Potential Protective Effects of Ethanolic Extract of Butterfly Pea *(Clitoria ternatea* Linn) Flower Against Carbon Tetrachloride-Induced Hepatotoxicity in Rats

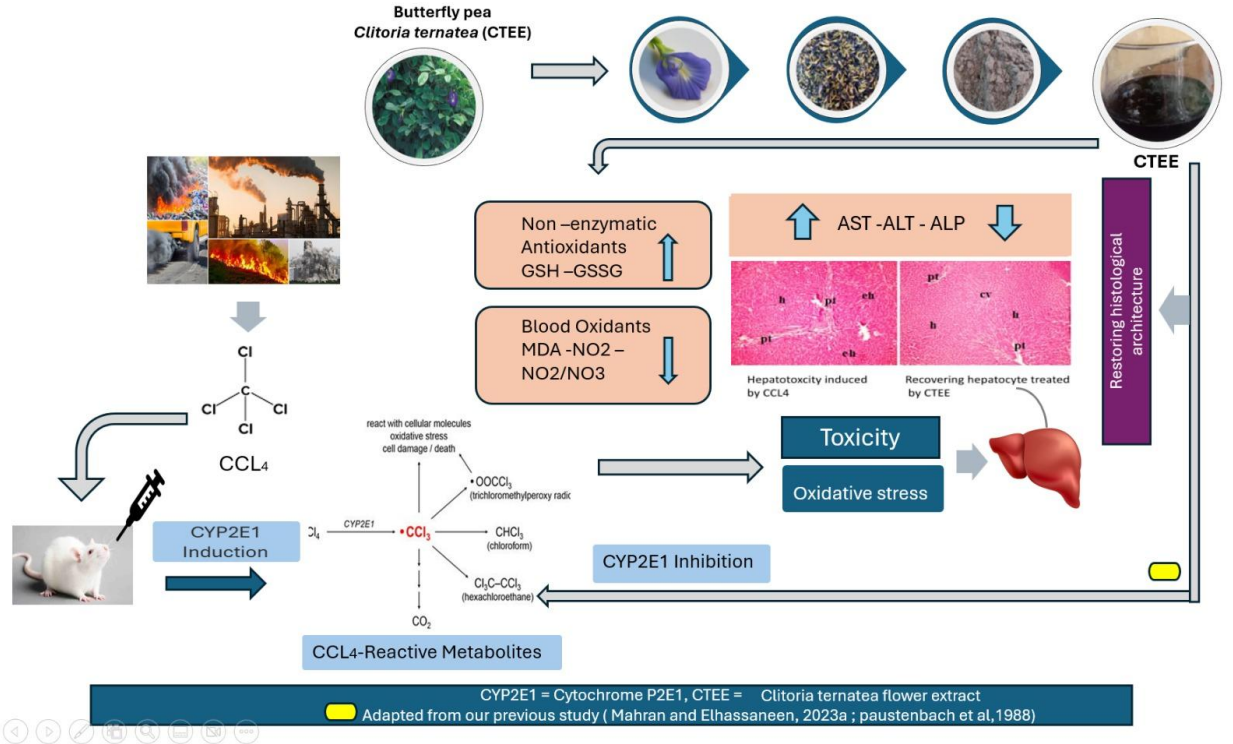
Neveen A. Elwardany [1] , Mariam A. Abdelkader[1]

ABSTRACT

The current study was carried out to explore the hepatoprotective properties of ethanol-derived *Clitoria ternatea* flower extract (CTEE) on liver injury induced by carbon tetrachloride in rats. It was observed that rats injected with carbon tetrachloride (CCl_4) exhibited significant ($p \leq 0.05$) reductions in various biological parameters like BWA, FI, and FER, with percentages of -42.86%, -36.99%, and -30.14%, respectively, compared to rats in the control group. Moreover, in comparison to the rats in the normal group, biochemical parameters like total bilirubin (TB), AST, ALT, ALP, and 129.90 liver enzyme activities showed significant ($p \leq 0.05$) elevations at 77.28, 129.90, 158.72, and 412.84%, respectively. Along with a decrease in the level of non-enzymatic antioxidants (GSH and GSSG) and an increase in the level of oxidants (MDA and NO_2), this was also correlated with an imbalance in certain antioxidant parameters and oxidants in the blood. Each of these parameters suggested that CCl_4 was the cause of the liver injury. Rat protocol feeding with CTEE intervention produced a significant ($p \leq 0.05$) improvements in all previous biological, biochemical, and oxidant/antioxidant status markers, indicating protection against hepatotoxicity. Furthermore, a dose-dependent pattern was seen in the rate of improvement for each of these parameters. In conclusion, CTEE demonstrated efficacy in preventing liver disorders caused by CCl_4. It is therefore advised that we include such plant parts (flowers of *Clitoria ternatea*) in our daily diets, beverages, and pharmaceutical formulae at concentrations of up to 400 mg/kg bw/day.

Keywords: ***Clitoria ternatea*, body weight, liver functions, glutathione fractions, malonaldehyde, nitrite.**

Graphical abstract



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[1]Home Economics Dept, Fac, of Specific Education, Alexandria Univ, Egypt.

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INTRODUCTION

Originally from equatorial Asia, which includes parts of South and Southeast Asia, *Clitoria ternatea* (family: *Fabaceae*) is also known as butterfly pea. It has been introduced to Africa, Australia, and the Americas. With elliptic, obtuse leaves, it is a perennial herbaceous plant (Patel, 2023). The flower of this plant is its most valuable component because it is used in ayurvedic medicine and as a natural food coloring for desserts and glutinous rice (Vuong and Parichat, 2021). Typically, dried lemongrass and other herbs are added to alternative flowers to make butterfly pea flower tea. When lemon juice turns purple, the mixture's color varies based on what is added to the liquid (Pantazi, 2016). For instance, to boost the acidity and give the beverage a purplish-pink hue, blue butterfly pea flower tea is typically combined with honey and lemon in Thailand and Vietnam. This results in a beverage that is typically served hot or cold after supper.

This plant has been linked to a number of different effects in traditional medicine, including sedative, antidepressant, anticonvulsant, anxiolytic, memory-enhancing, nootropic, and anti-stress properties. in conventional Chinese medicine (Chakraborthy *et al.*,2018). Additionally, studies on plant extracts have demonstrated a strong ability to lessen the severity of behavior induced by acetylcholine and serotonin (Neeti *et al.*,2003). Additionally, numerous biological and pharmacological activities were observed in its extracts, such as wound healing activity, inhibition of platelet aggregation, antimicrobial, antipyretic, anti-inflammatory, analgesic, diuretic, local anesthetic, anti-diabetic, and its use as vascular smooth muscle relaxing properties (Solanki & Jain, 2012; Nithianantham *et al.*,2013 and Sreeharini *et al.*,2013). Consequently, the herb has been used for a very long time in conventional therapies to treat a wide range of illnesses, such as kidney, blood, and diabetes (Pulok *et al.*,2008). Numerous prior investigations have ascribed the majority of *C. ternatea's* biological effects to a variety of bioactive substances, including flavanol glycosides, anthocyanins, steroids, triterpenoids, and nucleotides, which are cyclic peptides (Mukherjee *et al.*,2008). Moreover, previous reports (Gupta *et al.*,2010 and Pendbhaje *et al.*,2011) described the isolation and identification of phenolics, flavonoids, and different kaempferols from *C. ternatea* flowers. These substances are said to be in charge of their scavenging and antioxidant properties (Kuppan *et al.*,2013). The presence of multiple anthocyanins, the most significant of which are ternatins, polyacylated derivatives of delphinidin 3,3', 5'-triglucoside, accounts for the blue color of this flowering plant as well (Vidana Gamage *et al.*,2021).

The synthesis of the factors that required to prevent blood bleeding, the production of bile to aid in the absorption of nutrient, the processing of numerous products released into the bloodstream (such as glucose, plasma proteins, and urea), and the storage of various products (such as glycogen, fat, vitamins, and minerals) are just a few of the critical roles played by the vital organ, the liver (Kebamo *et al.*,2015). Furthermore, it is essential for the removal, metabolism, and biotransformation of foreign substances as well as for preserving the biological equilibrium of the human body (Grażyna *et al.*,2020 and Yu *et al.*,2020). Accordingly, harm to the liver can result in a variety of conditions, from cirrhosis and potentially fatal liver failure to a brief increase in liver enzymes (Bera *et al.*,2012). Numerous substances that are frequently used can harm liver cells and metabolism (Meyer and Kulkarni, 2001). Carbon tetrachloride (CCl_4) is the most widely used chemical. Based on studies indicated evidence of carcinogenicity in experimental animals, it is expected to be a human carcinogen. It also has hepatotoxic effects by triggering steatosis and centrilobular necrosis (WHO, 1999). According to reports, the cytochrome P-450 system biotransforms CCl_4 to create trichloroethylene free radicals, which is the mechanism of CCl_4-induced hepatic injury. The trichloroethylene peroxy radicals that are created when these free radicals react with oxygen act on the endoplasmic reticulum's lipid membrane to cause lipid peroxidation (Weber *et al.*,2003 and Wang *et al.*,2024). All of those earlier investigations, in addition to others, suggested that CCI_4 is one of the most effective models of liver injury. Liver disease continues to be a global health concern even with the amazing advancements in research. As a result, the ongoing and significant task of finding novel pharmaceuticals was undertaken (Nithianantham *et al.*,2011). To support these claims, numerous scientific studies and research are required. In this context, plant and natural products have been used in traditional treatments throughout the world to prevent or treat liver diseases. These assertions require a great deal of scientific investigation (Lahon et al.,2011). As far as we know, though, there hasn't been any evidence of the hepatoprotective potential of *C. ternatea* flowers against liver damage caused by carbon tetrachloride in rats. Thus, the goal of the current investigation was to determine whether ethanolic *C. ternatea* flower extract could shield rats' livers from carbon tetrachloride-induced damage.

Fig.1 Whole plant of Clitoria ternatea

Fig. 2 *C. ternatea* L. The Morphoforms: A, Single form

Botanical description of a plant

Clitoria ternatea displays twining fine stems that can reach a length of 0.5-3 m. The leaves exhibit a pinnate arrangement, consisting of 5-7 elliptic to lanceolate leaflets measuring 3-5 cm in length and featuring a short pubescence underneath (Fig. 1). The flowers are solitary, showcasing hues of deep blue to blue mauve, with very short pedicels and a length of 4-5 cm (Fig. 2). Its pods are beaked flat, linear, 5-10 cm long, 0.6-1.4 mm wide, and mildly pubescent, containing up to 10 seeds. These seeds are olive and brown in color, often displaying a mottled pattern, measuring 4.0-8 mm in length and 3.2-4.5 mm in width (Mehmood *et al.*,2019; Sarma *et al.*,2023 and Arya *et al.*,2022).

MATERIALS AND METHODS

Materials

The dried flowers of *Clitoria ternatea* Linn, commonly known as butterfly pea, were procured from Bab ElKhalk, Cairo, Egypt's El Misryia Company for Trading Herbs and Medical Plants. Taxonomic verification of Clitoria ternatea was conducted by the University of Alexandria's Faculty of Agriculture's Agricultural Plant Department, which is situated in Alexandria, Egypt. Casein was sourced from Morgan Chemical Co. in Cairo, Egypt. Carbon tetrachloride (CCl_4), in the form of a 10% liquid solution, along with all necessary chemicals, buffers, and solvents, were supplied by ElGhohorya Company for Trading Drugs, Chemicals, and Medical Instruments in Cairo, Egypt. The assays included in the kit for assessing Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and malondialdehyde (MDA) were acquired from (BIODIAGNOSTIC in Dokki). Additionally, albumin (Alb) assays were obtained from (El-Nasr Pharmaceutical Chemicals Company), while (GSH, and GSSG) assays were procured from (Nawah Scientific in Almokattam, Cairo, Egypt).

Methods

Preparation of *Clitoria ternatea* flowers ethanol extract (CTEE)

Parimaladevi *et al.*,(2003) and Elhassaneen *et al*,. (2023) instructions were followed in the preparation of the *Clitoria ternatea* flowers ethanol extract (CTEE). To put it briefly, samples of dried flowers from *Clitoria ternatea* were brought into the lab. After being manually removed, the flowers were sieved to remove any foreign objects. The flowers were ground into a powder (20 mesh) and subsequently combined following grinding at a high speed using a miller (Moulinex Egypt, Al-Araby Co., Egypt) to produce uniform samples. A quantity of twenty grams of dried Clitoria ternatea powder was subjected to extraction with 80% ethanol for a duration of 5–6 hours (20 ± 3 minutes per cycle) utilizing a Soxhlet apparatus. The final step involved the evaporation of the solvent under reduced pressure in order to extract the dry solvent, a process carried out using a (rotary evaporator) (**Buchi R-210, Switzerland**). Subsequently, the extract was stored at a temperature of 4 ºC until required. The total yield of *Clitoria ternatea* ethanol extract (CTEE) was determined to be 5.61% (w/w) in terms of yield.

Proximate analysis of *Clitoria ternatea* flower extract (CTEE)

The method described by Official Agricultural Chemists (AOAC) International (1990) was used to determine Proximate Analysis. Moisture, ash, fat, protein concentration, fiber, and carbohydrates are also included in the six analyses.

Biological (in-vivo) experiments

Ethical approval

Biological experiments conducted for this research were granted ethical approval by (The Institutional Animal Care and Use Committee (IACUC) at Alexandria University, Egypt). Approval ID/number for this study is ALEXU/.011/ 2023.

Animals

The study utilized animals, specifically adult male albino rats (180±6.4 g each), procured from (The Research Institute of Ophthalmology, Department of Medical Analysis, located in Giza, Egypt). The formulation of the Basal Diet (BD) includes (10%) Protein, (10%) corn oil, (1%) vitamin mixture, (4%)mineral mixture, (0.2%) choline chloride, (0.3%) methionine, (5%) cellulose, and corn starch (69.5%), as outlined by AIN (1993). The same prior reference was used to formulate the components of vitamin and salt mixtures.

Induction of hepatotoxic rats

Using the technique developed by Khan *et al.,* ,(2012), Forty-two (Albino rats, male) were administered intraperitoneal (IP) injections of tetrachlorocarbon (CCl_4) diluted in olive oil at a concentration of (50% V/V)-(2 ml/kg bw) twice weekly for a duration of two-week to induce chronic liver injury. A biochemical analysis of three rats chosen at random from the experimental group of animals and confirmed with histological study under light microscopy illustrated the presence of liver damage.

Experimental design

The Commission on Life Sciences, the National Research Council, and the Institute of Laboratory Animal Resources all issued guidelines that were adhered to in biological experiments (NRC, 1996). A total of 42 rats were housed individually in wire cages under standard conditions with a 12-hour light-dark cycle, temperature maintained at 25 ± 2ºC, and relative humidity at 52 ±3%. Before commencing the study, all rats were fed BD for a two-weeks acclimatization period. Subsequently, the rats were randomly allocated into two primary groups. The initial group of six rats served as the negative control (normal) and received BD as food along with a paraffin oil injection (5 ml/kg bw) as a vehicle for CCl_4 treatment. The second main group comprised (36) rats injected with CCl_4 to induce hepatotoxicity; these rats were further segregated into sex-matched subgroups (Groups 3–7) and administered CTEE orally at doses of (25, 50, 100, 200, and 400) mg/kg bw/day. A positive control group (model) receiving BD was assigned as group 2. Each group was accommodated in a single cage for 28 days, as depicted in Table (1). The selection of CTEE concentrations was guided by acute toxicity studies conducted by Sreeharini *et al,*. (2013), which demonstrated the safety of CTEE up to 5000 mg/kg bw via oral administration. Rat weights were recorded at the beginning of the experiment, weekly thereafter, and upon the conclusion of the study period.

Table 1. Experimental design to assess the potential protective effects of CTEE against CCl_4 induced hepatotoxicity in rats

Groups		Interventions
Group I Negative control (-Ve)	Normal control	BD for 28-day
Group II Positive control (+Ve)	CCl_4 control	BD +5 ml/kg bw paraffin oil injection for 28-day
Group III	CCl_4+ CTEE 25 mg/kg BW/day)	2 mg/kg/BW of CCl_4 in olive oil at a 50% V/V for 28-day+ 25 mg/kg BW/day of CTEE
Group IV	CCl_4+ CTEE 50 mg/kg BW/day)	2 mg/kg/BW of CCl_4 in olive oil at a 50% V/V for 28-day+ 50 mg/kg BW/day of CTEE
Group V	CCl_4+ CTEE 100 mg/kg BW/day)	2 mg/kg/BW of CCl_4 in olive oil at a 50% V/V for 28-day+ 100 mg/kg BW/day of CTEE
Group VI	CCl_4+ CTEE 200 mg/kg BW/day)	2 mg/kg/BW of CCl_4 in olive oil at a 50% V/V for 28-day+ 200mg/kg BW/day of CTEE
Group VII	CCl_4+ CTEE 400 mg/kg BW/day)	2 mg/kg/BW of CCl_4 in olive oil at a 50% V/V for 28-day+ 400 mg/kg BW/day of CTEE

* CCl_4 : carbon tetrachloride; CTEE: *Clitoria ternatea* flower extract

Biological evaluation

Every day of the 28-day experiment was monitored for diet consumption, and every week, body weight was recorded. In accordance with Chapman *et al.,* (1959), calculations were made for weight body gain (BWG%), intake food (FI), and efficiency food ratio (FER) using specific equations: BWG (%) = (Final weight - initial weight) / initial weight × 100, and FER (grams) = feed intake (grams/28 days) / grams of body weight gain.

Sampling collection

Rats underwent a 12-hour fasting period before blood samples were collected at the conclusion of the 28-day experimental period. Subsequently, the rats were euthanized under ether anesthesia and dissected.

Blood sampling

Following established procedures (Parasuraman *et al.*,2010 and Drury & Wallington, 1980), blood samples collected from the abdominal aorta were transferred to sterile, dry centrifuge tubes. The samples were kept at room temperature to clot (25°C) before centrifugation for 10 minutes at 3500 rpm to separate the serum. After centrifugation, the serum was carefully aspirated and transferred to sterile capped tubes for storage at -80°C until further analysis.

Liver sampling

Following blood collection, the rats were rendered unconscious during blood sampling, and their liver was immediately removed, perfused with ice-cold 0.9% normal saline, dried, and weighed using an electronic balanceiver organ was extracted, and the relative organ weight (percentage of body weight) was calculated according to Mee-Young *et al.,* (2013)'s formula. The liver organ was rinsed in 10% neutral formalin for preparing paraffin sections to study the histopathological changes under light microscopy.

Hematological Biochemical analysis

Liver functions

Being serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels quantified in serum in the assay implemented by Gella *et al.,* (1985) and Tietz *et al.,* (1994), the instrumental solution was adopted for the accomplishment of the assay. Also, entrusted with the kinetic activity of ALP was done with the modified method invented by Vassault *et al.,* (1999).

Biological - Antioxidant enzymes

The concentrations of reduced glutathione (GSH) and oxidized glutathione (GSSG) were determined in serum samples using established colorimetric methods, as outlined by Ellman (1959).

Biological oxidants

Using a flourometric method similar to that detailed by Misko *et al.,* (1993), serum nitrite (NO_2) was measured. The method of thiobarbituric acid (TBA) is used to measure the amount of serum malondialdehyde (MDA), as described by Buege and Aust (1978).

Histopathological technique

The liver fixed in 70%, 80%, 95%, and absolute alcohol for dehydration, followed by clearing in xylen. Subsequently, the liver tissue underwent impregnation in molten wax to create a paraffin block. These paraffin blocks were then sliced into 5µm thick sections, which were placed on pristine slides and incubated in a 37°C oven. The sections were deparaffinized in xylene, rehydrated gradually in alcohol, and finally stained with Haematoxylin and Eosin. Post-staining, they were dehydrated in increasing alcohol concentrations and cleared in xylene. Following this, the slides were covered with coverslips, examined, and photographed under light microscopy at the Histochemistry and Cell Biology department of the Medical Research Institute, Alex. Univ.

Statistical Analysis

A computerized Computer Software Package SPSS® version (18.0.) program was used to statistically analyze all the data using one-way ANOVA. The means ± Standard Deviation (SD) were reported for the results. $P \leq 0.05$ was used to determine the significance of differences between treatments (Snedecor and Cochran, 1967).

RESULTS AND DISCUSSION

Proximate analysis of *Clitoria ternatea* flower extract (CTEE)

Table (2) indicated the results of the proximate analysis including moisture content, crude protein, ash, crude fat, and crude fiber. Moisture 93.04, Crude protein 0.35, Ash 0.42, Fat 2.53, Crude fiber 2.21, Carbohydrate 1.45. There was an obvious quantity of fat (2.53 ± 0.1) and crude fiber (2.21 ± 0.2) in the *C. ternatea* flowers. The results agree with Neda *et al.,* (2013) and Muhammad Ezzudin & Rabeta (2018) who found that higher protein content in the leaves and flower of *C. ternatea* could be useful against muscle wasting.

Table 2. Proximate analysis of *Clitoria ternatea* flower extract (CTEE)

Consitituent	Percentage (%)
Moisture	93.04 ±0.22
Protein	0.35 ±0.17
Ash	0.42 ±0.11
Fat	2.53±0.09
Crude fiber	2.21±0.04
Carbohydrate	1.45 ±0.08

Biological evaluation

Effect of intervention with (CTEE on BWG, FI and FER) of hepatotoxic rats induced by (CCl_4)

The influence of CTEE interfere (BWG, FI, and FER) on rats subjected to CCl_4-induced hepatotoxicity is presented in Table (3). Upon analysis of this data, it was noted that rats treated with CCl_4 exhibited a notable decrease at ($p \leq 0.05$) in BWG (-42.86%), FI (-36.99), and FER (-30.14) compared to the control group. Conversely, in rats undergoing a 28-day feeding regimen, the administration of CTEE at varying doses of 25, 50, 100, 200, and 400 mg/kg bw/day a significantly ($p \leq 0.05$) elevated the levels of BWG, FI, and FER by -40.66, -34.07, -16.48, -10.99, and -8.79%; -30.41, -25.47, -16.30, -8.62, and -6.41%; and -27.40, -20.55, -15.03, -12.49, and -6.90%, respectively, in comparison to the normal animals.

Furthermore, there was a dose-dependent pattern to the rate of increase in each of these parameters. In related investigations, multiple researchers discovered that CCl_4 significantly reduced the rats' BWG, FI, and FER, and that feeding interventions containing various plant parts significantly increased these parameters as well (ElSamouny, 2021 and Elhassaneen *et al.*,2021 a,b). According to Hamzawy *et al.*, (2013) and Abd El-Rahman (2021), hepatotoxic rats showed a notable decrease in body weight and feed intake in this regard. Additionally, Ross *et al.*, (2020) and Weber *et al.*, (2003) demonstrated that hepatotoxic treatment can cause malnutrition, characterized by inadequate dietary intake (FI), impaired digestion, and subsequent malabsorption of nutrients. Additionally, this condition may involve anomalies in the storage and metabolism of nearly all nutrients, which are the main causes of malnutrition in hepatotoxic patients. Additionally, Boushey *et al.*, (2001) and Khan *et al.*, (2012) noted that the majority of hepatotoxic patients lose weight and are sick.

Table 3. Effect of CTEE intervention on (BWG., FI., and FER.) of hepatotoxic rats induced by CCl_4

Value	Normal cont.	Model cont.	CTEE intervention (mg/kg bw/day)				
			25	50	100	200	400
			BWG (%)				
Mean	0.94[a]	0.54[d]	0.56[d]	0.62[c]	0.78[bc]	0.83[b]	0.85[b]
SD	0.03	0.05	0.03	0.04	0.09	0.04	0.05
% of change	0.00	-42.86	-40.66	-34.07	-16.48	-10.99	-8.79
			FI (g/day/rat)				
Mean	13.14[a]	8.28[d]	9.15[c]	9.80[c]	11.00[bc]	12.01[b]	12.30[b]
SD	0.31	0.42	0.51	0.62	0.70	0.51	0.49
% of change	0.00	-36.99	-30.41	-25.47	-16.30	-8.62	-6.41
			FER				
Mean	0.075[a]	0.053[d]	0.055[d]	0.060[cd]	0.064[c]	0.066[bc]	0.070[b]
SD	0.004	0.006	0.003	0.005	0.003	0.006	0.01
% of change	0.00	-30.14	-27.40	-20.55	-15.03	-12.49	-6.90

Each value represents the mean ±SD (n = 6). Means in the same column with different superscript letters indicate the significant differences at $P \geq 0.05$. Natural control, healthy rats with no intervention; form control, carbon tetrachloride induced hepatotoxic rats without intervention; CTEE, *Clitoria ternatea* flower ethanolic extract; CTEE intervention, carbon tetrachloride-induced hepatotoxic rats with CTEE intervention; bw, body weight; BWG, body weight gain; FI, feed intake; FER, feed efficiency rate.

At last, a number of authors discovered that rats given CCl_4 injections showed decreased BWG, FI, and FER, and that these levels could be raised by consuming or interacting with various plant parts that contain bioactive compounds, like those in CTEE (Mansour, 2017; Tahoon, 2019; ElSamouny, 2021 and Elhassanen *et al.*,2021 a,b).

Biochemical Finding

Effect of intervention with CTEE on the liver functions of the hepatotoxic rats induced by CCl_4

Table (4) illustrates the effects of CTEE intervention on a hepatic function of CCl_4-induced hepatotoxic rats. comparing the CCl_4-treated rats companion to the control group, the results indicated a substantial elevation ($p\leq0.05$) in AST (77.28%), ALT (129.90%), ALP (158.72%), and TB (412.84%). However, following CTEE intervention (25, 50, 100, 200, and 400 mg/kg bw/day) in rats subjected to a 28-day dietary regimen, the levels of AST, ALT, ALP, and TB exhibited a significant decline compared to the control animals ($p\leq0.05$). The reductions were recorded as 68.62, 52.80, 42.21, 23.25, and 16.25%; 129.51, 110.88, 97.28, 67.45, and 28.17%; 153.00, 141.95, 119.53, 77.92, and 42.40%; and 366.22, 306.76, 239.86, 177.70, and 64.86%, respectively. Furthermore, a dose-dependent relationship was observed in the rate of decrease for each of these parameters. According to several researchers (Elhassanneen *et al.*,2012 and Susilo *et al.*,2019), CCl_4 - induced liver injury is regularly employed to investigate the hepatoprotective effects of pharmaceuticals and natural substances. CCl_4 is metabolized by liver cytochrome P450 into trichloromethyl (CCl_3-) radicals, which then combine with O_2 to create trichloromethyl peroxyl (CCl_3OO-) radicals, leading to liver damage. These free radicals disrupt the formation and purpose of intracellular organelles and cell membranes in liver cells, causing the peroxidation of membrane-bound fatty acids.

Table 4. Effect of intervention with CTEE on the liver functions of the hepatotoxic rats induced by CCl_4

Value	Normal cont.	Model cont.	CTEE intervention (mg/kg bw/day)				
			25	50	100	200	400
Serum Aspartate aminotransferase (AST) activity (U/L)							
Mean	38.58 ^{c}	68.39 ^{a}	65.05 ^{a}	58.95 ^{b}	54.86 ^{b}	47.55 ^{c}	44.85 ^{c}
SD	3.88	5.13	4.60	6.03	4.40	3.84	5.26
% of change	0.00	77.28	68.62	52.80	42.21	23.25	16.25
Serum alanine aminotransferase (ALT) activity (U/L)							
Mean	26.78 ^{e}	61.57 ^{a}	61.47 ^{a}	56.48 ^{ab}	52.83 ^{b}	44.84 ^{c}	34.33 ^{d}
SD	2.26	6.34	3.99	3.76	4.12	2.99	3..45
% of change	0.00	129.90	129.51	110.88	97.28	67.45	28.17
Serum alkaline phosphatase (ALP,U/L)							
Mean	100.96 ^{e}	261.19 ^{a}	255.41 ^{a}	244.26 ^{ab}	221.63 ^{b}	179.62 ^{c}	143.76 ^{d}
SD	9.32	19.14	13.66	15.85	9.10	11.32	11.09
% of change	0.00	158.72	153.00	141.95	119.53	77.92	42.40
Bilirubin (TB, mg/L)							
Mean	1.48 ^{e}	7.59 ^{a}	6.90 ^{ab}	6.02 ^{b}	5.03 ^{bc}	4.11 ^{c}	2.44 ^{d}
SD	0.23	0.62	0.41	0.38	0.46	0.29	0.21
% of change	0.00	412.84	366.22	306.76	239.86	177.70	64.86

Each value represents mean ±SD (n=6). Means on the same column with different superscript letters explain the significant difference at $P\leq 0.05$. Normal control, healthy rats without intervention; Model control, carbon tetrachloride-induced hepatotoxic rats without intervention; CTEE, *Clitoria ternatea* flower ethanolic extract; CTEE intervention, carbon tetrachloride-induced hepatotoxic rats with CTEE intervention.

The elevated levels of serum markers AST, ALT, ALP, and TB in this study indicate significant liver damage induced by CCl_4 injection. According to Shanmugasundaran *et al.,* (2010) and Nithianantham *et al.*, (2011), these variable assays are frequently regarded as sensitive markers for assessing the progression of liver damage since they contain cytoplasm, which promotes blood flow following liver cell damage.According to data from a recent study, CTEE feeding intervention rats' levels of aminotransferases (AST and ALT), ALP, and TB were significantly decreased, demonstrating that it could prevent cell damage. The CTEE content of a number of significant bioactive substances, such as steroids, anthocyanins, flavonol glycosides, triterpenoids, and nucleotides cyclic peptides may be responsible for these protective effects (Mukherjee *et al.*,2008 and Nithianantham *et al.*,2011). Moreover, earlier reports on CTEE included phenolics, flavonoids, and different kaempferols (Gupta *et al.*,2010 and Pendbhaje *et al.*,2011). According to reports, these substances have antioxidant and scavenging properties, and they play a protective role against toxins like CCl_4 that can cause liver damage (Kuppan *et al.*,2013; Elhassaneen *et al.*,2016 and Elhassannen *et al.*,2021 a,b).

Effect of intervention with CTEE on glutathione fractions of hepatotoxic rats induced by -CCl_4

Table (5) demonstrates the impact of CTEE intervention on the levels of biological antioxidants, specifically glutathione (GSH) fractions, in rats with hepatotoxicity induced by CCl_4. The analysis allowed for the determination it is mean GSH and GSSG values in the normal animal group were 8.98 ±0.38 and 0.697 ± 0.067 µmol/L, respectively. A notable reduction in GSH (-45.43%) and GSSG (-18.16%) levels was observed in the CCl_4-treated rats when compared to the normal group ($p \leq 0.05$). Conversely, rats that were administered a diet containing different doses of CTEE (25, 50, 100, 200, and 400 mg/kg bw/day) for a duration of 28 days exhibited a significant increase ($p \leq 0.05$) in GSH and GSSG levels by -37.31, -30.03, -23.39, -14.37, and -8.13%, and -19.10, -13.63, -9.08, -8.20, and -2.56%, respectively. Furthermore, a dose-dependent pattern was observed in the rate of increase for each of these parameters. Considerable focus has been placed on the biosynthesis, regulation, and intracellular functions of the tripeptide-thiol glutathione, also known as α-glutamyl cysteinyl-glycine.

Table 5. Effect of intervention with CTEE on glutathione fractions of the hepatotoxic rats induced by CCl_4

Value	Normal cont.	Model cont.	CTEE intervention (mg/kg bw/day)				
			25	50	100	200	400
Reduced glutathione concentration, (GSH, µmol/L)							
Mean	8.98 [a]	4.90 [c]	5.63 [c]	6.28 [bc]	6.88 [b]	7.69 [ab]	8.25 [a]
SD	0.38	0.54	0.55	0.61	0.39	0.48	0.60
% of change	0.00	-45.43	-37.31	-30.03	-23.39	-14.37	-8.13
Oxidized glutathione concentration (GSSG, µmol/L)							
Mean	0.697 [a]	0.570 [c]	0.564 [c]	0.602 [c]	0.634 [b]	0.640 [b]	0.679 [a]
SD	0.067	0.094	0.033	0.042	0.069	0.122	0.044
% of change	0.00	-18.16	-19.10	-13.63	-9.08	-8.20	-2.56
GSH/GSSG ratio (%)							
Value	12.886 [a]	8.591 [d]	9.987 [c]	10.439 [b]	10.858 [b]	12.021 [a]	12.149 [a]

Each value represents mean ±SD (n=6). Means on the same column with different superscript letters explain the significant difference at $P \leq 0.05$. Normal control, healthy rats without intervention; Model control, carbon tetrachloride-induced hepatotoxic rats without intervention; CTEE, *Clitoria ternatea* flower ethanolic extract; CTEE intervention, carbon tetrachloride-induced hepatotoxic rats with CTEE intervention.

The primary function among these is the detoxification process, which is associated with phase II free radical metabolites that are subsequently eliminated in the urine as a result of drug use or chemical pollution (Elhassaneen, 1996 and Rao *et al.*,2013). Moreover, GSH has super antioxidant properties such as being a nonenzymatic oxyradical scavenger and playing a part in the activation of various antioxidant enzymatic systems, including GSH-Px and GSH-Rd (Almaadawy *et al.*,2016). The transfer of GSH fractions from the liver to the blood may be impeded by CCl_4, in accordance with a number of earlier authors, including the current study (Elhassaneen *et al.*,2012 and Abd Elalal *et al.*,2022). These conditions are attributed to lipid peroxidation, hepatocyte dysfunction, and/or decreased energy due to a decrease in liver glycogen content.

The bioactive substances of CTEE, including anthocyanins, flavonoids, triterpenoids, phenolics, and steroids, exhibit antioxidant properties and enhance the liver's release of GSH into the bloodstream (Mukherjee *et al.*,2008; Nithianantham *et al.*,2011 and Mahran & Elhassaneen, 2023). The results of this investigation also showed that a decline in the ratio of GSH/GSSG was observed in conjunction with a decrease in serum GSH fractions. GSH/GSSG ratios in normal liver cells are usually very high, i.e., >10 (Thilavech *et al.*,2021**)**. The rats given CCl_4 injection showed a significant ($p \leq 0.05$) decrease in their serum redox state (GSH/GSSG). However, after receiving CTEE for up to 400 mg/kg bw/day, the GSH/GSSG ratio increased and approached the value of the rats in the normal group. Therefore, it is suggested that CTEE suppress oxyradical fluxes induced by CCl_4, which could cause the GSH/GSSG ratio to decrease.

Effect of intervention with CTEE on oxidative stress of the hepatotoxic rats induced by- CCl_4

Table (6) illustrates the impact of CTEE intervention on oxidative stress, malondialdehyde (MDA), and nitrite (NO_2) in hepatotoxic rats induced by CCl_4. From these data, it was attainable to determine that the normal animal group's mean MDA and NO_2 values were 0.401± 0.024 and 4.95± 0.59 nmol/mL, respectively. When contrasted to the normal group, the CCl_4-treated rates showed a substantial ($p \leq 0.05$) increase in MDA (112.17%) and NO_2 (13.36%). However, after 28 days of feeding rats, CTEE intervention (25, 50, 100, 200, and 400 mg/kg bw/day) significantly ($p \leq 0.05$) reduced MDA and NO_2 levels relative to normal animals by 106.35, 96.30, 77.25, 40.74, and 16.40%, and 109.05, 87.93, 54.74, 33.62, and 21.55%, respectively. Furthermore, the rate of decline in each of the aforementioned variables showed a dose-dependent pattern, as Weber *et al.,* (2003) and Wang *et al.,* (2024) explained. CCl_4 causes liver damage by being biotransformed by the liver cytochrome P-450 into free radicals (trichloromethyl, CCl_3-, and trichloromethyl peroxyl (CCl_3OO-). All fatty acids that are bound to membranes experience lipid peroxidation as a consequence of these radicals. Malondialdehyde (MDA), a hazardous degradative product, can form in cell membranes as a result of lipid peroxidation (Cheeseman and Salter, 1993). Due to its association with nitrogenic bases in DNA, MDA has been shown in several studies to have mutagenic impacts (Cline *et al.*,2004 and Mahran & Elhassaneen, 2023). Moreover, MDA forms a cross- linking with the components of the membrane, which disrupts its fluidity, osmolality, and receptor inactivation. According to Nilanjana (2013), each of these conditions has the potential to damage cells and result in the formation of atherosclerotic plaques. inversely, l-arginine is enzymatically transformed into citrulline and the biologically active free radical nitric oxide (NO) by nitric oxide synthase in an inverse manner (Manahan, 1989). The detrimental effects of NO can manifest through multiple pathways: 1) conversion into nitrite (NO_2) and nitrate (NO_3) upon interaction with oxygen and water; 2) formation of nitrosylated compounds through interaction with amino and thiol groups of proteins; 3) reaction with hemoglobin leading to the creation of iron-nitrosyl adducts in the bloodstream; and 4) combination with superoxide anion (O_2-) resulting in the production of NO_3 (Manahan, 1989 and Misko *et al.*,1993). Various research studies (Jacob *et al.*,1992; Elhassaneen, 2004; Elhassaneen *et al.*,2020 and Ehassaneen *et al.*,2023) have indicated that these substances are synthesized by the body during the progression of diseases such as arthritis, hepatotoxicity, obesity, anemia, cardiovascular ailments, and diabetes. An important discovery that lends support to the research hypothesis, suggesting a correlation between elevated oxidative stress-related disorders and CCl_4- induced liver damage, is the notable levels of MDA and NO_2 observed in this particular study. As such, the protection against hepatotoxicity might also be influenced by the antioxidative properties, lipid peroxidation, and inhibition of nitric oxide synthase by CTEE. This is evidenced by the considerable reduction in the production of MDA and NO_2 in the serum following dietary intervention. Detecting minimal concentrations of MDA and NO_2 would likely pose a challenge without any modifications in the antioxidant defense mechanism in rats administered with CTEE, in our assessment. The result agreed with Chayaratanasin *et al.,* (2019) and Maneesai *et al.,* (2021).

Table 6. Effect of intervention with CTEE on oxidative stress of the hepatotoxic rats induced by CCl_4

Value	Normal cont.	Model cont.	CTEE intervention (mg/kg bw/day)				
			25	50	100	200	400
Malondialdehyde concentration (MDA, nmol/mL)							
Mean	0.189 [d]	0.401 [a]	0.390 [a]	0.371 [ab]	0.335 [b]	0.266 [c]	0.220 [e]
SD	0.024	0.052	0.011	0.028	0.042	0.030	0.022
% of change	0.00	112.17	106.35	96.30	77.25	40.74	16.40
Nitrite (NO_2, nmol/L)							
Mean	2.32 [d]	4.95 [a]	4.85 [a]	4.36 [b]	3.59 [c]	3.10[b]	2.82 [e]
SD	0.11	0.59	0.35	0.65	0.31	0.29	0.24
% of change	0.00	113.36	109.05	87.93	54.74	33.62	21.55

Each value represents mean ±SD (n=6). Means on the same column with different superscript letters explain the significant difference at P≤ 0.05. Normal control, healthy rats without intervention; Model control, carbon tetrachloride-induced hepatotoxic rats without intervention; CTEE, *Clitoria ternatea* flower ethanolic extract; CTEE intervention, carbon tetrachloride-induced hepatotoxic rats with CTEE intervention.

Correlation studies

Significant discrepancies were observed in the correlation analysis between liver functions (AST, ALT, and ALP) and oxidative stress parameters (MDA and NO_2) and non-enzymatic antioxidant (GSH) in hepatotoxic rats induced by CCl_4 and feeding intervention CTEE Table 7. Based on the available data, a highly significant ($p\leq 0.05$) inverse association was observed between the serum concentrations of MDA and GSH (r2 = - 0.947). Furthermore, a substantial negative correlation ($p\leq 0.05$) was observed between liver functions (AST, r2 = + 0.732; ALT, r2 = +0.6955; ALP, r2 = + 0.663 and TB, r2 = + 0.814) and MDA and NO_2 concentration in serum (r2 = + 0.903). The NO_2 evidence showed a similar pattern, though with lower values. A significant ($p\leq 0.05$) decrease in GSH, an antioxidant, and an increase in liver functions (AST, ALT, ALP, and TB) in rats serum as a result of hepatotoxic injury were found to be in opposition to an increase in oxidative stress parameters (MDA and NO_2). Argued alternatively, these correlations confirm to the issues of monitoring high concentrations of MDA and NO_2 in the absence of changes in the non-enzyme antioxidant (GSH) of hepatotoxic rats. In accordance with Mehmood *et al.,* findings from (2019), an extract derived from the blue butterfly pea flower (*Clitoria ternatea* L.), that contains biologically active antioxidant aspects, impeded lipid peroxidation in hepatotoxic rats through elevated MDA and NO. According to Mehmood *et al.,* (2019) and Abd Elalal *et al.,* (2022), there was a relationship between relatively low concentrations of enzymatic and non-enzymatic antioxidants, such as GSH, and high levels of lipid peroxidation, particularly MDA and NO_2, in the plasma of hepatotoxic rats.

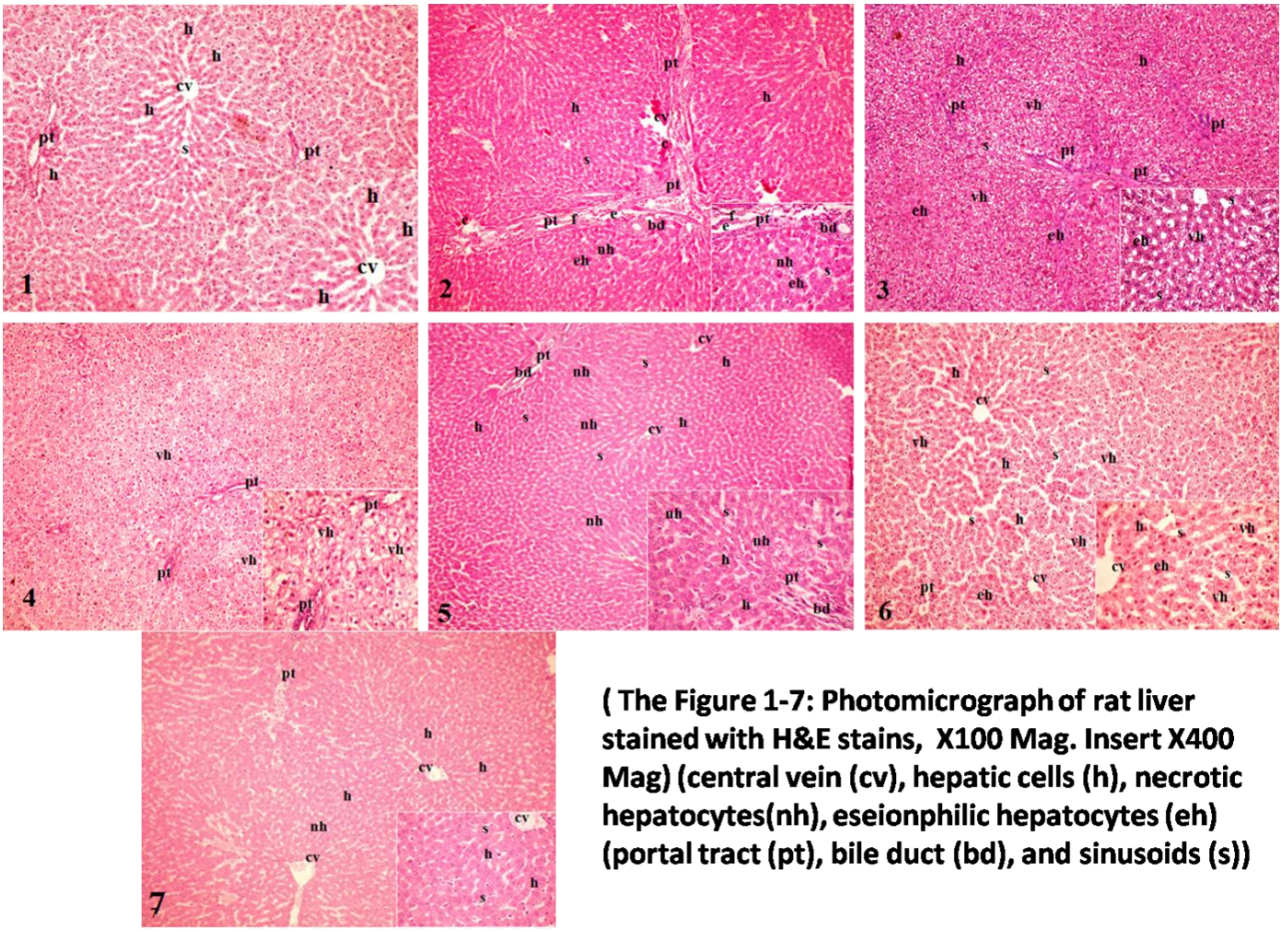
Histopathological Finding

Paraffin sections were stained with haematoxylin and eosin to study the histomorphological changes in rat live tissues in all groups. it was noticed the hepatocytes and stroma tissue have deep violet color of nuclei and pink cytoplasm. The white clear color may be indicted to the vacuolated cytoplasm or dissolving fats in the tissue section. The finding and discusion were illustrated as the following in Fig. 3(1-7):

Table 7. Correlation between oxidative stress and antioxidant parameters in hepatotoxic rats induced by CCl_4 and feeding intervention CTEE

Parameters	r^{2*}	Parameters	r^2
MDA/GSH	- 0.947	NO_2/GSH	- 0.891
MDA/NO_2	+ 0.903	NO_2/AST	+0.693
MDA/AST	+0.732	NO_2/ALT	+ 0.597
MDA/ALT	+ 0.695	NO_2/ALP	+ 0.610
MDA/ALP	+ 0.663	NO_2/TB	+ 0.764
MDA/TB	+ 0.814		

* $P \leq 0.05$



(The Figure 1-7: Photomicrograph of rat liver stained with H&E stains, X100 Mag. Insert X400 Mag) (central vein (cv), hepatic cells (h), necrotic hepatocytes(nh), eseionphilic hepatocytes (eh) (portal tract (pt), bile duct (bd), and sinusoids (s))

1- normal control rat, show portal tract and central vein. Radiated hepatocytes from central vein with round nuclei and homogenous cytoplasm, mild dilation of sinusoids. 2- Model control rat liver administrated CCl_4, note thick fibrotic area, three adjusting marked dilation congested portal tract with dilated bile duct attached with three congested centeral veins. There are infilterating lymphocytes associated with many fibrotic cells and edema. The proliferating and crowded hepatocytes have round nuclei, other with necrotic nuclei (nh) and eosinophilic cytoplasm, mild dilation sinusoids was seen. 3- Rat liver administrated CCL_4 plus fed 25 mg of CTEE, note mild reorganized liver tissue, dilation and congestion portal tract with dilated bile duct, The hepatocytes (h) loss its attachments and proliferating through central vein. They have round nuclei with vacuolated cytoplasm. Many necrotic hepatocytes and dilation of sinusoids was seen. 4- Rat liver administrated CCL_4 plus fed 50 mg of CTEE, note mild reorganized liver tissue, reorganized portal tract and central vein. The proliferating hepatocytes through central vein and portal tract with small round nuclei and vacuolated cytoplasm and dilation sinusoids. 5- Rat liver administrated CCL_4 plus fed 100 mg of CTEE, note mild reorganized liver tissue, a mild dilation of portal tract with mild dilated bile duct. The hepatocytes was radiating and proliferating through central vein, they have round nuclei with homogenous cytoplasm and many necrotic hepatocytes, mild dilation of sinusoids was seen. 6- Rat liver administrated CCL_4 plus fed 200 mg CTEE, show mild dilation central vein. Radiated hepatocytes from central vein with round dark nuclei and homogenous cytoplasm, few eosinophillic hepatocytes and vacuolated cytoplasm hepatocytes, mild dilation of sinusoids. 7- Rat liver administrated CCL_4 plus fed 400 mg CTEE in die, show recovering and regenerative of central vein and portal tract. The hepatic cells have round dark nuclei and homogenous cytoplasm. They have organized radiating from central vein and portal tract with few necrotic hepatocytes sinusoids.

The experiment revealed that the CCL_4 facilitates the degenerative of hepatic tissues which resulted in (Figure 3.2) creation of massive fibrous septa, separation, and accumulation of pseudolobes of more than three portal vein attached to the central vein and formed a large fibrotic area. This finding was similarly (Sahreen *et al.*,2011 and Veidal *et al.*,2011) to the reported of many authers In other hand the increased of the CTEE doses in fed illustrated the recovering of the toxic effect of CCL_4 which appeared as recovering of most liver cell and the reduction of the fiobrotic portal tract as well as the absent of infiltrating lymphocyte, fibrous cells and reduction of dilated portal tract and central vein and hemogenous of most hepatocytes at group diet with 400 mg CTEE (Figure 3. 7). Therefore, the present results revealed that the CTEE

have ability for ameliorate the toxic effect of CCL_4 induced in liver tissue and its potential protective roll in tissue.

Conclusion

A beneficial protection against liver harm caused by CCl_4 was provided by *Clitoria ternatea* flower ethanol extract (CTEE). Our theory that various classes of bioactive compounds (phytochemicals) present in CTEE can break the hepatotoxic effects of CCl_4 by one or more of the following mechanisms was validated by these results: One approach to improve serum antioxidant defenses is by inhibiting excessive enzymatic activity that is expressed in liver functions. Another way is by reducing the amount of oxidative stress (MDA and NO_2) while boosting the formation of non-enzymatic antioxidants (GSH). There was the histopathological finding confirmed the biochemical result and revealed liver fibrosis induced by CCL_4 in rats and the recovering of the liver tissues with the high fed of the 200 - 400mg CTEE. That's why we recommended including extracts of this plant (*Clitoria ternatea* flowers) in our regular meals, beverages, and pharmaceutical formulae at concentrations of up to 400 mg/kg bw/day.

Abbreviations

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BWG, body weight gain; CCl_4, carbon tetrachloride; CTEE, *Clitoria ternatea* flower ethanolic extract, FER, feed efficiency ratio; FI, feed intake; GSH, reduced glutathione; GSSG, oxidized glutathione; MDA, malondialdehyde; NO_2, Nitrite; TB, total bilirubin.

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الملخص العربي

التأثيرات الوقائية المحتملة للمستخلص الإيثانولي لزهرة البازلاء الفراشة (*Clittoria ternatea* Linn) ضد التسمم الكبدي الناجم عن رابع كلوريد الكربون في الفئران

نيفين الوردانى، مريم عبد القادر

أجريت الدراسة الحالية لاستكشاف التأثيرات الوقائية المحتملة للمستخلص الإيثانولي لزهرة بازلاء الفراشة (*Clitoria ternatea* Linn) ضد التسمم الكبدي الناجم عن رابع كلوريد الكربون في الفئران. ولقد اوضحت النتائج انه عند المقارنة بالفئران الطبيعية ، أظهرت الفئران المحقونة برابع كلوريد الكربون (CCl_4) انخفاضًا معنويا ($p\leq0.05$) في المعايير البيولوجية المختلفة بما في ذلك الزيادة فى وزن الجسم، المدخول من الغذاء، ونسبة كفاءة بمعدل –٤٢,٨٦%، و– ٣٦,٩٩ و–٣٠,١٤ .%على التوالى .كما أظهرت المؤشرات الكيموحيوية مثل نشاط إنزيمات الكبدAST ، ALT، ALP والبيليروبين الكلي ارتفاعاً معنوياً ($p\leq0.05$) عند مقارنتها بفئران المجموعة الطبيعية بمعدل ٧٧,٢٨، ١٢٩,٩٠، ١٥٨,٧٢ و٤١٢,٨٤% على التوالي .وقد تزامن ذلك أيضًا مع حدوث خلل في بعض مؤشرات الأكسدة/مضادات الأكسدة في الدم، أي انخفاض في مستوى مضادات الأكسدة غير الأنزيمية (GSH و GSSG) ،وزيادة في مستوى المواد المؤكسدة (MDA ، NO_2) وكل هذه العوامل تشير إلى أن إصابة الكبد بمركب رابع كلوريد الكربون. كما أظهرالتدخل بالمستخلص الإيثانولي لزهرة البازلاء الفراشة في بروتوكول تغذية الفئران تحسينات معنوية ($p\leq0.05$) كبيرة في جميع المقاييس البيولوجية والكيميائية الحيوية والمؤكسدات/مضادات الأكسدة السابقة والتي تشير إلى الحماية ضد السمية الكبدية في الفئران .كما أن معدل التحسن في جميع هذه العوامل أظهر بطريقة تعتمد على الجرعة . وفي النهاية ، كان المستخلص الإيثانولي لزهرة البازلاء الفراشة فعالا في الحماية من اضطرابات الكبد الناجمة عن رابع كلوريد الكربون ، ولذلك، توصى الدراسة بان يتم تضمين مثل هذه المستخلصات من أجزاء النبات (زهور *Clitoria ternatea*) بتركيزات تصل إلى ٤٠٠ ملجم/كجم من وزن الجسم/اليوم في وجباتنا الغذائية والمشروبات والتركيبات الدوائية اليومية.

الكلمات المفتاحية: زهرة بازلاء الفراشة ، وزن الجسم، وظائف الكبد، أجزاء الجلوتاثيون، المالونالدهيد، النتريت.