Evaluation of Methanolic Extract of *Clitoria ternatea* Hepatoprotective and Nephroprotective Activity in Rats

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ABSTRACT

Objective: This investigation was conducted to study the hepatoprotective and nephroprotective activities of methanolic extract of *Clitoria ternatea* in Cisplatin and CCl_4 induced in rats. **Methods**: The methanolic extract of the aerial part of *Clitoria ternatea* plant was investigated for its nephroprotective and hepatoprotective activities in animal experimental models. Nephrotoxicity was induced by 16 mg/kg BW of Cystone. The standard drug was Silymarin and the test drug was 0.5 and 1 g/kg BW of the methanolic extract of *Clitoria ternatea*. Hepatoxicity was induced by CCl_4. The standard drug was 100 mg/kg BW of cisplatin and the test drug was 0.5 and 1 g/kg BW of *Clitoria ternatea* extract. **Results:** In the hepatoprotective activity, the positive control group was provided by CCl_4 and increased levels of SGPT, SGOT, and ALP were observed compared to the negative control group, whereas Test (2) group was provided by 1000 mg/kg of the methanolic extract of *Clitoria ternatea*, that led to decreased levels of SGPT, SGOT, and ALP compared to the standard group. In the nephroprotective activity, the positive control group was provided by CCl_4 and increased levels of urea and creatinine were observed, whereas Test(2) group was provided by 1000 mg/kg of the methanolic extract of *Clitoria ternatea*, leading to decreased levels of urea and creatinine. **Conclusion:** On evaluating biochemical parameters it was found that 1000 mg/kg BW of the methanolic extract of *Clitoria ternatea* showed hepatoprotective and nephroprotective activities in rats.

Key Words: *SGPT, SGOT, ALP, Nephroprotective, Hepatoprotective.*

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INTRODUCTION

The liver is among the most complex and pivotal organs in the human body. It is a reddish-brown organ that lays under the diaphragm in the abdominal pelvic region of the abdomen. The liver has 4 lobes of the same shape and size. It is the largest gland and internal organ of the human body [1]. It is connected to 2 large blood vessels, portal vein and hepatic artery. It constituents about 2.5% of an adult's body weight. It produces bile that is an alkaline compound, helping to digest foods through the emulsification of lipids. Two major types of cells i.e. parenchyma and nonparenchyma cells populate the liver

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lobes. Sinusoidal endothelial cells, kuffer cells, and hepatic stellate cells are some of the nonparenchyma cells that line hepatic sinusoid. The liver is involved in regulating several physiological processes. It also plays a role in various crucial functions including storage, secretion, and metabolism. It has a great capacity to synthesize useful principle and detoxicate toxic substances. It helps in regulating, performance, and maintenance of the body's homeostasis. It plays role in almost every biochemical pathway including reproduction, energy provision, nutrient supply, fight against diseases, and growth. It aids the storage of vitamins; bile secretion; and the metabolism of protein, fat, and carbohydrate detoxification. Hepatotoxins are chemicals that lead to liver injury [2, 3]. Certain medicinal agents can damage the organ in case of overdose and even within the therapeutic ranges. Other chemical agents like those used in industries (like arsenic, Lead), laboratories (eg. CCl_4, Paracetamol), natural chemicals (like microcystins, aflatoxins) IFN, and herbal medicines (ephedra, *Cascara sagrada*) can also lead to hepatotoxicity. These chemical agents are converted to metabolites, which are chemically reactive in the liver that can interact with cellular macromolecules namely protein, lipids, and nucleic acids, leading to protein dysfunction, lipid peroxidation, DNA damage, and oxidative stress. This damage to cellular function can lead to cell death and possibly liver failure [4]. In this study, we induced hepatoxicity by using carbon tetrachloride (CCl4), which is a major inducer of hepatic damage that undergoes metabolic activation in a step dependent on cytochrome P-450 to produce free radicals [5]. In the modern treatment strategy, Silymarin is associated with nausea, vomiting, and headache, so, it has some limitations. Hence we found a good rationale beyond probing for the hepatoprotective and nephroprotective activity in our pipeline drug that is *Clitoria ternatea* [6].

MATERIALS AND METHOD:

Animal Husbandry and Statutory Approval

Healthy female and male rats (Wistar albino) aged 4-8 weeks were selected from Institutional Animal Ethics Committee of Gupta College of Technological Sciences, Asansol after behavioral and physical veterinary evaluations. The weight range was within ±20% of the mean body for each sex at the time of initiation of treatment. All animal experiments were approved by IAEC and the ethical conduct in animal handling. The animals were housed under acclimatization for at least five days before the initiation of dosing. Each 6 rats were kept in one standard polypropylene cage with stainless steel top grill. Paddy husk was autoclaved and used as bedding. The paddy husk was changed at least 3 times/week. The rats were kept in a clean place with 12L/12D light cycle. The air was conditioned at 22 ± 3 °C and the relative humidity was maintained between 55-65% with 100% exhaust. The standard pelleted diet was supplemented by ad libitum access during the study, except the overnight fasting before blood collection and was offered the feed immediately after completion of blood collection of all the animals. Moreover, rats were given ad libitum access to water in polypropylene bottles with a stainless steel slipper tube throughout the study period [7].

Collection of plant:

The leaves of *Clitoria ternatea* were collected from Rampurhat (W.B) India in August 2018 and were authenticated by the Head of Botany Department of Government College, Rampurhat, Birbhum, West Bengal.

Authentication:

A herbarium sheet was prepared and it was sent to the head of Botany Department of Government College, Rampurhat, Birbhum, West Bengal, for authentication of study the plant. Ref No: Rph/BOT/2018/42.

Extraction:

Extraction is the treatment of a plant or animal tissue with solvent, in order to the medicinally active ingredients (menstruum) be dissolved and most of the inert matter (marc) remain undissolved . The effective extraction of leaves depends largely on solubility and functional group consideration [8].

Soxhlet Extraction:

Soxhlet extraction (soxhlation) is used where a small amount of hot menstruum is passed over the drug repeatedly to dissolve the active ingredients until the drug is exhausted. The Soxhlet apparatus, required for the hot percolation is made of a very high grade of glass and consists of three parts (a) a flask in which the menstruum is boiled, (b) an extracting chamber in which drugs are filled and fitted with the a siphon and side tube, and (c) a condenser. The drugs to be extracted, in an appropriately comminuted form was unusually packed in a thimble made of filter paper, which was then placed into the wider part of the extractor. The thimble was used to prevent choking of the lower part of extraction by drug particles. Menstruum was put into the flask and boiled, the steam was allowed to pass through the side tube to the condenser where it was condensed and dropped onto the packed drug, through which it extracted out the active constituent. The liquid level in the siphoned out into flask as the menstruum volume increased in the extractor. With further heating, the menstruum vaporized and the dissolved active constituents remained behind in the flask. The extract discharged from the extractor was continuously refilled until the drug was exhausted. Thus, the same amount of menstruum was repeatedly made to Soxhlet through the drug about 14-15 times and the active ingredients were collected in the flask. This process is not

proper for drugs containing thermolabile active ingredients [9].

Successive Solvent Extraction:

After the selection, collection, and drying the leaves of *Clitoria ternate* Linn extraction was done. In pharmacy, menstruum is the solvent, which is used to extract a constituent and marc to the residue left after extracting the constituent. The effective extraction of plant materials depends largely on solubility and functional group consideration. The powder rhizomes were subjected to cold maceration and successive Soxhlet extraction using various solvents of increasing polarity namely petroleum ether, chloroform, acetone, and methanol. Each time before extracting, the next solvent powder was dried in an oven under 50 ºC. The solvent was distilled off to concentrate the extract and then the extract was put in a water bath to dry [10].

Extractive Value:

The amount of active ingredients in a specific amount of medicinal plant material is determined by the method used. It is used for plant materials with no biological or chemical assay method. The extraction of each crude medicine with a specific solvent yields a solution with various phytocompounds. The composition of these phytocompounds depends on the solvent used and drug nature. Using a single solvent can be a means of providing basic information about the quality of a specific drug [11].

Table 1: Extractive Values of leaves of *Clitoria ternatea* in different solvents

Solvent Used	Extractive value (%w/w)
Distilled water	12.46
Petroleum ether (60-80)	2.46
Chloroform	6.88
Ethyl acetate	9.84
Methanol	14.42

Preliminary Phytochemical Screening:

Preliminary tests were performed for the absence or presence of phytoconstituents like sterols, alkaloids, saponins, carbohydrates, terpenes, flavonoids, tannins, and glycosides in all the extracts by using the above four solvents individually. The description of methods adopted for performing the tests is summarized below.

Test for Alkaloids:

A part of the extract acidified using dilute sulfuric acid. This portion was divided into 2 parts and tested with the following precipitating reagents:

Mayer's Reagent:

1.36 g $HgCl_2$ dissolved in 60 ml water was added to a solution of 5g KI in 20 ml distilled water. They were mixed properly and the volume reached 100 ml with distilled water. The buff-colored precipitate was considered to be a positive test [12].

Dragendorff Reagent:

1 g of bismuth subnitrate in 20 ml of acetic acid was added to 20 g KI dissolved in 100 ml water. An Orange or brown-colored precipitate was considered a positive test [13].

Test for Carbohydrates:

Molisch's Test:

It was performed for the confirmation of carbohydrates. 1 ml of 10% α -naphthol was added to the extracts and mixed. Then 1 ml concentrated sulfuric acid was carefully poured along the sides of the test tubes. The appearance of a violet ring at the juncture of the two layers was considered the positive test [14].

Test for Glycosides

Kedde's Test (For Aglycone):

The extract was evaporated to dryness and 2 drops of 2% 3,5 dinitrobenzoic acid in 90 % alcohol and 1 drop of 90% alcohol were added and the above mixtures were made alkaline with 20 % Sodium hydroxide to get the purple color. The appearance of the purple color showed the presence of free aglycone moiety [15].

Keller –Killani Test (For Sugar):

0.4 ml of glacial acetic acid that contained a trace of ferric chloride was added to the dried extract. Then, 0.5 ml of concentrated H_2SO_4 was added to the mixture. The appearance of a green-blue color in the upper acetic acid layer indicated the presence of sugar moiety [16].

Test for Flavonoids:

1. Magnesium (dust) and concentrated HCl were used to treat the extract. The appearance of pink tomato color was indication of flavonoids.

Shinoda's Test:

2. 5-10 drops of dilute HCl were added to 0.5 ml of the extract. A trace magnesium was added to it. The appearance of pink, reddish-pink or brown colors was considered a positive test. The appearance of yellow, orange-red or brick color precipitate with lead acetate demonstrates the presence of flavonoids [17].
3. 10 ml of the extract was hydrolyzed with dilute sulfuric acid. This was extracted with ether and divided into 2 portions. 1 ml of dilute ammonia solution was added to one portion, a greenish-yellow color demonstrated the flavonoids. To the other portion, 1ml of dilute sodium bicarbonate solution was added. A pale-yellow color confirmed the presence of flavonoids.

Test for Sterols and Terpene:

Benedict's Test:

Few drops of the extract were added to 5 ml of Benedict's reagent and boiled in water-bath for 5min. The appearance of green, orange-red or yellow precipitate confirmed the presence of reducing sugar [18].

Fehling's Test for Reducing Sugars:

2 ml of extract was added to the equal volume of Fehling A and Fehling B mixtures and boiled for 5min in a water bath. The appearance of red precipitate proved was indication of reducing sugar.

Test for Saponins:

Foam Test:

A little amount of the dried extract was boiled with water and allowed to cool. Then, it was vigorously shaken for one minute. The formation of persistent honeycomb-like forth was taken as a positive result for saponins [19].

Test for Tannins:

5% ferric chloride solution was added to a small portion of the extract. The appearance of green to blue color is a positive test for tannins.

A creamy precipitate with lead acetate was considered a positive test for tannins.

Table 2: The result of Preliminary Phytochemical Screening of the Extracts for Various Phytoconstituents

Extracts	Alkaloid	Carbohydrates	Flavonoid	Glycosides	Red sugar	Saponins	Sterols	Terpenes	Tannins
Petroleum ether	--	--	--	++	--	--	++	++	--
Choloroform	+	--	--	+	++	-	+	--	+
Ethyl acetate	--	+	+++	+	--	+	--	--	--
Methanol	+++	+	+++	++	++	+	+	+++	--
Water	+++	+++	+++	++	++	--	--	--	++

+++ Prominently present, ++ Moderately present, +Slightly present, -- Absent

Acute Toxicity and Gross Behavioral Studies:

Acute toxicity studies were conducted for methanolic extract using Acute Toxic Method as described in OECD (Organization of Economic Co-operation and Development) Guide Line No: 423. Animals were given increasing doses of 30, 100, 300, 600, and 1000 mg/kg p.o of the methanolic extract suspended in 2 % tween-80 solution. Then, they were continuously observed for 2h for gross behavioral changes and intermittently every 2h and finally at the end of 24 and 72h to record any toxic sign [20].

Experimental Design:

Group I: Negative control (Normal Saline)

Group II: Positive control (CCl_4 1ml/kg/day) 9 days

Group III: Standard (Silymarin 100 mg/kg/day + CCl_4 1ml/kg/day) 9 days

Group IV: Test(1) (Methanolic extract of *Clitoria ternatea* 500 mg/kg/day + CCl_4 1ml/kg/day) 9 days

Group V: Test(2) (Methanolic extract of *Clitoria ternatea* 1000 mg/kg/day + CCl_4 1ml/kg/day) 9 days

Assessment of Liver Function:

The hepatoprotective effect of the extract was evaluated by the assay of liver function biochemical parameters namely ALP, SGPT, SGOT, and total SB according to the standard methods.

Histopathological Studies:

Formalin-fixed tissues were subjected to graded dehydration in ascending strength of alcohol 70%, 80%, 90%, and 100%, and a subsequent wash with xylene. Then, the tissues were embedded in liquid paraffin to facilitate the preparation of histopathological blocks, which are essential for imparting strength to the tissue, so that it can withstand the abrasive force of the blade while being sectioned. The 5 µm sections, obtained by trimming with manual rotary microtome were subjected to staining by Harris' hematoxylin and counterstained by Eosin. The sections were viewed under a trinocular microscope of different magnifications, which were photographed by Motif software inbuilt in the systems [21].

Statistical Analysis:

The results have been expressed as mean±SEM. One-way ANOVA has been employed for comparing the majority of parameters. Post hoc analyses were used for the identification of groups with significant differences for one-way ANOVA. Turkey's Multiple Range Test was used for comparisons. Whereas for two-way ANOVA, Bonferroni's test was used for the post hoc analysis. The significant groups were identified in the figure by designated alphabets [22].

Nephroprotective Activity:

Nephrotoxicity is a renal dysfunction or disease that arises as an indirect or direct result of exposure to drugs, and environmental or industrial chemicals. Drug nephrotoxicity is, therefore, any renal dysfunction attributed to drugs. Drug nephropathies are not limited to just one type of renal injury. Medicines target at least one discrete anatomic region of the kidney and may affect only one cell type, resulting in a range of nephropathies that cannot be distinguished from those with no chemical causes. Nephron is the structural and functional unit of the kidney with a continuous tube of specialized heterogeneous cells that exhibit sub-specialization between the nephrons or along their length. It is the main organ of homeostasis and excretion of water-soluble molecules since it is a metabolically active organ, it can actively concentrate on certain substances. Moreover, its

cells can metabolically activate various compounds and bioconvert chemicals. The kidney is usually exposed to high concentrations of medicines and their metabolites, because it excretes various types of them [23]. In addition, this organ has various mechanisms for the accumulation of nephrotoxins. It is highly vascular, that receives about 1/4 of the resting cardiac output. A large area is provided by the proximal renal tubule in order to bind to nephrotoxin and transport it into the renal epithelium. The glomerular filtrate reabsorption gradually increases the concentration of intraluminal nephrotoxin, while in the kidney, specific transport pathways may cause site-specific toxicity. Free radicals are highly-reactive substances that are produced in the body as a result of metabolic processes [24]. Many of these molecular species are oxygen (and sometimes nitrogen) centered free radical and its nonradical products. The term reactive oxygen species (ROS) denotes oxygen centered radicals (superoxide and hydroxyl radicals) collectively and also nonradical species derived from oxygen including singlet oxygen (O) and hydrogen 1 peroxide. The increased ROS production appears to be associated with most forms of tissue damage. Free radical can also react with DNA, proteins or lipids in the cell membrane and cause damage. ROS is involved in various chronic diseases and also aging. The defense provided by the antioxidant system is important for the survival of organisms. Detoxification of ROS in the cell is provided by both enzymatic and nonenzymatic systems, which constituent the antioxidant defense system. Many plants have antioxidant compounds, which can protect cells against the damage of reactive oxygen species like peroxynitrite, hydroxyl radicals, peroxyl radicals, superoxide, and singlet oxygen. These compounds or antioxidant that scavenge free radicals have crucial roles in improving the conditions of disease [25, 26].

Experimental Design:

Group(1): Negative control (Normal Saline)

Group(2): Positive control Cisplatin (16mg/kg BW single dose i.p)

Group(3): Standard Cystone (5ml /kg) + Cisplatin (16 mg/kg BW single dose i.p)

Group (4): Test(1) Methanolic extract of *Clitoria ternate* (500 mg/kg/day, p.o) + Cisplatin (16 mg/kg BW single dose i.p)

Group (5): Test(2) Methanolic extract of *Clitoria ternatea* (1000 mg/kg /day, p.o) + Cisplatin (16 mg/kg BW single dose i.p)

Assessment of Renal Function:

The nephroprotective effect of the extract was evaluated by the assay of kidney function biochemical parameters such as kidney weight as % of the total body weight, serum creatinine level, blood urea level, and histopathological evaluation of the kidney [27].

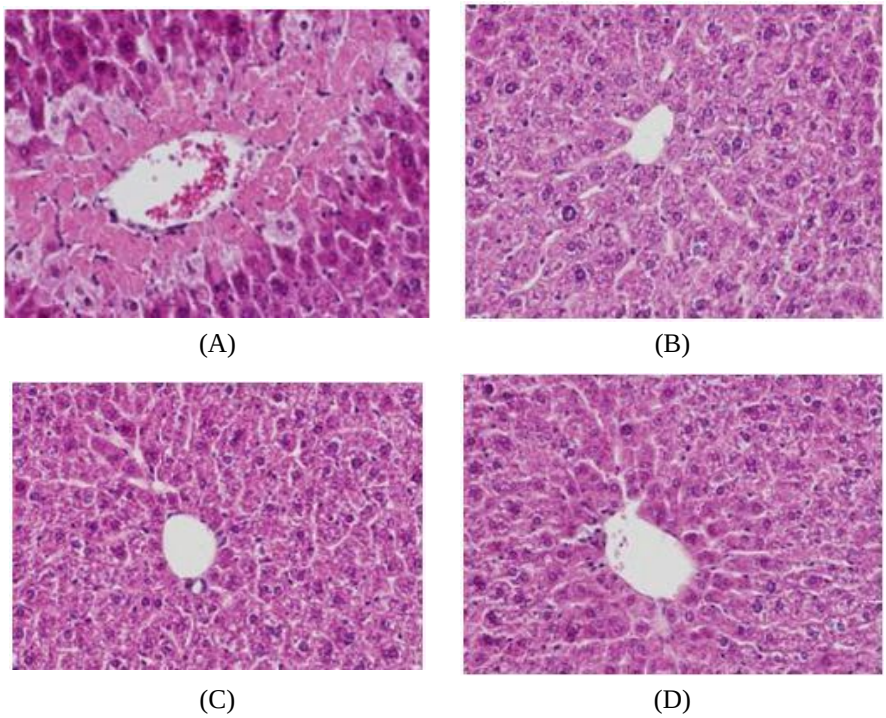
Statistical Analysis:

The results are as mean±SEM. One-way ANOVA was employed for comparing the majority of parameters. Post hoc tests were dine for the identification of groups with significant differences for One-way ANOVA. Turkey's Multiple Range Test was used for comparisons. Whereas for Two-way ANOVA Bonferroni's test was used for the post hoc analysis. The significant groups were identified in the figure by designated alphabets [28-30].

Table 3: Serum Biochemical Analysis Result of Hepatoprotective Activity

Group	Treatment region	SGPT(IU/L)	SGOT(IU/L)	ALP(IU/L)	Serum bilirubin(IU/L)
1	Normal saline	62.58±4.12	155.45±6.32	264.35±8.22	0.42±0.04
2	CCl_4	240.58±13.54^{a}	490.68±15.58^{a}	534.82±19.43^{a}	1.42±0.32^{a}
3	Silymarin+ CCl_4	73.42±5.45***	172.43±6.75***	283.76±10.41***	0.45±0.04***
4	MCT 500 mg/kg+ CCl_4	115.45±7.56***	226.43±8.68***	324.96±12.47***	0.62±0.05***
5	MCT 1000 mg/kg+ CCl_4	92.65±7.42***	205.54±8.62***	301.82±10.32***	0.52±0.05***

The values are as mean±SEM (n=6) and analyzed by using ANOVA followed by Bonferroni's multiple comparison test. The percentage change of protective effect compared with the CCl_4 treated control is expressed within brackets. P: ^{a}< 0.001 vs. vehicle control,^{ns}> 0.05, <0.05, <0.01, <0.001 vs CCl_4 treated control.



(A) (B)

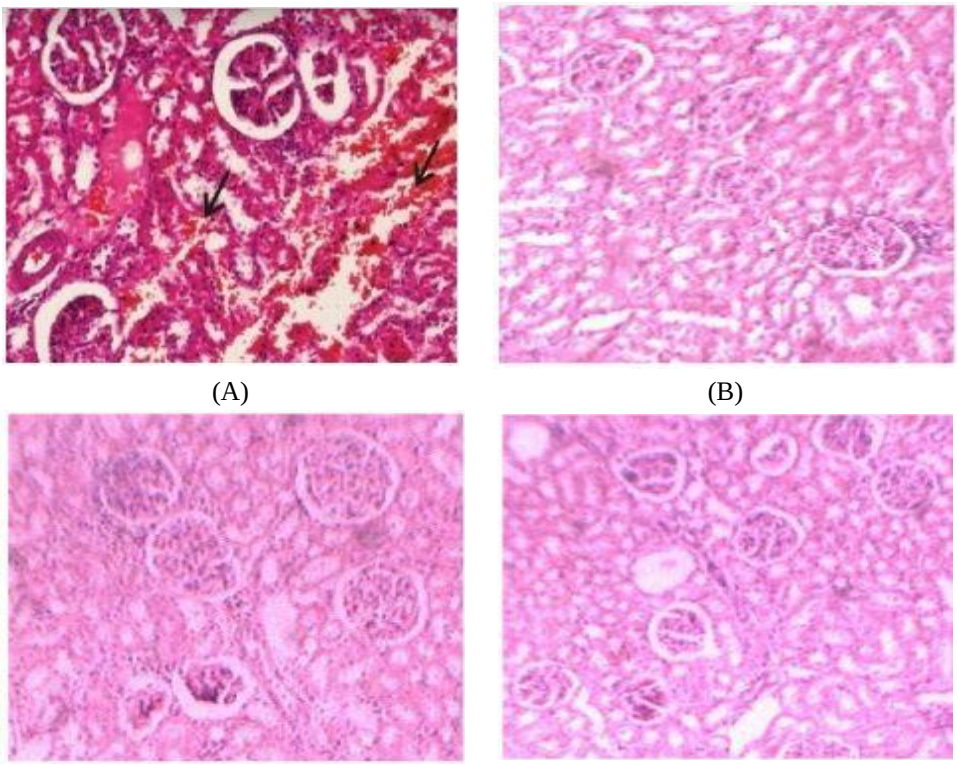
(C) (D)

Figure 1: Histopathological evaluation of Hepatoprotective Activity
(A) Group1: Negative control (Normal saline)
(B) Group 2: Positive control (CCl_4)
(C) Group 3: Standard (Silymarin + CCl_4)
(D) Group 4: Test(1) (Methanolic extract *Clitoria ternatea* 500 mg/kg + CCl_4)
(E) Group 5: Test(2) (Methanolic extract of *Clitoria ternatea* 1000 mg/kg + CCl_4)

Table 4: Serum biochemical analysis result for Nephroprotective activity

Group	Treatment Regimen	Urea(mg/dL)	Creatinine(mg/dL)
1	Normal Saline	38.76±3.76	1.06±0.40
2	Cisplatin	75.71±7.50^{a}	2.51±0.88^{a}
3	Cystone 5ml/kg + Cisplatin 16 mg/kg BW	52.57± 4.56***	1.36±0.62*
4	*Clitoria ternatea* 500mg/kg +Cisplatin 16mg/kg i.p	68.74 ± 5.46***	1.82±0.83^{ns}
5	*Clitoria ternatea* 1000 mg/kg/day + Cisplatin16mg/kg BW.single dose i.p	57.43±7.34***	1.55±0.42*

Values are as mean±SEM (n = 6) and analyzed by using ANOVA and Bonferroni's multiple comparison test. Percentage change of protective effect compared with the Cisplatin treated control is expressed within brackets. p: ^{a} < 0.001 vs vehicle control, ^{NS} > 0.05, *< 0.05, ** <0.01,***< 0.001 vs. Cisplatin treated control.



(A) (B)

(C) (D)

Figure 2: Histopathological evaluation of Nephroprotective Activity
(A) Group1: Negative control (Normal saline)
(B) Group2: Positive control (Cisplatin 5 ml)
(C) Group3: Standard (Cystone5 ml + Cisplatin 16 mg/kg)
(D) Group4: Test(1) (Methanolic extract of *Clitoria ternatea* 500 mg/kg+ Cisplatin16 mg/kg)
(E) Group 5: Test(2) (Methanolic extract of *Clitoria ternatea* 1000 mg/kg+ Cisplatin16 mg/kg)

DISCUSSION:

In the present study, it was found that the methanolic extract of *Clitoria ternatea* can modulate the nephrotoxicity induced by CCl_4. The hepatotoxicity of CCl_4 is due to the formation of the highly reactive trichloro (CCl_3) free radical, which alters the function of endoplasmic reticulum and causes peroxidative degradation of the lipid membrane of adipose tissue leading to the loss of the metabolic enzymes located within the cell structures. Furthermore, several phytoconstituents can induce microsomal enzymes either by inhibiting the CCl_4-induced peroxidation of lipids or by accelerating the CCl_4 excretion. Phytoconstituents namely alkaloids, saponins, triterpenoids, and flavonoids have hepatoprotective activities.

In this study, liver damage induced by CCl_4 was characterized by an increased level of SGPT, SGOT, ALT, and SB. Pre-treatment of animals with silymarin 100 mg/kg p.o. could reduce the level of SGPT, SGOT, ALP, and SB. Similar results were obtained by pre-treatment of the animal with methanolic extract of *Clitoria ternatea* (500 mg/kg and 1000 mg/kg p.o) in comparison to the group treated with CCl_4 . However, the efficacy of the extract at the dose levels tested was less than the standard hepatoprotective drug used in the study, silymarin.

It has been observed that silymarin, with very low toxicity and a suitable safety profile, has a laxative effect at high doses due to increased bile flow and bile secretion that is an adverse effect in patients in a clinical trial. Serious and rare adverse effects include gastroenteritis associated with allergy and collapse. Thus, combination therapy may partially reduce these adverse effects of silymarin.

It is evident from the studies carried out by earlier researchers that the tuber of the plant contains higher flavonoid and phenolic contents and scavenging activities. The qualitative phytochemicals investigations carried out on the methanolic extract of *Clitoria ternatea* has significant hepatoprotective activity, which is probably due to the high content of flavonoid.

In the present study, it was found that the tuber extract of *Clitoria ternatea* can modulate the nephrotoxicity induced by cisplatin. Cisplatin is a potent drug used to manage different types of cancer. However, the severe toxic side effects are the major limitation in its usage although the mechanism of cisplatin-induced nephrotoxicity is not exactly known. Many investigations have shown that lipid peroxidation and free radicals induce nephrotoxicity. Besides, various antioxidants and free radical scavengers

have been suggested to have a protective effect against nephrotoxicity and acute renal failure induced by cisplatin. In this study, the cisplatin-induced kidney damage was characterized by a significant increase in serum creatinine ($p < 0.01$) and urea ($p < 0.001$) in comparison to the control group. Pre-treatment of animals with cystone (5 ml/kg p.o) for 6 consecutive days and a single dose of cisplatin (16 mg/kg i.p) prevented the elevation of serum creatinine as well as urea as compared with cisplatin-induced nephrotoxicity group. However, pre-treatment of animals with methanolic extract of *Clitoria ternatea* (500 mg/kg) for 6 consecutive days and a single dose of cisplatin caused no significant reduction of serum creatinine as compared with the cisplatin-induced nephrotoxicity group. The histopathological evaluation of the kidney preparations in the treatment group also revealed a decreased level of induced tubular congestion, tubular cast, blood vessel congestion, epithelial desquamation, glomerular congestion, and inflammatory cells.

CONCLUSION:

1000 mg/kg of the methanolic extract of *Clitoria ternatea* leaves was found to possess hepatoprotective and nephroprotective activity. The activity was less compared to the standard drugs used in the study. The studies were done using the crude lyophilized extract.

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