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## Original Article

# Hepatoprotective activity of Chhit-Chan-Than extract powder against carbon tetrachloride-induced liver injury in rats



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## ABSTRACT

The capability of Chhit-Chan-Than extract powder (CCTEP, 10% aqueous *Ocimum gratissimum* L. extract) to protect against carbon tetrachloride (CCl<sub>4</sub>)-induced oxidative stress and hepatotoxicity *in vivo* was investigated. Wistar rats were divided into five groups. Group A was a normal control group given only vehicle; Group B, the hepatotoxic group, was injected intraperitoneally twice a week with repeated 8% CCl<sub>4</sub>/olive oil (0.1 mL/100 g of body weight); Groups C–E, extract-treated groups received CCl<sub>4</sub> and different doses of CCTEP (100 mg/kg and 200 mg/kg) or silymarin (200 mg/kg of body weight) daily by gavage for 8 weeks, respectively. The results showed that the CCl<sub>4</sub>-induced histopathological changes may be prevented by CCTEP through reducing the intercellular collagen stack, dropping blood serum alanine aminotransferase and aspartate aminotransferase levels, and restoring the catalase activity and glutathione content. The hepatoprotective properties were further confirmed by the marked improvement in histopathological examination

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*Ocimum gratissimum*  
Oxidative stress

and by quantitative steatosis-fibrosis scoring. The above results suggest that CCTEP is able to prevent the liver inflammation and fibrosis induced by repeated CCl<sub>4</sub> administration, and the hepatoprotective effects might be correlated partly with its antioxidant and free radical scavenging effects.

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## 1. Introduction

Chronic liver diseases are common worldwide and are caused by long-term exposure to toxic chemicals, drugs, alcohol, and/or viral infection, which are characterized by a progressive evolution from steatosis to chronic hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma [1,2]. Iterative hepatic injury induces chronic inflammation associated with dynamic healing response, ultimately leading to cirrhosis and its life-threatening complications [3]. In the absence of an effective pharmacological therapy for such pathological processes, the research of herbal drugs or plant extracts for the treatment of liver diseases has increased all over the world [4]. For example, silymarin, a flavonolignan mixture of milk thistle (*Silybum marianum*), is one such popular hepatoprotective herbal drug. Silymarin exhibits significant hepatoprotective effects, which have been well demonstrated using several *in vitro* and *in vivo* models [5,6], and is thus often used as a standard reference in this field. Clearly, there exists a critical need to validate the efficacy of traditional herb medicine and plant extracts using scientific evidence and experimental models in order to explore novel or alternative approaches for liver diseases.

The *Ocimum* species are widely distributed in tropical and subtropical regions and commonly used as a food spice and traditional herb in many countries. Among the *Ocimum* species, *Ocimum gratissimum* L. is a well-known medicinal plant since ancient times. It is popularly known as *Vana Tulsi* in India [7,8], *Chhit-Chan-Than* in Taiwan [9,10] and *clove basil* in most Western countries [11]. The leaf of *O. gratissimum* has a strong-smelling aromatic flavor and is rich in antioxidants and phytoconstituents. It has been prepared in a variety of forms for consumption and is known for its multiple pharmacological properties including anti-inflammation [10,12], analgesic and spasmolytic activities [13], antidiarrheal activity [14], antitumor activity [15,16], antiviral activity [17], anti-hyperglycemic activity [18], and improvement of phagocytic function [19]. Our research group demonstrated the beneficial effects of the aqueous *O. gratissimum* extract (OGE), which exerts potent antioxidant action in *in vitro* and *in vivo* studies [12,20]. Using an *in vitro* model, OGE also exerts hepatoprotective effects against oxidative stress [21]. OGE pretreatment, in a dose-dependent manner, restored the decrease in cell viability in H<sub>2</sub>O<sub>2</sub>-treated human hepatoma HepG2 cells. Herbal extracts of *Ocimum* species have also been shown to inhibit liver cirrhosis using the animal models [22,23]. Nevertheless, the *in vivo* hepatoprotection of OGE underlying its antioxidant property remains sketchy.

Carbon tetrachloride (CCl<sub>4</sub>) is an extensively used hepatotoxic agent in preclinical animal studies [24]. It is the best-characterized chemical for study of xenobiotic-induced free

radical mediated acute liver injury in rats [25]. CCl<sub>4</sub>-induced liver damage results in increased necrosis, steatosis, and foamy degeneration followed by the progression of fibrosis [26]. During hepatocellular damage, varieties of enzymes normally located in the cytosol are released into the blood flow, where their quantification in plasma is a useful biomarker. The metabolism of CCl<sub>4</sub>, catalyzed by liver microsomal cytochrome P450, rapidly overproduces free radicals that deplete hepatic glutathione (GSH) and initiates a chain lipid peroxidation of the hepatocyte membrane. CCl<sub>4</sub>-induced generation of peroxy and superoxide radicals result in the inactivation of catalase (CAT) and superoxide dismutase. These phenomena ultimately result in oxidative stress and hepatocyte injuries. Liver injury induced by CCl<sub>4</sub> is a classical system of xenobiotic-induced hepatotoxicity and has been used extensively for decades for the screening of anti-hepatotoxic/hepatoprotective activities of different drugs [27].

Blocking the CCl<sub>4</sub>-induced peroxidation chain reaction that results in hepatotoxicity is one of the potential strategies by which functional food could exert a hepatoprotective effect. The most remarkable pathological characteristics in the liver of CCl<sub>4</sub>-treated animals resemble closely the processes of chronic hepatitis and cirrhosis in human [28]. Thus, male Wistar rats challenged by CCl<sub>4</sub> were used as the animal model of chemical-induced hepatotoxicity in our recent studies [20] and also in the present study. The purpose of this project was to evaluate the hepatoprotective effect of *Chhit-Chan-Than* extract powder (CCTEP, containing 10% OGE) against acute CCl<sub>4</sub>-induced hepatic damage.

## 2. Methods

### 2.1. Preparation of extract powder and composition analysis

The CCTEP used in this study was made with 10% aqueous OGE and 90% dextrin as an excipient. Aqueous extract of *O. gratissimum* leaf was prepared as described elsewhere [12,16,20,21,29]. Briefly, leaves of *O. gratissimum* were harvested and washed with distilled water, followed by homogenization with distilled water using polytron. The homogenate was incubated at 95°C for 1 hour and then filtered through two layers of gauze. The filtrate was centrifuged at 20,000g at 4°C for 15 minutes to remove insoluble pellets and the supernatant was thereafter collected as OGE solution. Subsequently, the aqueous solution was mixed with water-soluble dextrin as an excipient. The powders obtained by spray drying were packaged in sealed metallized bioriented polypropylene bags at 24 ± 2°C prior to the subsequent

analyses. The total polyphenol and flavonoid contents were determined by the Folin–Ciocalteu method [30,31] and Lamaison method [32], respectively. The polyphenol content of OGE was analyzed as previously described [12,16,20,21,29] and revealed that the final product of CCTEP contained 1.77% polyphenolic acid and 0.69% flavonoids.

## 2.2. Treatment of animals

Male Wistar rats weighing 200–240 g were housed in conventional cages with free access to water and rodent chow at 20–22°C with a 12-hour light–dark cycle. All procedures involving laboratory animal use were in accordance with the guidelines of the Instituted Animal Care and Use Committee of Chung Shan Medical University (Taichung, Taiwan, R.O.C.) for the care and use of laboratory animals. The experimental setting is Group A were treated with olive oil (1.0 mL/kg) by intraperitoneal injection, and Groups B–E were treated with 8% CCl<sub>4</sub>/olive oil (1.0 mL/kg) by intraperitoneal injection, twice every week (Monday and Thursday) for 8 weeks, as described by Hernández-Muñoz et al [33] with some modifications. On Tuesday, Wednesday, Friday, and Saturday, Groups A and B were treated with water, Groups C and D were treated with low dose (100 mg/kg) and high dose (200 mg/kg) of CCTEP, respectively, and Group E was treated with silymarin (200 mg/kg); all by oral gavage. The mortality rate and body weight were recorded daily. At the end of the experiment, blood and organs were immediately collected after the animals were sacrificed. Liver tissue samples were taken from the left liver lobe and cut into two pieces. One piece was fixed in formalin for pathological examination. The other piece was subjected to biological analyses. The blood samples and organ tissues were all collected and stored at –80°C until use.

## 2.3. Biochemical assays of serum

All biochemical assays were conducted in the clinical laboratories of the Kuang Tien General Hospital, Taichung, Taiwan. The levels of aspartate transaminase (AST) and alanine transaminase (ALT) were measured on a Beckman Coulter Synchron Clinical Systems Analyzer (LX20PRO Autoanalyzer; Beckman Coulter Inc., Taipei, Taiwan). Specifically, AST was measured by an oxaloacetate/malate dehydrogenase method, whereas ALT was measured by a pyruvate/lactate dehydrogenase method. Serum total cholesterol and triglyceride were measured on a Hitachi 7170 autoanalyzer (Hitachi, Tokyo, Japan).

## 2.4. Preparation of liver homogenate and assay of protein content

A 1 g sample of liver tissue in 10 mL ice-cold potassium phosphate buffer (pH 7.0) was homogenized in an IKA-WEAR homogenizer (RW 20 DZM; IKA, Staufen, Germany). The homogenate solution was centrifuged at 12,000g for 30 minutes. The supernatant was collected and stored at –80°C for protein content assay, GSH and the antioxidant enzyme activities in the liver. The supernatant was collected and assayed for protein content by the Bradford method, using a

protein assay kit supplied by Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA), with bovine serum albumin as a standard.

## 2.5. Determination of GSH in the liver

GSH was determined by titration with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent) [34] as described previously [35], with some modifications. Proteins of 0.4 mL liver homogenates were precipitated by the addition of 0.4 mL a metaphosphoric acid solution (1.67 g metaphosphoric acid, 0.20 g EDTA, and 30.0 g NaCl in 100 mL H<sub>2</sub>O). After 40 minutes, the protein precipitate was recovered by centrifugation at 12,000g in a microcentrifuge (Microfuge; Beckman) at 4°C for 30 minutes. A 100 µL aliquot of the supernatant was combined with 0.7 mL 300 mM Na<sub>2</sub>HPO<sub>4</sub>, and the absorbance at 412 nm was read against a blank consisting of 0.1 mL supernatant plus 0.7 mL H<sub>2</sub>O. A 100 µL aliquot of DTNB (0.02%, w/v 1% sodium citrate) was then added to the blank and sample. Absorbance of the sample was read against the blank at 412 nm. The GSH content was determined using a calibration curve prepared with an authentic sample. An aliquot of lysate was used for the determination of protein content. GSH values are expressed as nmol/mg protein.

## 2.6. CAT assay

CAT activity was measured by the method of Kawamura et al [36]: 0.1 mL supernatant was added to cuvette containing 1.9 mL 50 mM phosphate buffer (pH 7.0). Reaction was started by adding 1.0 mL freshly prepared 30 mM H<sub>2</sub>O<sub>2</sub>. The rate of decomposition of H<sub>2</sub>O<sub>2</sub> was measured spectrophotometrically at 240 nm. Activity of CAT was expressed as U/mg of protein.

## 2.7. Histopathologic study

A portion of the liver was fixed in 10% formalin, processed using routine histology procedures, embedded in paraffin, cut in 5 µm sections, and mounted on a slide. The sections were then stained with hematoxylin–eosin dye and studied for histopathological changes (40×), i.e., necrosis, fatty changes, ballooning degeneration, and lymphocyte infiltration. Masson stain was used for liver steatosis and fibrosis scoring. Liver steatosis was graded on a 3-point scale: 1+, hepatocytes in the area of one-third of the lobules showing fatty accumulation; 2+, for two-thirds; and 3+, for all hepatocytes. The criteria used for scoring fibrosis severity were as follows: 0, normal; 1+, fibrosis present (collagen fiber present that extends from portal triad or central vein to peripheral region); 2+, mild fibrosis (collagen fiber present with extension without compartment formation); 3+, moderate fibrosis (collagen fiber present with some pseudo lobe formation); and 4+, severe fibrosis (collagen fiber present with thickening of the partial compartments and frequent pseudo lobe formation).

## 2.8. Statistical analysis

The experimental results are expressed as the mean ± standard deviation. Data were assessed using analysis of variance (ANOVA). Student t test was used in the

comparison between groups. A  $p$  value  $<0.05$  was considered statistically significant.

### 3. Results

#### 3.1. Clinic observation, body weight, and organ weight change

No dead rat was observed during the 8-week test of hepatoprotective effect of CCTEP. However, in the  $\text{CCl}_4$ -treated only group the rats look emaciated at Week 7 or Week 8, possibly due to the liver damage.

The change of body weight may be in response to the rat growth rate and physiologic conditions. Fig. 1 shows the change of body weight during the 8-week test of hepatoprotective effect of CCTEP. The  $\text{CCl}_4$ -treated control group rats (B, average weight  $382.1 \pm 53.9$  g) had a reduced mean body weight ( $p < 0.01$ ) as compared with those of normal control group (A, weight  $440.8 \pm 35.0$  g). The weight gain in Group A ( $240.8 \pm 25.8$  g) administrated with olive oil and water was the most obvious, but is small in Group B (average  $180.0 \pm 37.1$  g) administrated with  $\text{CCl}_4$ . In the interventional groups, supplement of silymarin or CCTEP with  $\text{CCl}_4$  did not adequately resume the lost weight caused by  $\text{CCl}_4$ . In Group C ( $440.0 \pm 12.9$  g), Group D ( $392.1 \pm 19.1$  g), and Group E ( $398.3 \pm 41.3$  g) administrated with different dose of CCTEP and silymarin, the scope of weight gain has restored, but was not statistically significant.

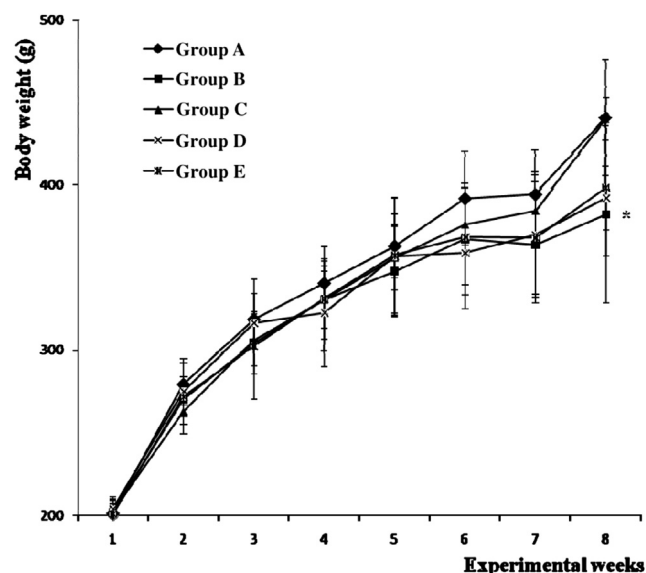


Fig. 1 – Effects of Chhit-Chan-Thau extract powder (CCTEP) on body weight after  $\text{CCl}_4$  treatment in rats. CCTEP and  $\text{CCl}_4$  were given as described in “Methods”. (A) Normal control group; (B)  $\text{CCl}_4$ -treated control group; (C)  $\text{CCl}_4$ - and 100 mg/kg CCTEP-treated group; (D)  $\text{CCl}_4$ - and 200 mg/kg CCTEP-treated group; and (E)  $\text{CCl}_4$ - and 200 mg/kg silymarin-treated group. Data are presented as mean  $\pm$  standard deviation,  $n = 8$ . \* $p < 0.05$  as compared with the normal control group.

Table 1 – The effects of Chhit-Chan-Thau extract powder on the ratio of organ to body weight (BW) in  $\text{CCl}_4$ -induced liver injury rats.

Group	Ratio of liver to BW	Ratio of spleen to BW	Ratio of kidney to BW
A	$2.63 \pm 0.04$	$0.20 \pm 0.02$	$0.67 \pm 0.02$
B	$3.79 \pm 0.12^*$	$0.25 \pm 0.02$	$0.85 \pm 0.09^{**}$
C	$3.33 \pm 0.21^{**}$	$0.22 \pm 0.03$	$0.68 \pm 0.01^{***}$
D	$3.09 \pm 0.11^{***}$	$0.21 \pm 0.01$	$0.70 \pm 0.03^{***}$
E	$3.39 \pm 0.31^{**}$	$0.21 \pm 0.01$	$0.74 \pm 0.00$

The ratios of organ to body weight are expressed as mean  $\pm$  SE,  $n = 8$ .

\* $p < 0.01$  as compared with the control group.

\*\* $p < 0.05$  as compared with the control group.

\*\*\* $p < 0.05$  as compared with the  $\text{CCl}_4$  only group.

A = control group; B =  $\text{CCl}_4$ -treated group; C =  $\text{CCl}_4$ - and 100 mg/kg Chhit-Chan-Thau extract powder-treated group; D =  $\text{CCl}_4$ - and 200 mg/kg Chhit-Chan-Thau extract powder-treated group; E =  $\text{CCl}_4$ - and 200 mg/kg silymarin-treated group.

After the final experiment, the rats were sacrificed, and the liver, kidney, and spleen were weighed. Table 1 shows that the ratio of organ weight in Group B increased, but has restored in Groups C–E. The ratio of liver weight to body weight in Group B significantly increased as compared with Group A ( $p < 0.01$ ), whereas that in Groups C and D also slightly increased ( $p < 0.05$ ). However, in Group D, the ratio of liver weight to body weight restored as compared with Group B ( $p < 0.05$ ). The ratio of kidney weight to body weight in Group B significantly increased as compared with Group A ( $p < 0.05$ ), and restored in Groups C and D ( $p < 0.05$ ). The ratio of spleen weight to body weight did not show any remarkable difference in each group.

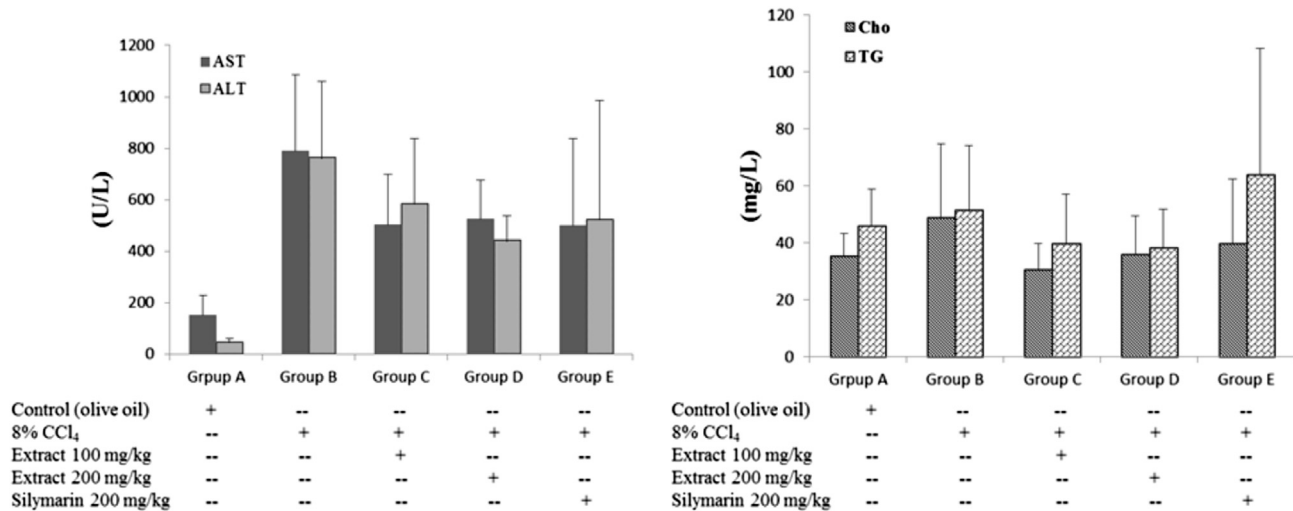
#### 3.2. Serum biochemistry examination

Measurements of serum biochemical markers were taken at the end of the experiment. Fig. 2 shows that the levels of triglycerine and cholesterol did not alter in all groups at 8 weeks. AST and ALT in the  $\text{CCl}_4$ -treated group increased markedly to  $761 \pm 149$  U/L and  $788 \pm 148$  U/L as compared with the control group at  $46 \pm 10$  U/L and  $151 \pm 45$  U/L, respectively. When cotreated with 100 mg/kg CCTEP, 200 mg/kg CCTEP (Fig. 2), or 200 mg/kg silymarin, the AST levels were restored to  $583 \pm 128$  U/L,  $440 \pm 50$  U/L, and  $522 \pm 267$  U/L, respectively, whereas the corresponding ALT levels were restored to  $504 \pm 98$  U/L,  $525 \pm 76$  U/L, and  $500 \pm 196$  U/L, respectively.

#### 3.3. Anti-oxidative enzyme activity

Effects of CCTEP on hepatic CAT and GSH levels for all experimental groups are shown in Table 2.  $\text{CCl}_4$  treatment caused a significant decrease of GSH level in liver tissue homogenates compared with the normal group. Pretreatment of 100 mg/kg CCTEP (C) enhanced the level of GSH compared with  $\text{CCl}_4$ -treated group. Treatment with CCTEP (200 mg/kg, D) prior to the  $\text{CCl}_4$  treatment elevated the GSH level. Similar results were obtained with silymarin (200 mg/kg, E).

The activity of liver CAT in the  $\text{CCl}_4$  treated rats (Group B) decreased significantly as compared with the control (Group



**Fig. 2** – The effects of *Chhit-Chan-Thhan* extract powder on serum AST, ALP, TG, and cholesterol in rats treated with CCl<sub>4</sub> for 8 weeks. *Chhit-Chan-Thhan* extract powder and CCl<sub>4</sub> were given as described in “Methods”. Data are presented as mean ± standard deviation, n = 8. \*p < 0.05 as compared with the normal control group. ALT = alanine amino transferase; AST = aspartamine amino transferase; Cho = cholesterol; TG = triglyceride.

A; p < 0.01; Table 2). The activity of liver CAT was restored in rats treated with CCTEP (100 mg/kg, Group C), CCTEP (200 mg/kg, Group D), and the silymarin (200 mg/kg, Group E).

### 3.4. Histopathologic study

The general liver morphological changes and fibrosis induced by CCl<sub>4</sub> administration were evidenced by both qualitative and quantitative histopathological examination. As compared with the normal rat liver, the tissue section in Figs. 3 and 4 show that the CCl<sub>4</sub>-induced chronic liver injury was concave on the liver surface and had lymphocyte infiltration in the

central vein. The hepatic cells were found to be cloudy, swelling, and necrotic, cytoplasmic vacuolization and fatty degeneration were observed in the centrilobular zone and mid zone. Masson trichrome stain clearly demonstrated that the CCl<sub>4</sub>-intoxicated liver became fibrotic with fiber extension and collagen accumulation (Fig. 5). These pathological changes (such as fibrosis and inflammation) were ameliorated by cotreatment of silymarin or CCTEP, which showed a dose-dependent effect (Figs. 4 and 5). The severity of the liver morphological changes and fibrosis induced by CCl<sub>4</sub> treatment were scored and summarized in Table 3. As shown, four out of eight CCl<sub>4</sub>-exposed rats had liver morphological change greater than Grade 3+, and five out of eight had liver fibrosis and necrosis greater than Grade 2. The CCTEP exposure greatly improved liver morphological changes, fibrosis, and necrosis. The average severity scores of the CCTEP-treated rats were markedly reduced in a dose-dependent manner.

**Table 2** – Effects of *Chhit-Chan-Thhan* extract powder on the liver glutathione levels and catalase activity after CCl<sub>4</sub>-treated in rats.<sup>a</sup>

Group	Glutathione (nmol/mg protein)	Catalase activity (IU/mg protein)
A	7.7 ± 0.4	8.2 ± 1.6
B	5.8 ± 0.4**	4.6 ± 0.1*
C	8.7 ± 0.9****	5.4 ± 0.3****
D	9.1 ± 0.4****	6.1 ± 0.3***
E	8.8 ± 0.9****	5.3 ± 0.2****

The liver GSH value and catalase activity are expressed as mean ± SE, n = 8.

\*p < 0.01 as compared with the control group.

\*\*p < 0.05 as compared with the control group.

\*\*\*p < 0.01 as compared with the CCl<sub>4</sub>-treated group.

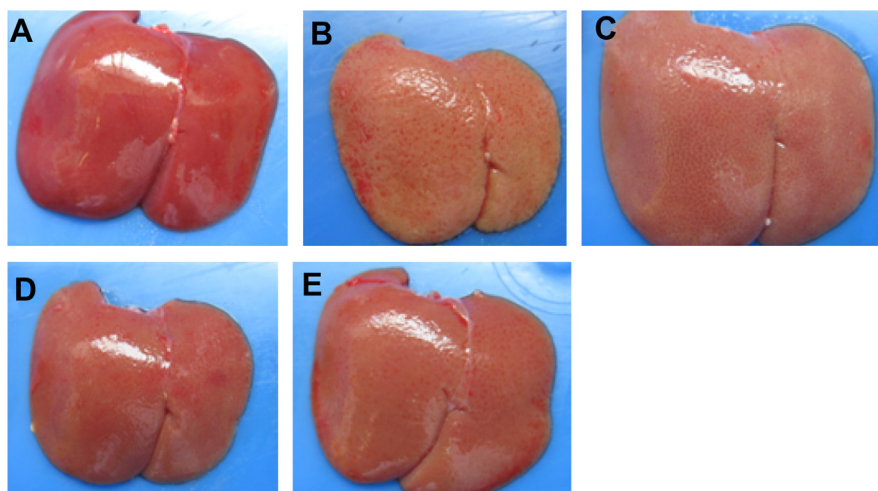
\*\*\*\*p < 0.05 as compared with the CCl<sub>4</sub>-treated group.

A = control group; B = CCl<sub>4</sub>-treated group; C = CCl<sub>4</sub>- and 100 mg/kg *Chhit-Chan-Thhan* extract powder-treated group; D = CCl<sub>4</sub>- and 200 mg/kg *Chhit-Chan-Thhan* extract powder-treated group; E = CCl<sub>4</sub>- and 200 mg/kg silymarin-treated group.

<sup>a</sup> *Chhit-Chan-Thhan* extract powder and CCl<sub>4</sub> are given as described in “Methods” in the main text.

## 4. Discussion

CCl<sub>4</sub> has been widely used in animal models to investigate chemical toxin-induced liver damage and as an excellent model to evaluate the efficacy of hepatoprotectants [37–39]. The ability of a hepatoprotective drug to reduce the injurious effects or to preserve the normal hepatic physiological mechanisms that have been disturbed by a hepatotoxin, is the index of its protective effects [40]. The purpose of this research program is to evaluate the hepatoprotective effects of CCTEP in CCl<sub>4</sub>-induced liver damage in Wistar rats. In this study, rats treated with CCl<sub>4</sub> developed significant hepatic damage as manifested by a significant increase in plasma activities of AST and ALT, which are indicators of hepatocyte damage and loss of functional integrity. The CCTEP treatment stimulated hepatoprotective effects that were proven by attenuating

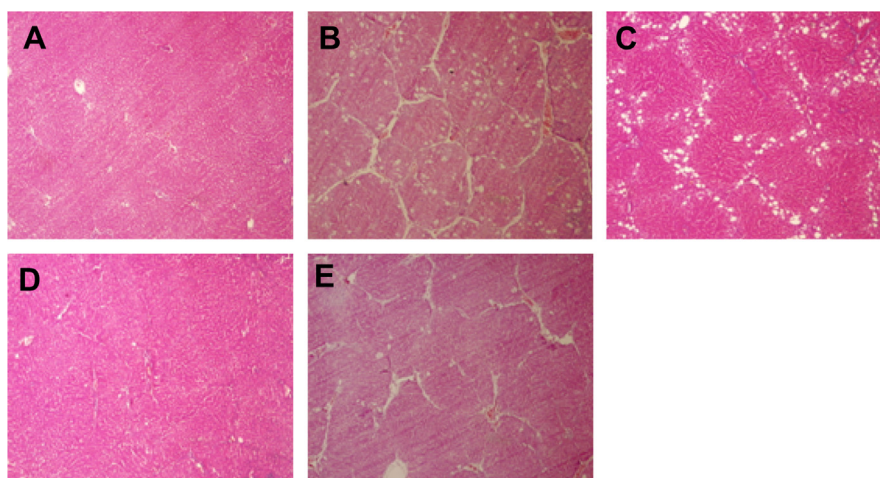


**Fig. 3** – Livers from  $\text{CCl}_4$ - and/or *Chhit-Chan-Than* extract powder (CCTEP)-treated rats. CCTEP and  $\text{CCl}_4$  were given as described in “Methods”. (A) Normal control group; (B)  $\text{CCl}_4$ -treated control group; (C)  $\text{CCl}_4$ - and 100 mg/kg CCTEP-treated group; (D)  $\text{CCl}_4$ - and 200 mg/kg CCTEP-treated group; and (E)  $\text{CCl}_4$ - and 200 mg/kg silymarin-treated group.

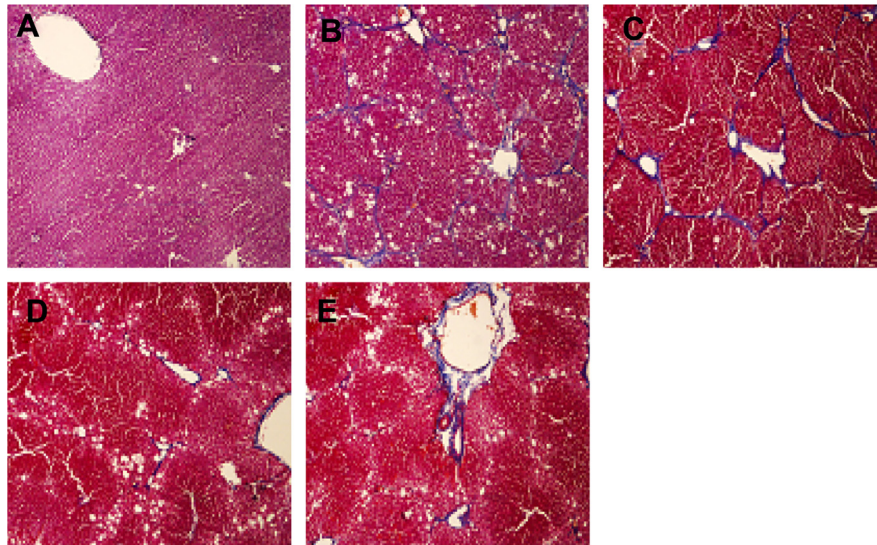
serum AST and ALT activities. The antioxidant activities of the CCTEP can ameliorate oxidative stress contributing to the amount of intracellular antioxidant enzymes, which was confirmed by GSH and CAT assay. Centrilobular necrosis, lymphocytes infiltration and steatosis were apparent in  $\text{CCl}_4$ -treated group, whereas administration of the CCTEP significantly reverses these abnormal indexes. This indicates that CCTEP can ameliorate oxidative stress to preserve hepatic function and hepatic injury induced by  $\text{CCl}_4$ .

Free radicals/reactive oxygen species (ROS) and oxidative stress play a central role in liver disease pathology and progression [1,2]. Cellular injury occurs when ROS generation exceeds the cellular capacity of removal [41]. The cleavage of  $\text{CCl}_4$  leads to the formation of highly unstable free radicals ( $\text{CCl}_3\bullet$  or  $\text{CCl}_3\text{O}_2\bullet$ ), to initiate peroxidation [26]. The oxidant species may in turn trigger the signal transduction pathways

involved in the antioxidant responses. Antioxidant enzymes (CAT, superoxide dismutase, glutathione peroxidase, etc.) represent one protection system against oxidative tissue-damage [42]. CAT is known to break  $\text{H}_2\text{O}_2$  down to  $\text{H}_2\text{O}$  and  $\text{O}_2$  and can be found in the peroxisome and mitochondria, especially in liver. Superoxide dismutase removes superoxide radicals by converting them into  $\text{H}_2\text{O}_2$ , which, in turn, can be rapidly converted into water by catalase and glutathione peroxidase. Thus, the inhibition of free radicals generation retards  $\text{CCl}_4$ -induced lipid peroxidation. In the present study, antioxidant enzymes activities in the liver of  $\text{CCl}_4$ -treated group rats were significantly lower than those in the normal control group. Previous studies have indicated that  $\text{CCl}_4$  reduces the activities of antioxidant enzymes and causes hepatopathy [26]. Interestingly, impaired hepatic antioxidant enzymes activities were brought back via the treatment of



**Fig. 4** – Effects of *Chhit-Chan-Than* extract powder (CCTEP) on liver pathologic analysis by hematoxylin–eosin stain after  $\text{CCl}_4$  treatment in rats. CCTEP and  $\text{CCl}_4$  were given as described in “Methods”. (A) Normal control group; (B)  $\text{CCl}_4$ -treated control group; (C)  $\text{CCl}_4$ - and 100 mg/kg CCTEP-treated group; (D)  $\text{CCl}_4$ - and 200 mg/kg CCTEP-treated group; and (E)  $\text{CCl}_4$ - and 200 mg/kg silymarin-treated group (original magnification: 40 $\times$ ).



**Fig. 5** – Effects of *Chhit-Chan-Than* extract powder (CCTEP) on liver pathologic analysis by Masson's stain after  $\text{CCl}_4$  treatment in rats. CCTEP and  $\text{CCl}_4$  were given as described in "Methods". (A) Normal control group; (B)  $\text{CCl}_4$ -treated control group; (C)  $\text{CCl}_4$ - and 100 mg/kg CCTEP-treated group; (D)  $\text{CCl}_4$ - and 200 mg/kg CCTEP-treated group; and (E)  $\text{CCl}_4$ - and 200 mg/kg silymarin-treated group.

CCTEP to  $\text{CCl}_4$ -treated rats. Our present works clearly showed an elevation of CAT level in liver, suggesting that it can restore these antioxidant enzymes and/or activate enzyme activity in the damage caused by  $\text{CCl}_4$ .

GSH is believed to be a highly effective extra- and intracellular antioxidant compound that neutralizes  $\text{H}_2\text{O}_2$  and hydroperoxides by its scavenging and antioxidant properties [43]. In this study, GSH content in the liver of  $\text{CCl}_4$ -intoxicated rats was declined by  $\text{CCl}_4$  treatment and its subsequent enhancement revealed antioxidant effects of CCTEP. A number of studies have revealed that GSH conjugates play a major role in eliminating the  $\text{CCl}_4$ -induced toxic metabolites that are the main cause of liver injuries. The maintenance of sufficient glutathione level is important for the prevention of  $\text{CCl}_4$ -induced damages. Toxicants that deplete GSH or influence the

activity of GSH-dependent enzyme(s) may result in toxic responses. The study herein presented was instigated by other reports [44] where  $\text{CCl}_4$  administration caused depletion in GSH contents. The mechanism of hepatoprotection by CCTEP against the  $\text{CCl}_4$  toxicity might be partially due to the restoration of GSH concentration in liver.

$\text{CCl}_4$  is metabolized by cytochrome P450 2E1 to the trichloromethyl radical ( $\text{CCl}_3\bullet$ ), which is assumed to initiate free radical-mediated lipid peroxidation leading to the accumulation of lipid-derived oxidation products that cause liver injury [45]. Alternatively, oxidative stress generated from cytochrome P450-catalyzed metabolism of xenobiotics can result in protective cellular responses, leading to the enhancement of cell survival upon noxious stimuli [46]. Studies have indicated that some phytochemicals are found to regulate

**Table 3** – The effects of *Chhit-Chan-Than* extract powder on histopathological evidence of  $\text{CCl}_4$ -induced liver fibrosis rats.<sup>a</sup>

Group	Severity score of fatty metamorphosis (incidence)					Severity score of fatty hepatic fibrosis (incidence)						
	N	0	1	2	3	Mean ± SE	0	1	2	3	4	Mean ± SE
A	8	8	0	0	0	0.0 ± 0.0	8	0	0	0	0	0.0 ± 0.0
B	8	1	2	1	4	2.0 ± 0.4*	0	1	4	3	0	2.3 ± 0.3****
C	8	1	1	3	3	2.0 ± 0.4*	2	4	2	0	0	1.0 ± 0.3****
D	8	3	3	2	0	2.0 ± 0.4*	5	2	1	0	0	0.5 ± 0.3***
E	8	2	4	2	0	1.0 ± 0.3*	4	3	1	0	0	0.6 ± 0.3***

Data represent the number of rats rated with a given level of fatty metamorphosis or hepatic fibrosis; see "Methods" in the main text for additional details.

\* $p < 0.01$  as compared with the control group.

\*\* $p < 0.05$ .

\*\*\* $p < 0.01$  as compared with the  $\text{CCl}_4$  only group by the Student t test.

\*\*\*\* $p < 0.05$ .

SE = standard error.

<sup>a</sup> *Chhit-Chan-Than* extract powder and  $\text{CCl}_4$  are given as described in "Methods" in the main text.

cytochrome P450 enzymes and demonstrate hepatoprotective effects, especially on the specific protein activity by mRNA expression [47,48]. The present study has found that CCTEP contributes to the amount of catalase and GSH inside the liver after chronic CCl<sub>4</sub> treatment (Table 2). Catalase is known to breakdown H<sub>2</sub>O<sub>2</sub>, the downstream product of free radical metabolic cascade, and thus protect liver from oxidative stress. In a previous study, we clearly demonstrated *in vitro* OGE's powerful antioxidant activity with 80% scavenging effect, which is as effective as  $\alpha$ -tocopherol, as well as lowering thiobarbituric acid-reactive substances (TBARS) in H<sub>2</sub>O<sub>2</sub>-treated HepG2 cells [21]. Taken together with the present study, it can therefore be claimed that CCTEP lowers oxidative stress-induced liver injury by increasing antioxidants inside the liver. Induction of GSH levels may be due to the activation of GSH synthesizing enzymes such as  $\gamma$ -glutamylcysteine synthetase and GSH synthetase, which are key enzymes in the biosynthesis of GSH [49]. These results could indicate that CCTEP either inhibits ROS induced lipid peroxidation, or directly lowers ROS production, possibly through the inhibition of CYP2E1 activity. Some antioxidants, such as Vitamin C and E or garlic acid, are shown to possess similar mechanisms [50,51].

For the therapeutic strategies of liver injury and disease, it is important to find antioxidant compounds that are able to block liver injuries through free radicals generated by toxic chemicals. Therefore, the present study speculated that the aqueous *O. gratissimum* extract protects against diseases that are caused by oxidative stress because it is rich in polyphenols and has excellent radical scavenging ability. Our preliminary phytochemical screening discovered a significant quantity of simple phenols and flavonoids inside OGE, including catechin, caffeic acid, epicatechin, and rutin [12,16,20,29]. Other researchers have reported that green tea catechin is capable of inhibiting the activation of hepatic stellate cells (HSCs), which leads to liver fibrosis by lowering the secretion of liver fibril-forming collagen [52], rutin is capable of lowering liver oxidative damage [53–55], and caffeic acid is capable of treating liver cirrhosis by suppressing H<sub>2</sub>O<sub>2</sub> formation [56]. Primarily, the hepatoprotective effects of these phytoconstituents may inhibit lipid peroxidation in the liver, which suggests that it may prevent free radical damage and thus protect the liver from oxidative stress [57].

In the present study, although CCTEP could not completely repair the hepatic injury induced by CCl<sub>4</sub>, it is possible that the CCl<sub>4</sub> dose at the late stage was too high so that the liver damage could not be restored. The trend of protective effects on AST and ALT levels between CCl<sub>4</sub>-control group (Group B) and interventional groups (Groups C–E) is clearly demonstrated in Fig. 2 and described in the Section 3.2. "Serum biochemistry examination". However, the evident reduction of values cannot reach significance by ANOVA analysis (due to the small experimental animal number and data variance). Thus, the transaminase-restored effects of CCTEP and silymarin were hard to clarify completely in this study. Fortunately, the diagnostic value of histopathologic morphology in long-term hepatic injury is more meaningful than that of AST or ALT in the acute stage. In a chronic CCl<sub>4</sub>-treated rat model, progressive scarring of the liver (fibrogenesis) is characterized by an excessive deposition of extracellular matrix (ECM)

proteins (predominantly types I and III collagen) due to the activation of HSCs. Our laboratory also demonstrated that OGE possesses an antiliver fibrosis property by inhibiting *in vitro* HSCs activation, as well as effectively suppressing the expression of  $\alpha$ -smooth muscle actin and type I collagen using an *in vivo* model (unpublished data).

CCTEP used in this study was developed from aqueous OGE for further commercial products such as oral capsules or beverage power package. The powder was made with 10% OGE and 90% dextrin as an excipient. Several products and phytoconstituents deriving from *O. gratissimum* have been investigated by different models and protocols *in vitro* or *in vivo* in our laboratory [12,16,20,21,29,58]. The hepatoprotective efficacy of OGE has already been reported by our research group [21,29], as well as the proposal molecular mechanisms [29]. The motive of the present study was to explore the newly-developed product with similar protective property, but less molecular mechanism is discussed. The hepatoprotective effects of *O. gratissimum* extract were associated not only with antioxidative mechanisms but also with inflammatory pathways, cell death pathways, and activation of HSCs, which are discussed elsewhere. Our previous works [29] demonstrated the beneficial effects of OGE in acute hepatic injury *in vivo* and found that significantly decreased stress proteins including HSP70 and iNOS were observed. The antioxidant activities of OGE can directly ameliorate oxidative stress by inhibiting lipid peroxidation and contributing to the levels of intracellular antioxidant enzymes, which were confirmed by TBARS assay and antioxidant assay (unpublished data). The cytoprotective property may also be achieved by regulating Bcl-2 expression and Akt phosphorylation, and slightly affecting the phosphorylation of mitogen-activated protein kinases including p38MAPK and JNK that reduce proinflammatory mechanisms [12,21].

Studies indicate that different doses of drugs may have different physiologic or pharmacologic effects [59], and low doses may be ineffective due to low absorption in the gastrointestinal tract [60]. Previous studies have established that silymarin, the current leading hepatoprotective herbal extract, works best at 0.2 g/kg body weight [6,61]. By contrast, another study has shown that silymarin failed to reverse fibrosis significantly, which was previously established by prolonged chronic CCl<sub>4</sub> administration in rats when compared with an appropriate vehicle control [62]. This difference in results may be due to the diverse doses of herbal extract used. Previous studies reported that dried flower *Hibiscus sabdariffa* L. extract treatment works ideally by being mixed in with a regular diet at 5% of the diet [63], aqueous extract of *Anoectochilus formosanus* has been reported to work at 2.0 g/kg body weight [64], and *Ginkgo biloba* extract has shown promising results at 0.5 g/kg body weight [65]. In the present study, the suppression of CCTEP at 200 mg/kg body weight was similar to that of silymarin at the same dose. This demonstrates the potency of CCTEP and suggests that *O. gratissimum* may have the potential to be an herb choice in the treatment of chronic liver disease.

In conclusion, the present study showed that CCTEP (10% aqueous *O. gratissimum* L. extract) has a remarkable protective effect against CCl<sub>4</sub>-induced liver damage. The micronutrient functions of *O. gratissimum* may be as an effector of important



cellular stress response pathways that ultimately influence endogenous cellular antioxidant levels. The inhibitory effects of CCTEP may have therapeutic achievements in chemical-induced hepatic injury *in vivo*, and thus dietary CCTEP may be useful as a hepatoprotective agent against chronic liver disease in humans.

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