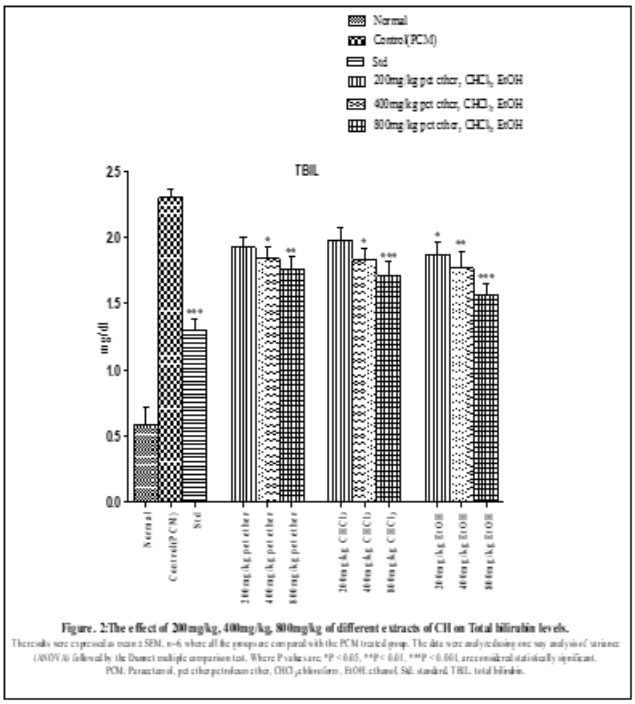

Figure. 2:The effect of 200mg/kg, 400mg/kg, 800mg/kg of different extracts of CH on Total bilirubin levels.

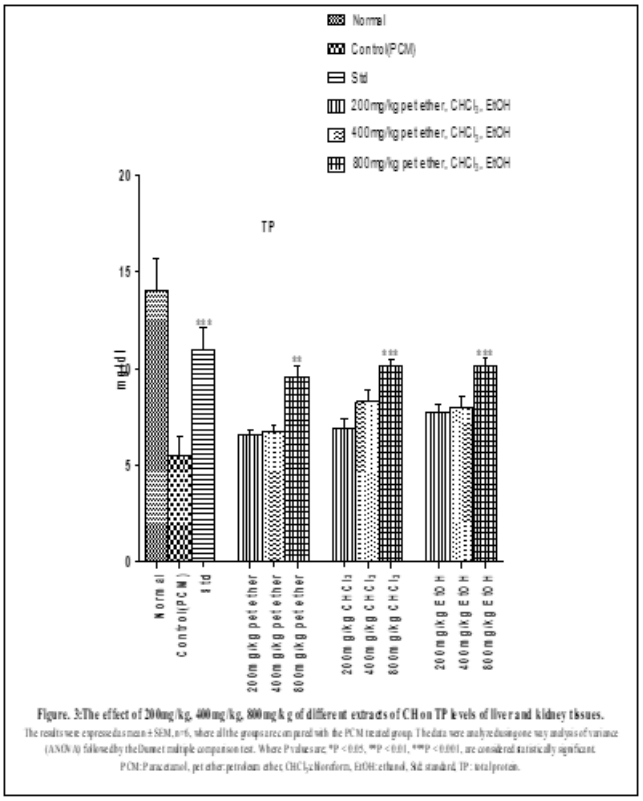

Figure. 3:The effect of 200mg/kg, 400mg/kg, 800mg/k g of different extracts of CH on TP levels of liver and kidney tissues.

The results were expressed as mean ± SEM, n=6, where all the groups are compared with the PCM treated group. The data were analyzed using one way analysis of variance (ANOVA) followed by the Dunnet multiple comparison test. Where P values are, *P < 0.05, **P < 0.01, ***P < 0.001, are considered statistically significant. PCM: Paracetamol, pet ether: petroleum ether, $CHCl_3$:chloroform, EtOH: ethanol, Std: standard, TP: total protein.

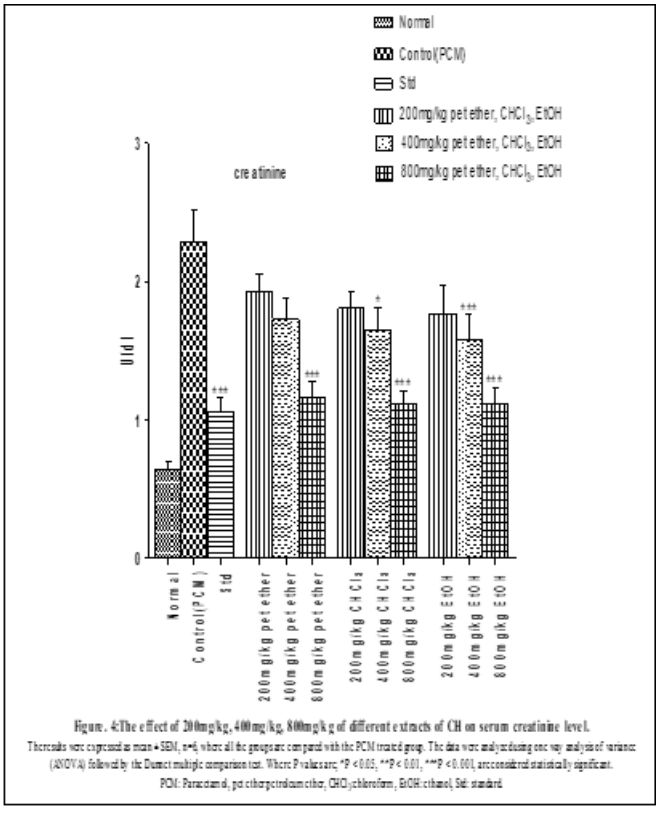

Figure. 4:The effect of 200mg/kg, 400mg/kg, 800mg/k g of different extracts of CH on serum creatinine level.

The results were expressed as mean ± SEM, n=6, where all the groups are compared with the PCM treated group. The data were analyzed using one way analysis of variance (ANOVA) followed by the Dunnet multiple comparison test. Where P values are; *P < 0.05, **P < 0.01, ***P < 0.001, are considered statistically significant. PCM: Paracetamol, pet ether:petroleum ether, $CHCl_3$:chloroform, EtOH: ethanol, Std: standard.

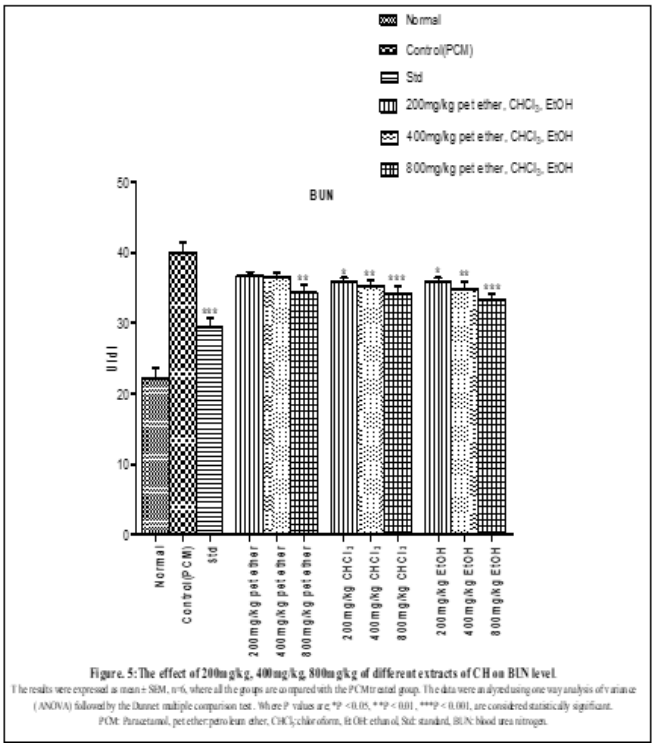

Figure. 5: The effect of 200mg/k g, 400mg/kg, 800mg/k g of different extracts of CH on BUN level.

The results were expressed as mean ± SEM, n=6, where all the groups are compared with the PCM treated group. The data were analyzed using one way analysis of variance (ANOVA) followed by the Dunnet multiple comparison test. Where P values are, *P < 0.05, **P < 0.01, ***P < 0.001, are considered statistically significant. PCM: Paracetamol, pet ether: petroleum ether, $CHCl_3$: chloroform, EtOH: ethanol, Std: standard, BUN: blood urea nitrogen.

Effect of different extracts of *Cardiospermum halicacabum* Linn. on GSH, SOD, catalase & MDA levels in Paracetamol induced hepatotoxicity and nephrotoxicity

Significant (***P < 0.001) decrease in GSH, SOD, catalase levels and increase in MDA levels is observed in PCM treated (Control) group whereas standard group significantly (***P < 0.001) restores all the antioxidant levels in the hepatic and renal tissue near to the normal group. Low dose & medium dose of pet ether extract is not significant (ns) and high dose of pet ether extract is significant (**P < 0.01) in restoring hepatic and renal tissue antioxidant levels when analyzed and compared with the negative control group. Low dose of $CHCl_3$ extract is not significant (ns), medium dose and high dose of $CHCl_3$ extract is significant (***P < 0.001) in restoring hepatic and renal tissue antioxidant levels when analyzed and compared with the negative control group. Low dose of EtOH extract is less significant (*P < 0.05), medium dose and high dose of EtOH extract is significant (***P < 0.001) in restoring hepatic and renal tissue antioxidant levels when analyzed and compared with the negative control group.

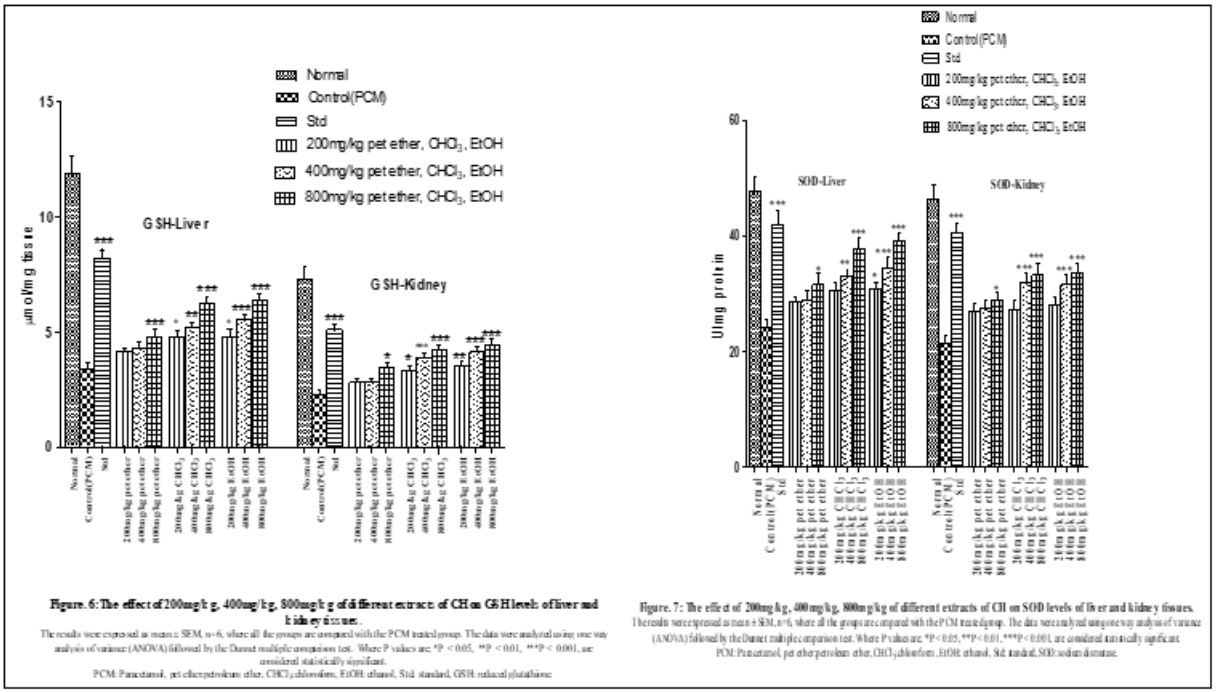

Figure. 6: The effect of 200mg/kg, 400mg/kg, 800mg/kg of different extracts of CH on GSH level: of liver and kidney tissues.

The results were expressed as mean ± SEM, n=6, where all the groups are compared with the PCM treated group. The data were analyzed using one way analysis of variance (ANOVA) followed by the Dunnet multiple comparison test. Where P values are: *P < 0.05, **P < 0.01, ***P < 0.001, are considered statistically significant.

PCM: Paracetamol, pet ether:petroleum ether, $CHCl_3$:chloroform, EtOH: ethanol, Std: standard, GSH: reduced glutathione

Figure. 7: The effect of 200mg/kg, 400mg/kg, 800mg/kg of different extracts of CH on SOD levels of liver and kidney tissues.

The results were expressed as mean ± SEM, n=6, where all the groups are compared with the PCM treated group. The data were analyzed using one way analysis of variance (ANOVA) followed by the Dunnet multiple comparison test. Where P values are: *P < 0.05, **P < 0.01, ***P < 0.001, are considered statistically significant.

PCM: Paracetamol, pet ether:petroleum ether, $CHCl_3$:chloroform, EtOH: ethanol, Std: standard, SOD: sodium dismutase.

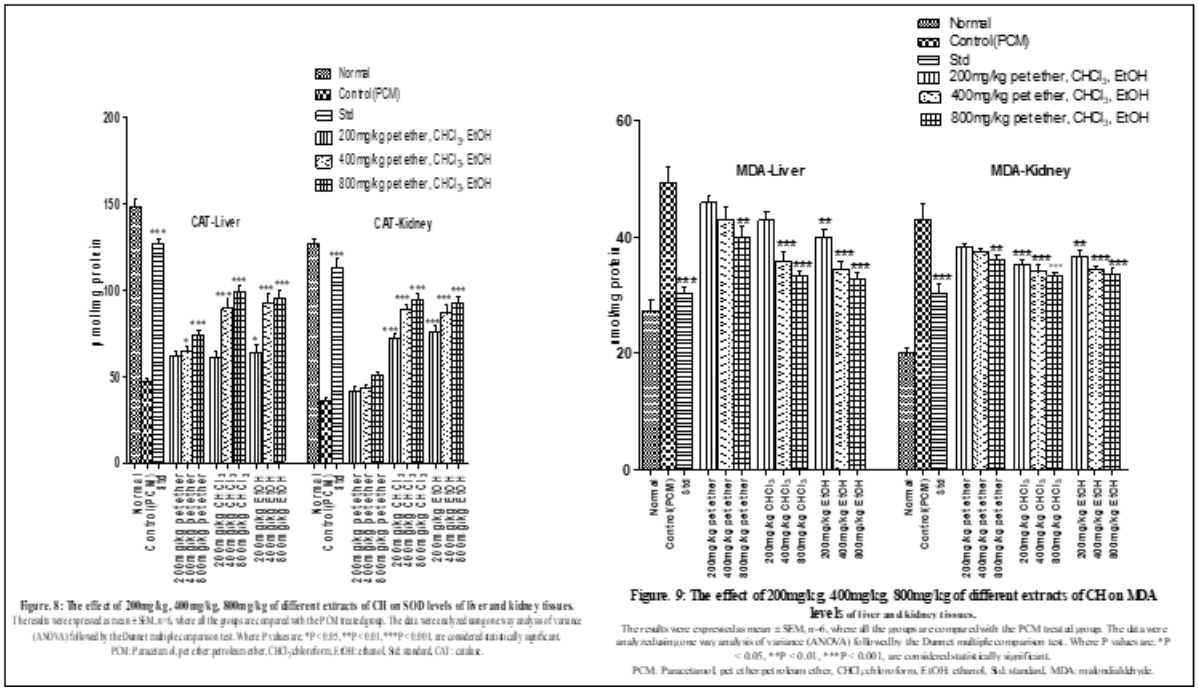

Figure. 8: The effect of 200mg/kg, 400mg/kg, 800mg/kg of different extracts of CH on SOD levels of liver and kidney tissues.

The results were expressed as mean ± SEM, n=6, where all the groups are compared with the PCM treated group. The data were analyzed using one way analysis of variance (ANOVA) followed by the Dunnet multiple comparison test. Where P values are: *P < 0.05, **P < 0.01, ***P < 0.001, are considered statistically significant.

PCM: Paracetamol, pet ether: petroleum ether, $CHCl_3$:chloroform, EtOH: ethanol, Std: standard, CAT: catalase.

Figure. 9: The effect of 200mg/kg, 400mg/kg, 800mg/kg of different extracts of CH on MDA levels of liver and kidney tissues.

The results were expressed as mean ± SEM, n=6, where all the groups are compared with the PCM treated group. The data were analyzed using one way analysis of variance (ANOVA) followed by the Dunnet multiple comparison test. Where P values are: *P < 0.05, **P < 0.01, ***P < 0.001, are considered statistically significant.

PCM: Paracetamol, pet ether:petroleum ether, $CHCl_3$:chloroform, EtOH: ethanol, Std: standard, MDA: malondialdehyde.

4. Discussion

The study is designed to induce hepatotoxicity and nephrotoxicity by PCM administration. After knowing the status of the liver and kidney by measuring biochemical markers serum ALT, AST, ALP, total bilirubin, total protein and serum creatinine and BUN, an estimation of endogenous antioxidant levels are done. Paracetamol, commonly known as acetaminophin is metabolized in the liver by glucuronide conjugation and sulfate conjugation. Nearly 90% of PCM undergoes glucuronidation and sulfation before elimination. A little portion of paracetamol is metabolized by cytochrome-P 450 enzyme system results in the formation of highly reactive radical *N*-acetyl-*p*-benzoquinoneimine (NAPQI). Usually NAPQI formed at therapeutic dose of PCM binds to intracellular GSH and gets detoxicified. Continuous increase in NAPQI formation by chronic use or overdose dose of paracetamol depletes GSH levels. Further NAPQI generates reactive oxygen species (ROS) by acylation or oxidation of cytosolic and membrane proteins. Eventually ROS oxidizes and damage important biomolecules DNA, proteins and lipids. The integrity of hepatic cell membrane is lost and cause leakage of hepatic contents into serum. ALT, AST and ALP in the serum, are significant biochemical markers increase in their levels indicates hepatotoxicity. ROS interacts with polyunsaturated fatty acids (PUFA) increases the level of malondialdehyde (MDA), increase in this aldehyde level is quantified by Thiobarbituric acid reacting substance (TBARS), which is notable biomarker helps to evaluate extent of lipid peroxidation and oxidative stress [26], [27], [28]. The sequence of events that causes hepatotoxicity may leads to nephrotoxicity. There is evidential observation even nephrotoxicity may occur independent of hepatic injury.

This depicts even though NADPH, cytochrome P-450 and oxygen are involved in the metabolism of paracetamol in liver and kidneys, but both the organs have different and independent metabolic capabilities [29]. Even though the mechanism of nephrotoxicity induced by PCM is not clear but several research reported that formation of reactive intermediate NAPQI during PCM metabolism is responsible for renal toxicity. Further NAPQI forms covalent adduct by binding to renal proteins and increases ROS generation. The generated ROS induces oxidative stress depletes GSH and other antioxidants cause lipid peroxidation that leads to apoptosis, tubular necrosis and renal toxicity [26], [30], [31]. Lorz C *et al*., studied the molecular aspects of paracetamol causing renal injury and reported about the cleavage of caspases-12 and expression of GADD153; a marker of endoplasmic reticulum (ER) stress and a transcription factor that triggers apoptosis of tubular epithelial cells [32]. A preliminary phytochemical investigation shows the presence of flavonoids, phenols, and alkaloids in ethanol and chloroform extract of plant. Flavonoids and Phenolic compounds quench the free radicals formed by oxidative stress and exhibits antioxidant property better than Vitamin C, Vitamin E, and carotenoids [33]. The mechanism of these compounds scavenges the free radicals is; either by decreasing ROS/RNS formation by forming chelation of the trace metals required for ROS/RNS formation or replenishing or upregulating the formation of antioxidants [34]. The study is evident by the observation of control group with comparison to the normal group there is increased levels of serum ALT, AST, ALP, total bilirubin and decreased protein levels by liver function tests and increase serum creatinine, blood urea nitrogen (BUN) levels by kidney function tests. Increased MDA levels and depleted antioxidant GSH, SOD and catalase in the control group is observed when compared to the normal group. Whereas in drug treated groups there is a significant dose dependent decrease in those serum indicators are observed where their levels are increased in hepatotoxicity and nephrotoxicity meanwhile increase in the antioxidant levels indicates hepatoprotective and nephroprotective effect of the plant extract. As the preliminary phytochemical investigation reveals chloroform extract and ethanol extract of *Cardiospermum halicacabum* possess good amount of polyphenols and flavonoids that can replenish the depleted antioxidant levels. Further investigation is needed which flavonoids and polyphenols is responsible for hepatoprotective & nephroprotective activity.

5. Conclusion

Among these extracts medium and high doses of ethanol extract and chloroform extract of the plant, CH shows significant antioxidant properties by replenishing antioxidant levels inhibiting lipid peroxidation induced by oxidative stress and decreasing reactive intermediates formed by the metabolism of PCM. It is quite relevant to correlate their antioxidant activity due to the presence of flavonoids and polyphenols in the chloroform and ethanol extract.

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Conflict of Interest
The authors declare that there is no conflict of interest.

Abbreviations
ANOVA: analysis of variance, **DNA:** deoxyribonucleic acid, **CPCSEA:** Committee for the Purpose of Control and Supervision of Experiments on Animals, **NADPH:** nicotinamide adenine dinucleotide phosphate hydrogen, **RNS:** reactive nitrogen species.

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