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Caffeic acid rich *Citrus macroptera* peel powder supplementation prevented oxidative stress, fibrosis and hepatic damage in CCl_4 treated rats

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Abstract

Background: *Citrus macroptera* has been used as a culinary fruit and medicinal plant in traditional medicine system in Bangladesh. The aim of the present study was to evaluate the presence of phenolic compounds in *Citrus macroptera* peel powder and the protective effect of *Citrus macroptera* against carbon tetrachloride (CCl_4)-induced liver injury in rats.

Methods: The hepatoprotective activity was assessed using various biochemical parameters such as liver marker enzymes (alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP)) and oxidative stress parameters. Histopathological changes in the liver of different groups were also studied.

Results: Administration of CCl_4 increased the serum ALT, AST, ALP enzymatic activities and lipid peroxidation products but decreased the cellular antioxidant activities and reduced glutathione (GSH) levels in rats which were brought back to near normal levels by the treatment with *Citrus macroptera*. *Citrus macroptera* administration has also shown to decrease the necrotic zones, fibrosis and inflammatory cell infiltration in CCl_4 treated rats. HPLC-DAD analysis of *Citrus macroptera* extract showed the great presence of caffeic acid and (−) epicatechin.

Conclusion: The results of this study suggest that *Citrus macroptera* exerts hepatoprotective activity via promoting the antioxidant defense against CCl_4-induced oxidative liver damage.

Keywords: *Citrus macroptera*, Oxidative stress, Fibrosis, Inflammation, Caffeic acid

Background

Hepatotoxicity is a growing public health concern in modern society due to the increasing incidence of alcoholism, cigarette smoking, drug abuse and other unhealthy lifestyle options such as consuming high fructose-containing beverages and high fat containing foods. Liver damage is characterized by a progressive development of steatosis to chronic hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma [1, 2]. Oxidative stress plays a central role in the development of liver diseases. Carbon tetrachloride (CCl_4) is a widely used solvent in chemical industries and well known for its hepatic and renal toxic actions. It is also used to establish experimental animal model of hepatic dysfunction in the laboratory [3, 4]. The metabolism of CCl_4 occurs mainly in the liver by CYP450 enzyme system into trichloromethyl ($^{\bullet}CCl_3$) and peroxy trichloromethyl ($^{\bullet}OOCCl_3$) free radicals which have been reported to cause hepatotoxic effects, like fibrosis, steatosis, necrosis, and hepatocarcinoma [3, 5]. Chronic insult to the liver due to hepatotoxins, alcohol consumption and high-calorie diet triggers inflammation and fibrosis [4]. Liver fibrosis is a dynamic process where oxidative stresses are responsible for the activation of hepatic stellate cells (HSCs) [6]. HSCs are the major cell types responsible for the deposition of a large amount of extracellular matrix

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(ECM) and collagen in liver [7]. CCl_4-mediated liver fibrosis is characterized by activation of Kupffer cells and induction of an inflammatory response by secreting cytokines, chemokines and other pro-inflammatory factors [8]. CCl_4 treatment also attracts more inflammatory cells in liver apart from Kuffer cells and further contributes to liver necrosis [8]. High level of lipid peroxidation products and decreased antioxidant levels were also noted in CCl_4 mediated hepatic dysfunction in animal [9]. As oxidative stress plays a central role in liver pathologies and their progression, the use of antioxidants would have been an alternative therapeutic approach to counteract the liver damage. Citrus fruits are a rich source of natural antioxidants and showed beneficial role in various degenerative diseases [10]. Caffeic acid was found in high amount compared to other phenolic compounds in *Citrus macroptera* peel which is a strong antioxidant compound [11]. Caffeic acid also showed hepatic protection and prevented fibrosis in various experimental animal models [12–14]. This hepatoprotective activity of caffeic acid is linked to improved antioxidant defense and inflammatory state in liver [11, 15].

Citrus macroptera is known as 'Satkara' in Bengali and 'Wild orange' in English. It is a semi-wild species of citrus fruits which is native to the regions of Southeast Asia and found in large amount in Sylhet Division of Bangladesh. The fruit is used as a cooking ingredient in different kinds of meats and as an aromatic vegetable in Bangladesh. Meats cooked with satkara are now served in many Bangladeshi/Indian restaurants in the United Kingdom. *Citrus macroptera* (Satkara) fruit is also used as an appetite stimulant and in the treatment of fever as reported in the traditional system of medicine in Bangladesh. The previous report suggested that *Citrus macroptera* contains mainly lupeol and stigmasterol [16]. Other studies conducted on the bark and leaves of the *Citrus macroptera* reported the antioxidant and antimicrobial activities [16, 17]. A recent investigation also suggests that *Citrus macroptera* extract showed in-vitro α-amylase inhibitory activity and hypoglycemic activity in normal rats [18]. We recently reported the hepatoprotective activity of *Citrus maxima* peel powder in CCl_4 treated rats [19]. However, limited data and few scientific literatures are available on any therapeutic effect of *Citrus macroptera* in liver diseases. Therefore, this current research was designed to understand the possible anti-inflammatory and anti-fibrotic activity of *Citrus macroptera* peel powder in hepatic dysfunction in CCl_4 treated rats.

Methods

Chemicals

Arbutin (AR), gallic acid (GA), hydroquinone (HQ), (+)-catechin hydrate (CH), vanillic acid (VA), caffeic acid (CA), Syringic acid (SA), (−)-epicatechin (EC), vanillin (VL), *p*-coumaric acid (PCA), *trans*-ferulic acid (FA), rutin hydrate (RH), ellagic acid (EA), benzoic acid (BA), rosmarinic acid (RA), myricetin (MC), quercetin (QU), *trans*-cinnamic acid (TCA), and kaempferol (KF) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC), methanol (HPLC), acetic acid (HPLC), and ethanol were obtained from Merck (Darmstadt, Germany).

Plant material

Citrus macroptera fruits were collected from Sylhet, Bangladesh and authenticated by Sarker Nasir Uddin, Senior Scientific Officer, National Herbarium, Mirpur, Dhaka. A voucher specimen (Acc. No. 40847) was deposited in the herbarium for future reference. The peels were removed and dried for further processing. The dried peels were grinded to fine powder using an electric grinder machine and mixed with the food directly as supplementation. A portion of the dried peel powder was used to prepare crude ethanol extract and used for polyphenol detection and quantification.

High performance liquid Chromatograpgy (HPLC) detection and quantification of polyphenolic compounds

Detection and quantification of selected phenolic compounds in the ethanol extract were determined by HPLC-DAD analysis as described by Ismet et al. [20] with some modifications. It was carried out on a Dionex UltiMate 3000 system equipped with quaternary rapid separation pump (LPG-3400RS) and photodiode array detector (DAD-3000RS). Separation was performed using Acclaim® C_{18} (5 μm) Dionex column (4.6 × 250 mm) at 30 °C with a flow rate of 1 ml/min and an injection volume of 20 μl. For the preparation of calibration curve, a standard stock solution was prepared in methanol containing arbutin, (−)-epicatechin (5 μg/ml each), gallic acid, hydroquinone, vanillic acid, rosmarinic acid, myricetin (4 μg/ml each), caffeic acid, Syringic acid, vanillin, *trans*-ferulic acid (3 μg/ml each), *p*-coumaric acid, quercetin, kaempferol (2 μg/ml each), (+)-catechin hydrate, ellagic acid (10 μg/ml each), *trans*-cinnamic acid (1 μg/ml), rutin hydrate (6 μg/ml) and benzoic acid (8 μg/ml). The UV detector was set to 280 nm for 22. 0 min, changed to 320 nm for 28.0 min, again change to 280 nm for 35 min and finally to 380 nm for 36 min and held for the rest of the analysis period while the diode array detector was set at an acquisition range from 200 nm to 700 nm.

Animals and treatment

Ten to 12 weeks old, 24 Long Evans female rats (150–170 g) were obtained from Animal breeding unit of Animal House at the Department of Pharmaceutical Sciences, North South University and were kept in individual cages

at room temperature of 25 ± 3 °C with a 12 h dark/light cycles. They had free access to standard laboratory feed and water, according to the study protocol approved by Ethical Committee of Department of Pharmaceutical Sciences, North South University for animal care and experimentation. To evaluate the hepatoprotective effect of *Citrus macroptera*, rats were equally divided into four groups (six rats in each group). The groups are as follows-

Group I- Animals were treated with 1 ml/kg of saline (0.85%) and olive oil (1 ml/kg) intragastrically twice a week for 2 weeks
Group II- Animals were treated with 1 ml/kg of saline (0.85%) and olive oil (1 ml/kg) intragastrically twice a week for 2 weeks. Animals of group II also received *Citrus macroptera* fruit peel powder supplementation mixed with food (0.5% of the diet, *w*/w).
Group III – Animals were treated with CCl_4 (1:3 in olive oil) at a dose of 1 ml/kg intragastrically twice a week for 2 weeks.
Group IV- Animals were treated with CCl_4 (1:3 in olive oil) at a dose of 1 ml/kg intragastrically twice a week for 2 weeks. Animals of group IV also received *Citrus macroptera* fruit peel powder mixed in food (0.5% of the diet, w/w) respectively, in addition to CCl_4 treatment, twice a week for 2 weeks.

Animals of group I were served as control animals. Animals were checked for the body weight, food and water intake on a daily basis. After 14 days, all animals were weighted and sacrificed, collected the blood in citrate buffer containing tubes and all the organs such as heart, kidney, spleen, and liver were havested. Immediately after collection of the organs, they are weighted and one part was stored in neutral buffered formalin (pH 7.4) for histological analysis and another part was kept in the refrigerator at − 20 °C for further studies. The collected blood was centrifuged at 8000 rpm and separated the plasma and stored in a refrigerator at − 20 °C for further analysis.

Assessment of hepatotoxicity

Liver marker enzymes (alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were estimated in plasma by using Diatec diagnostic kits (Hungary) according to the manufacturer's protocol.

Preparation of tissue sample for the assessment of oxidative stress markers

For determination of oxidative stress markers, 0.2 g of liver tissue was homogenized in 1.8 mL Phosphate buffer (pH 7.4) and centrifuged at 10000 rpm for 30 min at 4 °C. The supernatant was collected and used for the determination of protein and enzymatic studies as described below.

Estimation of lipid peroxidation

Lipid peroxidation in liver was estimated colorimetrically measuring thiobarbituric acid reactive substances (TBARS) followed by previously described method [21].

Assay of nitric oxide (NO)

Nitric oxide (NO) was determined according to the method described by Tracey et al. as nitrate [22]. In this study, Griess-Illosvoy reagent was modified by using naphthyl ethylenediamine dihydrochloride (0.1% *w*/*v*) instead of 1-naphthylamine (5%).

Advanced protein oxidation products (APOP) assay:

APOP levels were measured by a modification of the method of Witko-Sarsat et al. [23] and Tiwari et al. [24]. APOP concentrations were expressed as nmol·mL^{-1} chloramine-T equivalents.

Catalase assay (CAT)

CAT activities were determined using previously described the method by Chance and Maehly [25]. One unit of CAT activity was defined as an absorbance change of 0.01 as units/min.

Reduced glutathione assay (GSH)

Reduced glutathione was estimated by the method of Jollow et al. [26]. 1.0 ml sample of 10% homogenate was precipitated with 1.0 ml of (4%) sulfosalicylic acid. The samples were kept at 4 °C for 1 h and then centrifuged at 4000 rpm for 20 min at 4 °C. The total volume of 3. 0 ml assay mixture composed of 0.1 ml filtered aliquot, 2.7 ml phosphate buffer (0.1 M, pH 7.4) and 0.2 ml DTNB (5,5-dithiobis-2-nitrobenzoic acid), (100 mM). The mixture which was developed as yellow color, UV absorbance was taken immediately at 412 nm on a Smart SpecTM plus Spectrophotometer and the content of GSH was expressed as ng/mg protein.

Histopathological determination

For microscopic evaluation, liver tissues were fixed in neutral buffered formalin and embedded in paraffin, sectioned at 5 μm and subsequently stained with hematoxylin/eosin to see the architecture of hepatic tissue and inflammatory cell infiltration. Liver injury score in the Hematoxylin/eosin stained sections were evaluated as follows: 0, minimal or no evidence of injury; 1, mild injury consisting of cytoplasmic vacuolation and focal nuclear pyknosis; 2, moderate to severe injury with extensive nuclear pyknosis and loss of intercellular borders and 3, severe necrosis with disintegration of hepatic cords, hemorrhage and

neutrophil infiltration. All evaluations were made in 3 fields per section and 3 sections per liver.

Sirius red staining for fibrosis and Prussian blue staining for iron deposition were also done in liver sections. Sections were then studied and photographed under a light microscope (Zeiss Axioscope) at 40 magnifications. Collagen deposition was semiquantitatively measured using NIH Image J free software (Version 1.48v).

Statistical analysis

All values are expressed as a mean ± standard error of the mean (SEM). The results were evaluated by using the One-way ANOVA followed by Bonferroni test using Graph Pad Prism Software, version 6. Statistical significance was considered $p < 0.05$ in all cases.

Results

Effect on body weight, food, and water intake

Body weight of each rat was recorded every day during the experiment, and % change was calculated for all groups. It was found that the body weight decreased significantly in CCl_4-intoxicated rat group, which is a typical feature of chronic liver intoxication. On the other hand, treatment of CCl_4 treated group with *Citrus macroptera* markedly improved the weight loss of rats (Table 1). CCl_4 treated rats group showed significant decrease in food, and water intake compared to control rats. Reduction of food and water intake in CCl_4 treated rats group was further improved in *Citrus macroptera* treated group (Table 1).

Effect on organ wet weight

Table 1 shows the effect of various treatments on the rats' organs weight. The spleen wet weight was significantly ($p = 0.012$) increased in the CCl_4-treated rats compared to control rats. *Citrus macroptera* (0.5% per kg of diet) treatment did not significantly attenuate the wet weight of the spleen in the CCl_4-treated rats. CCl_4-treated rats also showed decreased in liver wet weight; however, *Citrus macroptera* supplementation did not change the wet weight of the liver compared to the control. Another crucial finding in this study was the reduction of kidney wet weight due to CCl_4 intoxication which was normalized by *Citrus macroptera* supplementation (Table 1). Wet weights of the heart were relatively unchanged among the groups tested in this study (Table 1).

Effect on biochemical parameter of liver functions

Biochemical assay of liver function markers revealed that CCl_4 administration in rats induced a significant ($p < 0.05$) increase in plasma AST ($p = 0.036$), ALT ($p < 0.0001$), and ALP ($p < 0.0001$) activity compared to control rats, respectively (Table 2). *Citrus macroptera* (0.5% of diet) supplementation concurrently with CCl_4 significantly ($p < 0.05$) counteracted the alteration in all hepatotoxicity indices compared to the CCl_4-treated group. In addition, supplementation with *Citrus macroptera* alone in the diet for 2 weeks in normal control rats did not show any significant change in liver enzymes compared to the control rats (Table 2).

Oxidative stress markers and antioxidant enzymes

To determine the oxidative stress in our study, we evaluated the MDA, nitric oxide and APOP concentration in plasma and liver homogenates. CCl_4 administration in rats showed an increased lipid peroxidation product, MDA concentration both in plasma ($p < 0.0001$) and in liver ($p = 0.0004$) homogenates significantly ($p < 0.05$) (Table 2). Additionally, *Citrus macroptera* (0.5% of diet) supplementation significantly ($p < 0.05$) reduced the level of lipid peroxides compared to CCl_4 intoxicated group.

CCl_4 administration in rats also increased APOP development in plasma and in liver compared to control rats significantly ($p < 0.0001$). *Citrus macroptera* (0.5% of diet) supplementation in CCl_4 intoxicated rats significantly ($p <$

Table 1 Effect of *Citrus macroptera* peel powder supplementation on body weight, food and water intake and organ weight of CCl_4 treated rats

Parameters	Control	Control+*Citrus macroptera*	CCl_4	CCl_4 + *Citrus macroptera*
Initial Bodyweight (g)	156.07 ± 2.38	165.63 ± 2.99 ns	162.92 ± 4.81nss	162.67 ± 1.52 ns
Final Bodyweight (g)	167.37 ± 4.05	182.40 ± 5.95 ns	165.92 ± 5.23 ns	174.20 ± 3.16 ns
Food intake/d (g/day)	16.49 ± 0.88	15.68 ± 0.97a	12.21 ± 0.54a	15.03 ± 0.57b
Water intake/d (ml/day)	18.85 ± 0.62	20.11 ± 1.20 ns	13.44 ± 0.73a	15.20 ± 0.78b
Liver wet weight (g/100 g of body weight)	3.67 ± 0.15	3.68 ± 0.06 ns	3.32 ± 0.09 ns	3.47 ± 0.05 ns
Kidneys wet weight (g/100 g of body weight)	0.62 ± 0.02	0.56 ± 0.04 ns	0.53 ± 0.02a	0.62 ± 0.01b
Heart wet weight (g/100 g of body weight)	0.34 ± 0.01	0.28 ± 0.00	0.31 ± 0.02	0.30 ± 0.01
Spleen wet weight (g/100 g of body weight)	0.31 ± 0.03	0.39 ± 0.02 ns	0.46 ± 0.04a	0.41 ± 0.02 ns

Values are presented as mean ± SEM. $N = 6$ in each group or otherwise specified. One way ANOVA with Bonferoni tests were done as post hoc test. Values are considered significance at $p < 0.05$. control vs CCl_4 significanltly different at a < 0.05. CCl_4 vs *Citrus macroptera* treatment which are significantly different at b < 0.05. ns- non significant

Table 2 Effect of *Citrus macroptera* peel powder supplementation on biochemical parameters in plasma and liver of CCl_4 treated rats

Parameters	Groups			
	Control	Control+*Citrus macroptera*	CCl_4	CCl_4 + *Citrus macroptera*
Plasma				
AST(U/L)	34.45 ± 3.85	35.89 ± 3.46 ns	65.46 ± 8.87a	47.37 ± 4.85b
ALT(U/L)	30.15 ± 2.94	31.58 ± 2.87 ns	66.04 ± 2.87a	43.07 ± 5.88b
ALP(U/L)	57.60 ± 1.38	33.76 ± 4.95b	80.35 ± 5.58a	37.57 ± 7.21b
MDA(nmol/mL)	7.27 ± 0.19	11.01 ± 0.47 ns	17.27 ± 1.79a	9.81 ± 0.42b
NO (nmol/mL)	3.87 ± 0.27	4.43 ± 0.70 ns	6.80 ± 0.65a	3.42 ± 0.61b
APOP (nmol/mL equivalent to Chloramine-T)	228.33 ± 10.66	326.35 ± 56.23 ns	736.27 ± 54.83a	347.38 ± 26.18b
GSH (ng/mg protein)	11.85 ± 0.51	11.25 ± 0.74 ns	7.50 ± 0.40a	13.63 ± 1.22b
Catalase (U/min)	7.17 ± 0.79	10.50 ± 1.20 ns	5.33 ± 1.05 ns	7.00 ± 1.06 ns
Liver				
NO (nmol/mL)	14.12 ± 0.91	12.35 ± 0.92 ns	15.87 ± 2.23 ns	12.47 ± 1.20 ns
MDA (nmol/mL)	38.77 ± 1.84	40.31 ± 2.02 ns	64.41 ± 4.89ba	51.33 ± 5.13 ns
APOP (nmol/mL equivalent to Chloramine-T)	632.06 ± 33.84	497.78 ± 29.20 ns	968.57 ± 70.27a	566.43 ± 48.60b
Catalase (U/min)	20.83 ± 3.52	24.17 ± 3.00 ns	14.17 ± 2.01a	23.33 ± 2.47b

Values are presented as mean ± SEM. N = 6 in each group or otherwise specified. One way ANOVA with Bonferroni tests were done as post hoc test. Values are considered significant at $p < 0.05$. Values are considered significance at $p < 0.05$. control vs CCl_4 significanltly different a < 0.05. CCl_4 vs *Citrus macroptera* treatment which are significantly different at b < 0.05. ns- non significant. APOP-Advanced protein oxidation product expressed as nmol/mL equivalent to Chloramine-T

0.05) reduced the APOP concentration in plasma and liver tissue homogenates significantly (Table 2).

NO measured as nitrate was also increased significantly ($p = 0.0027$) in plasma of CCl_4 administered rats compared to control rats (Table 2). *Citrus macroptera* (0.5% of diet) supplementation (Table 2) in CCl_4 administered rats normalized the elevated the NO level in plasma. However, NO level was not changed significantly in liver homogenates of CCl_4 administered rats compared to control rats. This effect could be attributed to the rapid excretion of nitrate into the plasma from liver tissues.

CCl_4 administration also decreased plasma antioxidant such as GSH concentration ($p = 0.0002$) compared to the control rats (Table 2). *Citrus macroptera* (0.5% of diet) supplementation in CCl_4 administered rat significantly counteracted the oxidative stress by restoring the antioxidant GSH concentration compared to the CCl_4 administered rats (Table 2). Moreover, catalase activity was decreased in CCl_4 administered rats compared to control rats. However, the changes were not statistically significant. *Citrus macroptera* (0.5% of diet) supplementation in CCl_4 administered rat increased the catalase activity compared to the CCl_4 administered rats (Table 2). *Citrus macroptera* (0.5% of diet) supplementation in control rats did not alter the catalase activity compared to control rats (Table 2).

Inflammation, fibrosis iron deposition in liver

Figure 1a showed the well-formed hepatocytes in an intact hepatic lobule of normal rat liver. However, inflammation was seen in rats treated with CCl_4. A massive surge of inflammatory cells was found in the centrilobular part of liver sections stained Researchers are still working on natural plants to discover new compounds of high biological activitystaining in CCl_4 treated rats group (Fig. 1c). Necrotized tissue scar in liver was also seen in the liver of CCl_4 treated rats (Fig. 1c). *Citrus macroptera* (0.5% of diet) supplementation attenuated the inflammatory cell infiltration and necrosis in the liver tissues of CCl_4 treated rats (Fig. 1d). Liver fibrosis was evaluated histologically by visualizing the red color of collagen fibers using Sirius red stain. No collagen deposition was observed in control rats (Fig. 2a). Collagen fibers were heavily deposited around portal tracts and central veins in CCl_4-intoxicated rat's liver and extended from central vein to portal tract resulting in the formation of pseudolobules (Fig. 2c). *Citrus macroptera* (0.5% of diet) supplementation prevented the deposition of collagen fibers in the liver of CCl_4 treated rats (Fig. 2d).

Histological staining also revealed that no free iron was deposited in the liver of control rats (Fig. 3a) whereas high amount iron deposition was found in liver section stained for the free iron depot in CCl_4 treated rats (Fig. 3c). *Citrus macroptera* supplementation decreased iron deposition in CCl_4 treated rats (Fig. 3d).

Analysis of ethanol extract of *Citrus macroptera* by HPLC-DAD

Identification and quantification of individual phenolic compounds in the ethanol extract of *Citrus macroptera*

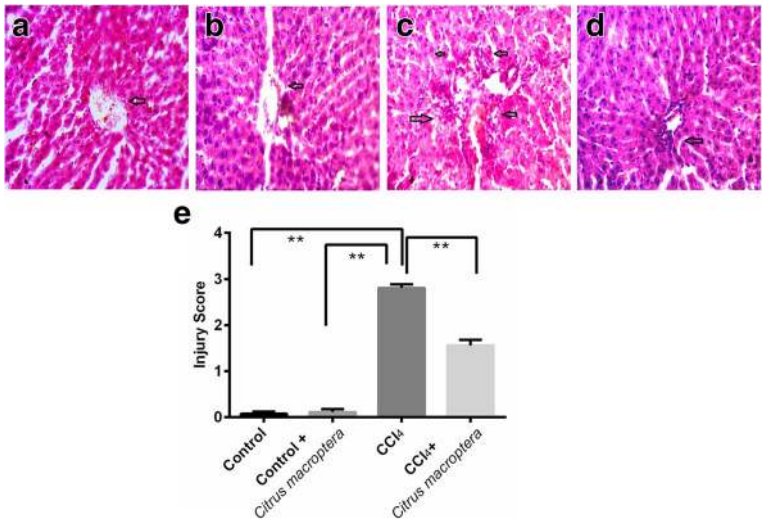

Fig. 1 Effect of *Citrus macroptera* peel powder on hepatic inflammation in CCl_4 treated rats. **a**, Control, showed normal architecture of hepatocyte with no nuclear pyknosis; **b**, Control + *C macroptera,* showed normal architecture of hepatocyte with no nuclear pyknosis; **c** CCl_4, showed severe necrosis with disintegration of hepatic cords, hemorrhage and neutrophil infiltration and **d**, CCl_4+ *C macroptera,* showed reduced necrosis, less hemorrhage and minimized neutrophil infiltration. Magnification 40×. *ic*-inflammatory cells. **e**, shows the injury scores in different experimental groups after CCl_4 chalenge and treatment

were analyzed by HPLC-DAD. The chromatographic separations of polyphenols in ethanol extract are shown in Fig. 4. The content of each phenolic compound was calculated from the corresponding calibration curve and presented as the mean of five determinations as shown in Table 3. Caffeic acid was detected as the most abundant phenolic compound (428.36 mg/100 g of dry extract) found in the ethanol extract.

Discussion

Currently, public awareness program against liver diseases such as fibrosis and cirrhosis are being aggressively focused [27]. The liver is the main organ where almost all kinds of toxins, foods as well as drugs are detoxified [28]. CCl_4 is metabolized in phase-I reaction and activates the ROS producing system [29]. In the current study, CCl_4 administration in rats developed hepatic damage by increasing lipid peroxidation and oxidative stress. CCl_4 administration in rats also increased inflammatory cells infiltration and fibrosis in liver tissues. Our investigation also suggests that *Citrus macroptera* peel powder supplementation ameliorated most of the deleterious effects developed in CCl_4 administered rats.

The active metabolite of CCl_4 lead to hepatocyte damage and may initiate the leakage of a lot of hepatic enzymes (AST, ALT, and ALP) in blood circulation which was associated with immune cells infiltration, massive centrilobular apoptosis, ballooning degeneration and finally cell death [30]. Elevated liver marker enzymes activities (AST, ALP, and ALT) indicate the dysfunction and damage in the liver during disease condition [31]. This study also revealed the increased liver marker enzymes (AST, ALP, and ALT) activities in CCl_4 administered rats which were decreased or normalized by *Citrus macroptera* supplementation. The previous study reported that reduction of liver enzyme activity would be beneficial in case of hepatic damage protection [32]. Earlier investigations also showed that derivative of

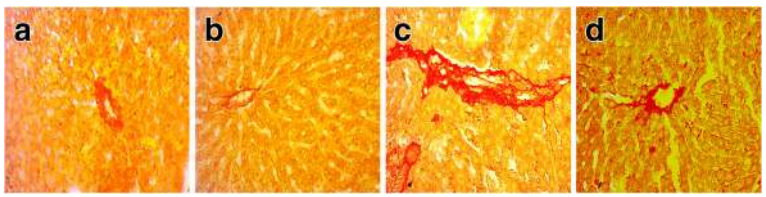

Fig. 2 Effect of *Citrus macroptera* peel powder on hepatic fibrosis in CCl_4 treated rats. **a**, Control, showed baseline collagen around the hepatic duct, no fibrosis developed; **b**, Control + *C macroptera,* showed baseline collgen around the hepatic duct, no fibrosis developed; **c**, CCl_4, Collagen deposition and fibrosis occured around the central vein and hepatic duct. **d**, CCl_4+ *C macroptera,* Collagen deposition was reduced significantly around the central vein and bile duct. Magnification 40×. *fb*- fibrosis

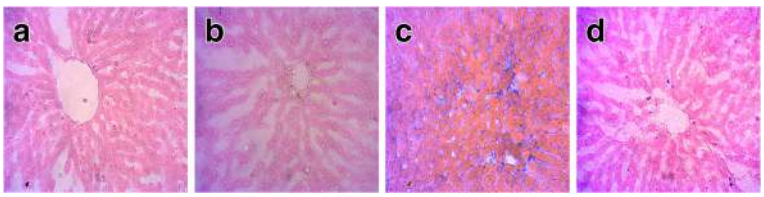

Fig. 3 Effect of *Citrus macroptera* peel powder on hepatic iron deposition in CCl_4 treated rats. **a**, Control, showed no free iron deposition as seen as blue color stain; **b**, Control + *C macroptera,* showed no free iron deposition as seen as blue color stain; **c**, CCl_4, showed free iron deposition as seen as blue color stain and **d**, CCl_4 + *C macroptera.* Magnification 40×

caffeic acid also prevented the liver damage and decreased the liver marker enzymes activities [33, 34].

One of the major markers of hepatic damage is oxidative stress-mediated lipid peroxidation which plays irreversible damage to hepatocellular components. MDA is the outcome of lipid peroxidation in cell membranes and damages the integrity of cells. MDA development also causes an imbalance to lysosomes and helps to oxidize the protein [35]. Our study showed that *Citrus macroptera* supplementation decreased lipid peroxidation level in plasma and liver tissue compared to CCl_4 administered rats. The strong antioxidant can scavenge the free radical and superoxide anions from a biological system. Caffeic acid is a strong antioxidant showed protection against free radical-mediated lipid peroxidation in experimental animals reported previously [34].

Previous studies also reported that anti-oxidant system can reduce inflammatory, pro-inflammatory signaling by inhibiting inducible nitric oxide synthase (iNOS) which is a key factor for immunity responder [36, 37]. iNOS is considered as the major source of NO in stress condition and generates nitrosative stress. NO is a vasodilator which maintains regular vascular tone in blood vessels. It also works as a signaling molecule to produce immunological substances when the body needed [38]. However, increased production of NO in oxidative stress may turn into peroxynitrite ($^{-}ONOO\bullet$) radical formation which further initiate nitrosative stress in tissues. In this study, we found that CCl_4 increased the level of NO in plasma but not in tissue, which was normalized by the supplementation of *Citrus macroptera* peel powder. These findings are also supported by a recent investigation, suggested that derivative of caffeic acid prevents NO production in liver [39].

Furthermore, free radicals-mediated oxidative stress also reduces the amount of natural anti-oxidant like SOD, catalase, and GSH which fights against any free radicals-mediated damage in tissues. It is well reported that superoxide radicals ($O_2^{\bullet-}$) are removed by SOD which produces hydrogen peroxide (H_2O_2) [40]. H_2O_2 then further converted into water by another antioxidant enzyme known as catalase [40]. Moreover, CCl_4 administration also depleted intracellular-reduced GSH levels, suggesting that GSH loss might result from detoxification of CCl_4 by GSH conjugation [40]. GSH also breaks H_2O_2, ROOH and helps against protein oxidation [41]. Our study revealed that administration of oral CCl_4 reduced the amount of anti-

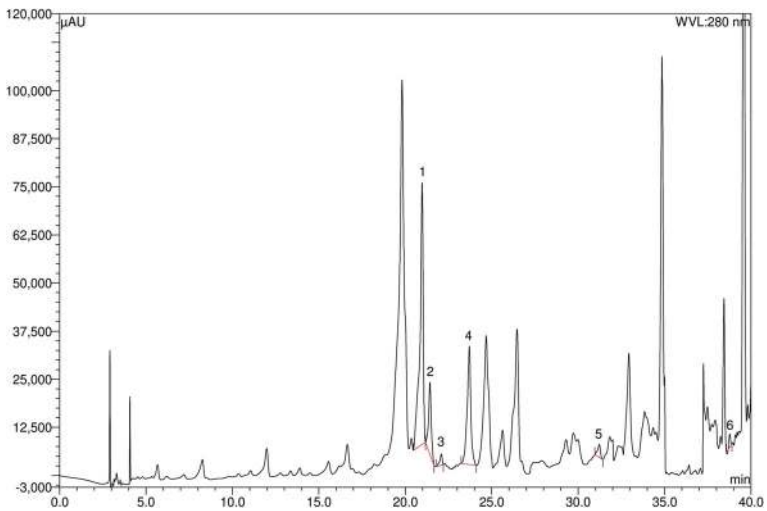

Fig. 4 HPLC chromatogram of *Citrus macroptera*. Peaks: 1, caffeic acid; 2, syringic acid; 3, (–)-epicatechin; 4, vanillin; 5, benzoic acid; 6, kaempferol

Table 3 Contents of polyphenolic compounds in the ethanol extract of *Citrus* ($n = 5$)

Polyphenolic compound	Ethanol extract of *Citrus macroptera*	
	Content (mg/100 g of dry extract)	% RSD
Caffeic Acid	428.36	1.91
Syringic Acid	81.91	0.53
(−)-epicatechin	33.87	0.16
vanillin	52.46	0.27
benzoic acid	47.84	0.36
kaempferol	2.12	0.03

oxidant capacity in both plasma and liver tissue which was restored by *Citrus macroptera* supplementation. Our previous study also demonstrated that iron overload could lead to cause hepatic damage. Free iron in tissues may start Fenton like reaction resulted in a hydroxyl free radicals generation [42]. Supplementation with *Citrus macroptera* peel powder supplementation reduced iron deposition in the liver of CCl_4 treated rats.

The most noteworthy pathological characteristics of CCl_4 intoxicated hepatotoxicity are inflammation and fibrosis. Hepatic fibrosis is usually initiated by hepatocyte damage, leading to the recruitment of inflammatory cells and platelets with the subsequent release of cytokines, chemokines, and growth factors [7]. Inflammatory cells infiltration further lead to activation of HSCs and their transformation into myofibroblast-like cells [43]. Chronically activated HSCs produce large amounts of ECM proteins and enhance fibrosis followed by TGF-β1 mediated signaling pathways [1]. Free radicals may also activate HSC which further help to secret collagenase-1 from local fibroblast [1, 44]. Our investigation showed that CCl_4 administration significantly enhanced inflammatory cell infiltration and fibrosis in the liver of rats. These findings are in agreement with previously published literature which showed that CCl_4 mediated oxidative stress is responsible for the inflammation and fibrosis in liver [45, 46]. Moreover, *Citrus macroptera* peel powder supplementation reduced inflammatory cells infiltration and fibrosis in the liver of CCl_4 treated rats. This antifibrotic activity of *Citrus macroptera* peel powder could be attributed to the presence of the antioxidant and anti-inflammatory compound, caffeic acid. Recent findings also suggested that caffeic acid administration in experimental animals may prevent hepatic fibrosis [14, 39].

Researchers are still working on natural plants to discover new compounds of high biological activity. It is reported by World Health Organization (WHO) that more than 80% people in developing countries basically depends on herbal medicines [47]. Researchers are more focusing on plant-derived polyphenols, seeing that the synthetic anti-oxidants have several adverse effects [48]. Our investigation showed that caffeic acid; syringic acid and vanillic acid are present in *Citrus macroptera* peel powder. The previous report also suggests that syringic acid and vanillic acid which are important polyphenols showed anti-oxidants, anti-inflammatory, anti-fibrogenic and hepatoprotective activities in CCl_4 induced rats [49]. *Citrus macroptera* or wild orange is occasionally used for increasing appetite, reducing fever and making meat preparation [50]. A recent investigation also suggests that *Citrus macroptera* is extremely safe, even methanolic extract at a dose of 1000 mg/kg did not produce toxic effects in rats considering biochemical as well as histological assessment [51]. Our investigation also supports this notions that *Citrus macroptera* peel powder supplementation in normal rats did not alter any of the biochemical as well as histological parameters compared to control rats.

Conclusion

The current study showed that *Citrus macroptera* peel powder possesses a good number of polyphenols such as vanillic acid, caffeic acid and syringic acid and benzoic acid. This study also pointed out that oxidative stress can be reduced by *Citrus macroptera* peel powder supplementation in CCl_4 treated rats. *Citrus macroptera* peel powder was able to reduce iron deposition, inflammation and fibrosis in the liver of CCl_4 treated rats. Further studies are required to assess efficacy in the clinical condition of liver damage.

Abbreviations

ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; APOP: Advanced protein oxidation product; AST: Aspartate aminotransferase; CAT: Catalase; CCl_3: Trichloromethyl free radical; CCl_3O_2: Trichloroperoxyl radical; CCl_4: Carbon tetrachloride; DTNB: 5,5-dithiobis-2-nitrobenzoic acid; ECM: Extracellular matrix; GSH: Reduced glutathione; H_2O_2: Hydrogen peroxide; HPLC-DAD: High performance liquid choromatography diod array ditector; HSCs: Hepatic stellate cells; iNOS: Inducible nitric oxide synthase; MDA: Malondialdehyde; MPO: Myeloperoxidase; NO: Nitric oxide; ONOO: Peroxynitrate; ROS: Reactive oxygen species; SOD: superoxide dismutase; TBA: Thiobarbituric acid; TCA: Trichloroacetic acid; TGF-β: Transforming growth factor-β; TNF-α: Tumor necrosis factor-alpha

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Availability of data and materials

Data generated in this study did not store in any public repositories but will be available upon request which is preserved in our lab computer hard disc.

Authors' contributions
MAA, MATS, and HMR designed the experimental design. MATS and NT carried out the animal care, treatment and data acquisition from the experiment. MAA, MATS, and NT performed all the biochemical analysis. MAA, MATS, and AU also performed the histological staining and analysis of tissues. MHH analyzed the extract for phenolic contents through HPLC-DAD system. MAA, AU, GMR and HMR took part in data analysis and manuscript writing. MAA, GMR and HMR checked and finalized the manuscript for submission. The contribution of MCS should be incorporated. All authors read and approved the final manuscript.

Ethics approval
The study protocol approved by Ethical Committee of Department of Pharmaceutical Sciences, North South University for animal care and experimentation.

Consent for publication
The authors declare that there is no conflict of interests regarding the publication of this paper.

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