

## Research Article



## Stress Inhibitory and Hepatoprotective Activities of *Clitoria ternata* against Paracetamol Induced Hepatotoxicity in Albino Rats

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### ABSTRACT

The present study mainly focuses on the evaluation of stress inhibitory (antioxidant), antimicrobial, anti haematotoxic and hepatoprotective activities of petroleum ether and chloroform extracts of *Clitoria ternata* against paracetamol induced toxicity. Phytochemical analysis was done qualitatively, antimicrobial activity was determined by agar well diffusion method, oxidative stress and hepatotoxicity was induced in rats by giving high doses of Paracetamol. Anti hepatotoxicity of *C.ternata* was measured by estimating the levels of SGOT, SGPT, ALP and total bilirubin. *In vivo* antioxidant analysis was carried by estimating the levels of MDA, SOD, Catalase. Amelioration of haematological parameters was also evaluated using % of Haemoglobin, RBC count, Total platelet count, Total count, Packed cell volume (PCV) and Acute eosinophilic count (AEC). Our results showed that both the extracts of *C.ternata* showed significant levels of antitoxic and antioxidant properties and exhibited hepatoprotective effect against paracetamol induced toxicity. In addition, *C.ternata* also involved in the amelioration of hematological parameters altered due to toxicity. The chloroform of extract of *C.ternata* was more potent and significant compared to petroleum ether extract. This study justifies the traditional use of *C.ternata* from their antioxidant and hepatoprotective activities and act as a good source for the preparation of herbal medicine.

**Keywords:** Antioxidant, Hepatoprotective activity, Silymarin, Zone of inhibition.

### INTRODUCTION

Aerobic organs generate a large number of reactive oxygen species commonly known as free radicals that induce oxidative tissue damage. These radicals, induce lipid peroxidation or cause inflammation, have been associated as important pathological mediators in many clinical disorders such as heart disease, diabetes, gout and cancer.<sup>1-3</sup> A major defense mechanism against radicals is the antioxidant system, which involves the conversion of reactive oxygen molecules into non-toxic compounds.

The liver is the prime organ meant for various states of metabolic and physiologic homeostasis of the organism.<sup>4</sup> Liver diseases are the major serious health problems and are mainly caused by toxic chemicals and drugs including excess consumption of alcohol, high doses of paracetamol, carbon tetrachloride etc. The drug-induced liver injury accounts for more than 50% of acute liver failure, including hepatotoxicity.<sup>5</sup> Paracetamol or Acetaminophen is an antipyretic drug, which can produce an electrophilic metabolite, Nacetyl parabenzoquinonimine (NAPQI), at high doses. The reactive metabolite can be conjugated with cellular glutathione (GSH) to cause extensive GSH depletion which leads to the cellular necrosis.<sup>6-7</sup> In spite of the tremendous advances made in allopathic medicine; no effective hepatoprotective drug is available. Hence a search for new pharmacologically active agents from plant sources led to the discovery of useful drugs that play a major role in the treatment of human diseases including liver ailments.<sup>8</sup> Numerous medicinal plants and their

formulations are used for liver disorders along the Ayurveda history. Herbs have recently attracted attention as health beneficial foods and as source materials for drug development.<sup>9-10</sup> One such herb with a high rate of medicinal importance including the treatment of liver damage is *Clitoria ternate* Linn.

*Clitoria ternate* Linn. is a member of the family Fabaceae; commonly known as 'Aparajita' or shankupushpi. It is a perennial climber widely used in the traditional Ayurvedic system of Indian medicine for treating a wide variety of ailments. *C.ternata* is a very bioactive plant and used in various diseases and exhibited various properties like anxiolytic, anti depressant, anti convulsant anti stress, anti-inflammatory, analgesic and antipyretic properties.<sup>11-14</sup> Recent study showed that malonylated flavonol glycosides, new anthocyanins, ternatins A3, B3, B2, C1-C5 and O3 and preternatins A3 and C4 were isolated from flowers of *C.ternata*.<sup>15-16</sup> The methanolic extract of leaf, root and flowers of *C. ternata* was reported to exhibit hepatoprotective activity.<sup>17</sup> *C. ternata* possesses various levels of phytochemical compounds and also exhibited *in vitro* antioxidant capacity was reported earlier.<sup>18-19</sup> Literature survey of this medicinal revealed that no extensive phytochemical and pharmacological investigations had been carried out. Hence, the present study is therefore, aimed to evaluate the levels of phytoconstituents, antimicrobial, anti haematotoxic, antioxidant and hepatoprotective effects of leaves of petroleum ether (PE) and chloroform extracts (CE) of *C. ternata* for treating dreadful diseases spending up new avenues in diseases management for humans.



## MATERIALS AND METHODS

### Soxhlet extraction and fractionation of the plant extract using petroleum ether and chloroform

The plants were air dried sun light in at 40°C. Leaves were separated and then ground into powder using a grinding apparatus and subsequently extracted in a Soxhlet apparatus by the serial extraction method with solvents based on the increase in polarity using petroleum ether, followed by chloroform. Solvent elimination under reduced pressure afforded the petroleum ether (2 % w/w yield) and chloroform extract (2.4 % w/w yield) respectively. The extracts were then kept in desiccators at room temperature (22-24°C) prior to use in our experiments.<sup>20</sup>

### Phytochemical evaluation (Qualitative methods)

Phytochemical examinations were carried out for all the extracts as per the standard methods.<sup>21-22</sup>

### Antimicrobial activity

#### Test microorganisms and growth media

The following Gram-positive and Gram-negative bacteria, yeasts, and molds were used for antimicrobial activities studies: Gram-positive bacteria included *Bacillus cereus*, *Bacillus subtilis* and *Staphylococcus aureus*; Gram-negative bacteria included *Escherichia coli*, *Pseudomonas aeruginosa*; Fungi included *Candida albicans* and *Saccharomyces cerevisiae*; molds include *Aspergillus niger*, were used in this study. The bacterial strains were grown in Mueller–Hinton agar (MHA) plates at 37°C, whereas the fungi such as molds were grown in Potato dextrose agar (PDA) plates media, respectively, at 28°C. The stock culture was maintained on nutrient agar slants at 4°C.

#### Determination of antibacterial activity by Agar well diffusion method

The antibacterial and antifungal activities of the leaf extracts was determined using agar well diffusion method following the published procedure with slight modification.<sup>23-25</sup>

### In vivo studies

#### Experimental animals

Wistar albino rats of both the sexes (weighing about 120-175gms) were used in the study were procured from Mahaveer enterprises, Hyderabad and were placed at random and allocated to treatment groups in clean polypropylene cages (38x23x10cms) with not more than four animals per cage. Animals were housed and maintained under standard laboratory conditions, at a temperature of 24±2°C and relative humidity of 30–70% with dark and light cycles (12/12hour). The animals were allowed free access to standard pellet diet (Hindustan lever, Kolkata, India) and water ad libitum during the course of study. The animals were acclimatized to laboratory conditions for 10 days before commencement of experiment. All the experimental procedures and

protocols used in this study were reviewed and approved by the Institutional Animal Ethics Committee (IAEC) and were in accordance with the guidelines of the IAEC.

### Hepatoprotective activity against Paracetamol induced toxicity

Paracetamol-induced hepatotoxicity in rats was carried by the method of Gupta.<sup>26</sup>

Healthy albino rats were divided into 6 groups of 4 animals in each.

Group-I, served as normal, which received normal saline (5ml/kg body weight).

Group-II treated as toxic control received paracetamol (500 mg/kg p.o) once daily for 7 days (Toxic control).

Group-III, received paracetamol (500 mg/kg, p.o.) and chloroform extract (100mg/kg p.o) simultaneously for 7 days.

Group-IV, received paracetamol (500 mg/kg, p.o.) and Petroleum ether extract (100 mg/kg p.o) simultaneously for 7 days.

Group-V received paracetamol (500 mg/kg, p.o.) and standard drug silymarin (25 mg/kg p.o) simultaneously for 7 days.

The biochemical parameters were determined after 18 h fasting of the last dose.

### Biochemical studies

The blood was obtained from all animals by puncturing retro-orbital plexus. The blood samples were allowed to clot for 45 min at room temperature. Serum was separated by centrifugation at 2500 rpm at 30°C for 15 min and utilized for the estimation of various biochemical parameters including determination of serum Bilirubin<sup>27</sup>, SGPT and SGOT<sup>28</sup> and Alkaline phosphatase.<sup>29</sup>

### Antioxidant studies

After collection of blood samples the rats were sacrificed and their livers excised, rinsed in ice cold normal saline followed by 0.15 M Tris-HCl (pH 7.4) blotted dry. A 10 % w/v of homogenate was prepared in 0.15 M Tris-HCl buffer and processed for the estimation of lipid peroxidation. The rest of the homogenate was centrifuged at 15000 rpm for 15 min at 4°C used for the estimation of levels of SOD<sup>30</sup>, Catalase<sup>31</sup> and total protein.<sup>32</sup>

### Determination of Thiobarbituric Acid Reactive Substances (TBARS)

Lipid peroxidation in liver tissues were estimated calorimetrically by measuring thiobarbituric acid reactive substances (TBARS).<sup>33</sup> To 0.2ml of sample, 0.2ml of 8.1% Sodium dodecyl sulfate, 1.5 ml of 20% acetic acid and 1.5 ml of 0.8% TBA were added. The volume of the mixture was made up to 4ml with distilled water and then heated at 95°C in a water bath for 60 min. After incubation the



tubes were cooled to room temperature and the final volume was made up to 5 ml in each tube. Then 5 ml of n-butanol:Pyridine mixture was added and the contents were vortexed thoroughly for 2 min. After centrifugation at 3000 rpm for 10 min the upper organic layer was taken and its OD was read at 532 nm against an appropriate blank without the sample. The levels of lipid peroxides were expressed as millimoles of thiobarbituric acid reactive substances (TBARS)/100gram of liver tissue using an extinction co-efficient of  $1.56 \times 10^5 \text{M}^{-1} \text{cm}^{-1}$ .

### Haematological studies

The Automated Haematologic Analyzer was used to analyze the hematological parameters such as % of Haemoglobin, RBC count, Total Platelet count, Total Count, Packed cell volume (PCV) and Acute Eosinophilic count (AEC). The analyses were carried out based on standard methods.<sup>34</sup>

### Histopathological studies

Small pieces of liver tissues in each group were collected in 10% neutral buffered formalin for proper fixation. These tissues were processed and embedded in paraffin wax. Sections of 5- 6 $\mu\text{m}$  in thickness were cut and stained with hematoxylin and eosin (H&E). These sections were examined photomicroscopically for necrosis, steatosis and fatty changes of hepatic cells.

### Statistical analyses

Experimental results are presented as the mean and standard deviation (SD) of three parallel measurements. All statistical analysis is performed using Minitab graphical computer software packages.

## RESULTS

### Phytochemical studies (qualitative analysis)

The results of the petroleum ether (PE) and chloroform (CE) extracts of *C.ternata* revealed the presence of phenolics, flavonoids, alkaloids and tannins. Our earlier reports shown that both PE and CE of *C.ternata* quantified with some levels of phytochemicals including tannins, phenolics, flavonoids and alkaloids.<sup>19</sup>

### Antimicrobial studies

The results shown in the table 1 clearly indicated that all the antibiotics used in the present study exhibited a significant level of zone of inhibition against all the tested gram +ve and -ve bacteria and fungi. In the present study, the crude PE and CE of *C.ternata* was evaluated for antibacterial activity against 3 strains gram +ve bacteria, namely *S. aureus*, *B. subtilis*, and *B.cereus* (table 1). The zones of inhibition ranging from 8.9 to 12.3mm. Among the 3 gram +ve bacteria, both CE and PE of *C.ternata* was highly effective against *S. aureus* followed by *B.cereus* and minimum zone of inhibition was noticed against *B. subtilis*. Both CE and PE of *C.ternata* also showed antibacterial property against gram -ve bacteria namely *E.coli* and *P.aeruginosa*. The zone of inhibition is in the range of 7.4 to 13.1 mm.

The anti fungal activity of PE and CE of *C.ternata* was also tested against three different fungi including *C.albicans*, *S.cerevisiae* and *A.niger* (table 1). The antifungal activity was found to be high against *A.niger* followed by *C. albicans* with and very low activity was observed in *S.cerevisiae*.

**Table 1:** Showed the antibacterial and antifungal activity of PE and of *C.ternata*

Type of the plant extract	Zone of Inhibition (in mm)							
	Gram +ve bacteria			Gram -ve bacteria		Name of the Fungi		
	<i>B.subtilis</i>	<i>B.cereus</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>C. albicans</i>	<i>S.cerevisiae</i>	<i>A. niger</i>
PE of <i>C.ternata</i>	8.9	9.1	9.9	9.1	7.4	8.0	6.8	9.1
CE of <i>C.ternata</i>	11.1	10.7	12.3	13.1	11.7	10.4	8.4	13.3
Chloramphenicol	20.4	20.7	22.2	19.8	23.1	NT	NT	NT
Tetracycline	16.9	17.8	19.2	18.1	16.5	NT	NT	NT
Pencillin	NT	NT	NT	NT	NT	13.1	12.8	17.1

Values are means of three replicate determinations; NT denotes not tested.

**Table 2:** Showed the Influence of PE and CE of *C.ternata* on serum SGPT, SGOT, ALP, TB and DB levels in rats toxicated with Acetaminophen (Paracetamol).

Name of the group	TB	DB	SGPT	SGOT	ALP
G-I: Normal	0.6+ 0.122	0.13+ 0.057	60+ 18.09	49.2+21.5	242.6+ 9.384
G-II: Paracetamol treated toxic	1.47+0.275*	0.65+0.191*	98.5+6.454*	82.5+9.327	416.2+22.86
G-III: Paracetamol + CE of <i>C.ternata</i>	0.8+0.1**	0.366+ 0.057**	75+1.0**	62.66+10.0**	251+3.655**
G-IV: Paracetamol + PE of <i>C.ternata</i>	1.13+ 0.208	0.633+0.115	87+2.645**	68.66+1.52**	313+32.04**
G-V: Paracetamol + Silymarin treated	0.53+ 0.152**	0.2+0.1**	53.6+4.613**	39.33+3.05**	202+6.557**

Values are means  $\pm$ Standard Deviation (no. of animals in each group=4). p values calculated by ANOVA and  $p < 0.05$  Values are considered statistically significant; \*\* $p < 0.05$  compared with Paracetamol control group; \* $p < 0.005$  compared with Normal group.



**Table 3:** Stress inhibitory (Antioxidant) activity of PE and CE of *C.ternata* on Paracetamol induced toxicity.

Name of the sample	SOD (U/mg of Protein)	CAT (U/mg of Protein)	TBARS (MDA Levels)	Total Protein
Normal	3.73±0.1527	226.0±3.32	1.91±0.136	8.12±0.150
Paracetamol	1.16±0.115*	156.8±2.81*	6.6±0.416*	5.0±0.020*
Paracetamol + CE of <i>C.ternata</i>	3.16±0.152**	194.9±4.31**	3.89±0.158**	6.16±0.070**
Paracetamol + PE of <i>C.ternata</i>	2.63±0.305**	169.7±7.60**	5.16±0.169**	5.58±0.263**
Paracetamol + Silymarin	3.86±0.152**	233.5±10.2**	1.82±0.073**	8.14±0.123**

Values are means ±Standard Deviation (n=4). p values calculated by ANOVA and p< 0.05 Values are considered statistically significant; \*\*p<0.05 compared with Paracetamol treated toxic group; \*p<0.005 compared with Normal group.

**Table 4:** Showed various Hematological parameters

Name of the sample	HG%	R.B.C (Million/Cu.mm)	Platelet Count (Lakhs)	T.C (Cells/Cu.mm)	AEC (Cells/Cu.mm)	P.C.V (%)
Normal	8.9±0.1732	5.94±0.245	3.55±0.203	7700±435.9	310± 43.59	35.9±0.81
Paracetamol	5.9±0.4583*	3.59±0.282*	2.75±0.124*	4400±1058.3	150± 26.46*	18.5± 0.96*
Paracetamol+ CE of <i>C.ternata</i>	7.2±0.360**	4.52±0.242**	2.91± 0.260	6200± 871.8	220± 17.32**	23.3± 2.4**
Paracetamol+ PE of <i>C.ternata</i>	6.5±0.8718	3.89±0.4451	2.82±0.043	5200± 360.6	180± 30	21.2±1.01**
Paracetamol+ Silymarin	8.1±0.9**	4.77±0.156**	3.11±0.589	7300± 500**	230± 17.32**	25.3± 0.3**

Values are means ±Standard Deviation (n=4). p values calculated by ANOVA and p< 0.05 Values are considered statistically significant; \*\*p<0.05 compared with Paracetamol control group; \*p<0.005 compared with Normal group.

### Hepatoprotective activity

Our main focus is to evaluate the hepatoprotective and antioxidant activities of PE and CE of *C.ternata* in acetaminophen (Paracetamol) induced liver toxicity. Rats were used to estimate the levels of liver markers like total bilirubin (both direct and conjugated) and enzymes like SGOT, SGPT and ALP as an indicator for liver protection against Paracetamol induced toxicity. Silymarin, a standard hepatoprotective drug was used as positive control. Results showed that high levels of all the hepatic biomarkers were observed on hepatotoxicity treatment group than normal control group (group-I and II). Treatment of Acetaminophen toxicated groups with CE and PE of *C.ternata* (Group-III and Group-IV) reduces all the elevated levels of SGOT, SGPT and ALP and also decrease in total bilirubin content, which seems to offer the protection. Group –V (standard treatment group) Silymarin, a standard hepatoprotective drug significantly decreased the elevated levels of serum enzymes and bilirubin and also increase the protein content in paracetamol toxicated groups.

### Antioxidant studies

The levels of antioxidant parameters were shown in table 3. It was observed that the increased MDA levels along with reduced levels of SOD, Catalase and serum total proteins in liver is an indication of increased lipid peroxidation induced by paracetamol (Group-II). Treatment of toxicated group with CE and PE of *C.ternata* (Groups III and IV) at 100 mg/kg of doses significantly decreased the MDA levels and also increased

the SOD, Catalase and total protein levels as shown in Table 3.

### Hematological studies

The blood samples of rats of the treatment groups were collected before sacrifice and stored along with anticoagulant, EDTA to evaluate the hematological parameters including % of hemoglobin (HG%), Erythrocyte count (RBC count), Total platelet count, Total count (TC), Acute Eosinophilic Count (AEC), Packed cell volume of the cell (PCV). The levels of hematological parameters were shown in table 4.

We noticed that the levels of HG%, RBC count, Total platelet count, TC, AEC and PCV were markedly decreased, after toxicated with paracetamol (Group-II) compared with the normal control group (Group-I). The oral administration of CE and PE of *C.ternata* resulted in amelioration of these parameters significantly and were likely to be approaching the normal values when compared with that of toxic group (Groups-III and IV).

### Histopathological studies

The histological appearance of hepatocytes reflects their conditions as in figure 1a-1e.

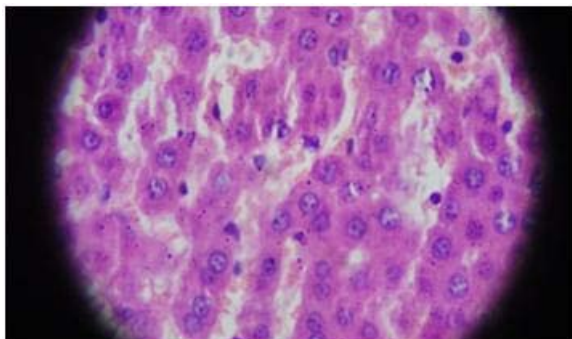
### DISCUSSION

An increase in antibiotic resistance in microorganisms and also the side effects caused by synthetic antibiotics, medicinal plants are gaining importance in the treatment of microbial infections and are considered as clinically effective and safer alternatives to the synthetic

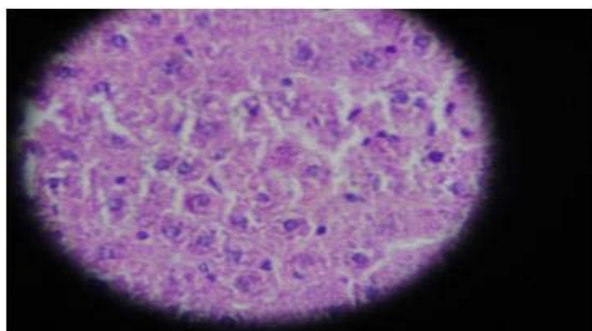




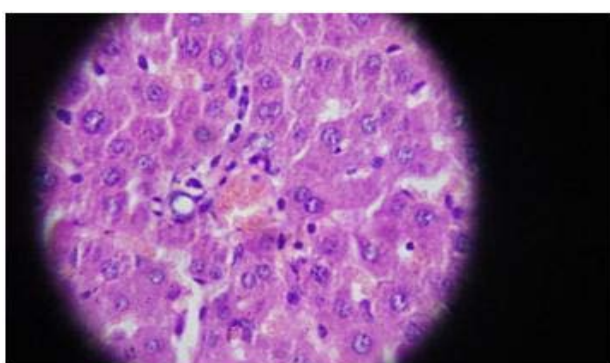
antibiotics, so in the present era, many medicinal plants have been widely used as antimicrobial agents and hence an attempt has been made to evaluate the antimicrobial potential of both PE and CE of *C.ternata* in the present study. Agar diffusion method was extensively used to investigate the antibacterial activity of natural substances and plant extracts.



**Figure 1a:** Showed the Group-I: Normal hepatic cells each with well defined cytoplasm, nucleus, nucleolus and well brought out central vein.



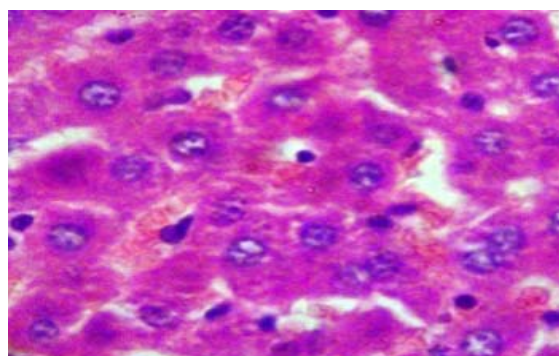
**Figure 1b:** Showed Group-II: Paracetamol intoxicated group animal showed total loss of hepatic architecture with centrilobular hepatic necrosis, damaged hepatic portal system, fatty changes and vacuolization.



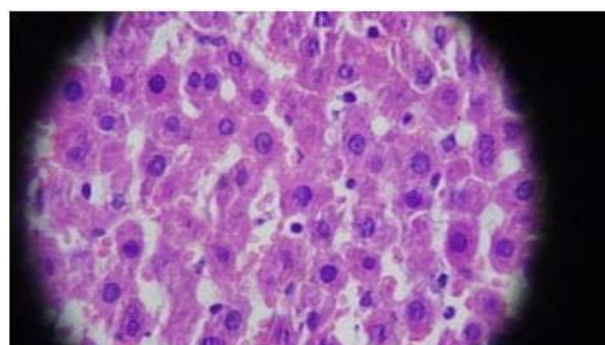
**Figure 1c:** Showed Group-III: Paracetamol intoxicated group animals treated with CE of *C.ternata*. This group showed very minimal inflammation in hepatocytes with moderate portal system and normal lobular architecture.

The inhibition of both CE and PE of *C.ternata* was found to be high in gram +ve than gram -ve bacteria, and the reason for different sensitivity between Gram +ve with -ve bacteria is probably interrelated with their cell wall structure<sup>35-36</sup> which may be due to the fact that the gram

negative bacteria with high content of cell wall lipopolysaccharides are inaccessible to some of the phytochemical bioactive principles.



**Figure 1d:** Showed Group-IV: Paracetamol intoxicated group animals treated with Petroleum ether extract of *C.ternata*. This group showed minimal inflammation with moderate portal system and their lobular architecture was nearly normal.



**Figure 1e:** Showed Group-V (Positive Control): Paracetamol intoxicated group animals treated with standard liver drug Silymarin. This group showed almost same with normal control properties having normal hepatic cells each with well defined cytoplasm, prominent nucleus, nucleolus and well brought out central vein.

The extent of hepatic damage induced by high doses of paracetamol was assessed by histological evaluation and the level of various biochemical markers. Treatment of animals with paracetamol resulted in a significant increase of liver enzymes like SGOT, SGPT and ALP were noticed due to damage of hepatocytes<sup>37</sup> and also raise in the levels of Serum bilirubin, leads to severe jaundice which is an implication of high liver damage as seen in Group-II. Treatment of paracetamol toxicated groups with CE and PE of *C.ternata* (Group-III and Group-IV) reduces all the elevated levels, which seems to offer the protection and maintain the functional integrity of hepatic cells with the healing of hepatic parenchyma and regeneration of hepatocytes<sup>38</sup> and also is an indication of stabilization of plasma membrane as well as repair of hepatic tissue damage.

As lipid peroxidation is a fixed mechanism of hepatotoxicity along with oxidative stress and other free radical damage occurred in the biochemical cascade, evaluation of antioxidant efficacy and lipid peroxidation

inhibition activity are regarded as extremely important parameter indicative of the possible mechanism of hepatoprotection. The thiobarbituric acid assay is the most popular method of estimation of malondialdehyde level, which is an indication of lipid peroxidation and free radical activity. Elevated MDA level and decreased levels of SOD, Catalase were observed in toxicated Group (group-II) indicated the lipid peroxidation which is also a hallmark sign of hepatic damage and necrosis, and also decreased total protein content may be due to the initial damage produced and localized in the ER results in the loss of P450 leading to its functional failure with a decrease in protein synthesis and accumulation of triglycerides leading to fatty liver<sup>39</sup>, further indicating the failure of antioxidant defense mechanism. On treatment of toxic groups with CE and PE of *C.ternata* (group-III and IV) showed an increase in the levels of SOD, Catalase and protein suggested that the repairment of antioxidant defense system, which plays an important role in hepatoprotection.

The significant reduction in the blood components is an indication that the oxygen carrying capacity of animal's blood would be reduced because of the fact that oral administration of high doses of paracetamol greatly affected hematological parameters due to anemia, leucopenia, hematotoxicity and thrombocytopenia. The decreased RBC count, total platelet count and Hb% leads to iron deficiency anemia, and also hyperactivity of bone marrow lead to production of damaged RBC integrity that are easily destroyed in the circulation which may be another reason for decreasing hematological values.<sup>40</sup>

## CONCLUSION

The potent antimicrobial, hepatoprotective and antioxidant properties and also the amelioration of haematological properties of PE and CE of *C.ternata* exhibited may be due to high and significant levels of bioactive phytochemical compounds as reported by our earlier studies. It provides experimental evidence that *C.ternata* improved the liver antioxidant enzymes level, preserved histoarchitecture and enhanced the performance of damaged liver following paracetamol administration. These protective activities of *C.ternata* might be due to the synergetic effect of phytochemical compounds present in them making them as good sources for the production of a stress inhibitory (antioxidant) and hepatoprotective herbal medicine. It was well known that, there was a close correlation between the antimicrobial, antioxidant and hepatoprotective properties and the amount of phytochemical compounds like polyphenols, flavonoids, tannins and alkaloids etc., present in the plant. Since polyphenols and their derivatives in plants and plant products play a vital role in anti-oxidization as well as in the various antitoxic biological functions, which may help in preventing the diseases. The identification of bioactive molecules with antitoxic properties may provide new

directions for identification of antioxidant and hepatoprotective drugs.

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