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Antioxidant Properties of *Citrus macroptera* Fruit and Its *in vivo* Effects on the Liver, Kidney and Pancreas in Wistar Rats

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ABSTRACT

In this study, the antioxidant potential of *Citrus macroptera* fruit was compared between its pulp (CMPU) and peel (CMPE). The biochemical effects of an ethanol extract of CMPU were investigated on major organs, including the liver, kidney and pancreas. Male wistar rats (n = 24) were randomly divided into four groups, including Group I (control), Group II (250 mg kg^{-1}), Group III (500 mg kg^{-1}) and Group IV (1000 mg kg^{-1}) and they were administered the extract for 28 days. The CMPE contained higher amounts of total polyphenols (620.91±7.75 mg), flavonoids (508.33±5.49 mg), tannins (585.99±4.46 mg) and protein (4.00±0.14 g) compared to the pulp (291.06±10.14, 145.02±0.36, 526.08±3.32 mg/100 g and 2.89±0.32 g/100 g, respectively). Similarly, CMPE also exhibited higher 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) scavenging activity and Ferric Reducing Antioxidant Power (FRAP) values than CMPU. However, the ascorbic acid content was higher in CMPU (120.83±0.0019 mg/100 g) than CMPE. *In vivo* studies confirmed that CMPU possessed a significant lipid lowering activity that occurred in a dose dependent manner and that it caused beneficial changes in several other biochemical parameters. Additionally, a significant diminution of lipid peroxidation in liver and kidney tissues was observed. It was concluded that *C. macroptera* possesses high antioxidant potential and is relatively safe. Its protective effects against various chronic diseases that are associated with oxidative stress should be further investigated.

Key words: *Citrus macroptera*, antioxidant, DPPH, biochemical effects, histopathology, lipid profile, toxicity

INTRODUCTION

Antioxidants are bioactive reducing agents that can prevent damage caused by Reactive Oxygen Species (ROS), including superoxide anion radicals, hydroxyl radicals and hydrogen peroxide. Therefore, they are protective against various chronic diseases, including cancer, coronary heart disease, autoimmune disease, diabetes, sclerosis, atherosclerosis, cataracts and chronic inflammation (Willcox *et al*., 2004). The extreme reactivity of ROS with lipids and proteins contributes to their rapid damaging capacity (Devasagayam *et al*., 2004). In recent years, there has been a worldwide trend of using naturally derived antioxidants from herbs, fruits and vegetables (Afroz *et al*., 2014) to avoid the

unwanted side effects that have been observed with the use of synthetic antioxidants (Tanvir *et al*., 2015a). The widespread use of diverse plant sources as antioxidants has led to other concerning issues, one of the most important of which is the varying biochemical effects that they can produce in different organs. Nevertheless, there is a lack of scientific data concerning the possible toxicities of many medicinal plants despite scientific evidence of their biological activities (Dias and Takahashi, 1994). Hence, further studies into the antioxidant potential of natural products and their biochemical effects on vital organs would be useful.

Citrus macroptera Montr. is a semi-wild species of the Rutaceae family and the citrus genus (Dreyer and Huey, 1973). It is commonly known as 'Satkara' in Bangladesh and is grown primarily in the northeastern part of the country in the Sylhet division. The English meaning of Satkara is 'Wild orange' (Manner *et al*., 2006). Although it is actually a type of fruit, the locals commonly consider it to be a vegetable. The fruit (i.e., the peel) is used as an ingredient in different types of meat and chicken dishes, as well as in the preparation of pickles (Rahmatullah *et al*., 2010). Traditionally, the fruit has been used to treat several diseases, such as hypertension, stomach pain and alimentary disorders (Grover *et al*., 2002; Malik and Chaudhury, 2006). Despite traditional claims, however, there is a lack of evidence delineating the *in vivo* effects of Satkara on major organs in the body, including the kidney, liver and pancreas and therefore it has been difficult to establish its safety profile.

Citrus macroptera has been reported to contain lupeol, stigmasterol, beta-pinene, limonene, beta-caryophyllene, geranial edulinine, ribalinine and isoplatydesmine (Gaillard *et al*., 1995; Rana and Blazquez, 2012; Waikedre *et al*., 2010). *Citrus macroptera* extracts have been reported to exert anti-diabetic effects, possibly by reducing fasting blood glucose and serum insulin levels, which has been shown to alleviate hyperglycemia-associated oxidative stress in experimental type 2 diabetic rats (Zheng *et al*., 2012). These effects are thought to occur via the saponins, steroids and terpenoids that have been identified in *C. macroptera* (Uddin *et al*., 2014b). Moreover, *C. macroptera* was shown to exhibit significant neuropharmacological effects (Rahman *et al*., 2014; Uddin *et al*., 2014b) against oxidative stress by increasing the levels of cellular antioxidant enzymes, such as superoxide dismutase and catalase, in rats (Rahman *et al*., 2014). The phytoconstituents of *C. macroptera*, such as polyphenols, flavonoids and organosulfur compounds have also been reported to contribute to its neuropharmacological effects (Yip and Dallman, 1988; Dallman *et al*., 1980).

Oxidative stress is one of the major causes of aging and chronic human diseases, including liver cirrhosis, diabetes, kidney failure and cardiac disorders (Tiwari, 2004). Although, the adverse effects of allopathic medicines have been well documented, the adverse effects of many of the phytomedicines that are increasing in popularity because of their safe profiles and economic nature (Parmar and Kar, 2008) are still underreported. In the present study, the antioxidant properties of *C. macroptera* fruit pulp and peel extracts were investigated to provide comprehensive data on their antioxidant potentials. Additionally, various biochemical parameters and histopathological characteristics of animals that were administered the fruit pulp extracts were investigated through a sub-acute toxicity study. *Citrus macroptera* fruit pulp is still considered to be a waste product due to its sour taste compared to the peel. There is also currently not enough information on its *in vivo* effects on major organs to recommend it for use against different types of chronic diseases that are mediated by ROS, including cirrhosis, diabetes and myocardial infarction. In this study, both the antioxidant properties of *C. macroptera* fruit and its *in vivo* effects on liver, kidney and pancreas in Wistar rats was investigated.

MATERIALS AND METHODS

Chemicals and reagents: The standards gallic acid, catechin, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,4,6-tris (2-pyridyl)-1,3,5-triazine (TPTZ) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Folin-Ciocalteu's phenol reagent, L-ascorbic acid and tannic acid were purchased from Merck Co. (Darmstadt, Germany). The 1,1,3,3-tetraethoxypropane was purchased from Nacalai Tesque, Japan. All of the reagents used were of analytical grade.

Fruit sample collection: Mature *C. macroptera* fruits were collected in June, 2014 from the Sylhet district in Bangladesh and were authenticated by Professor Nuhu Alam from the Department of Botany, Jahangirnagar University. Following collection, the fruits were packed in cardboard-like cases and were transported by road (with an approximate transportation time of between 5-6 h) to the Laboratory of Preventive and Integrative Biomedicine in the Biochemistry and Molecular Biology Department at Jahangirnagar University in Savar, Dhaka, Bangladesh. In the laboratory, the fruits were cleaned with sterile water under a laminar blower to remove any possible contamination and were subsequently stored in the refrigerator (4°C) for a maximum of one day before further processing.

Preparation of extracts: Fruit extracts were prepared according to a method described by Chew *et al*. (2011) with slight modifications. Fresh *C. macroptera* fruit samples were first divided into two parts, the "Pulp" and the "Peel" by separating the peels from the pulps using a sharp knife. The mature *C. macroptera* pulps and peels were cut into small pieces using a sterile, smooth steel knife and were dried under sunlight for 24 h. When the samples were free of moisture and had a crunchy-like appearance, they were separately

mashed into fine powders using a household blender (Jaipan Commando, Mumbai-63, India). To prepare a 20% ethanol extract, 20 g fractions of each powdered sample of pulp and peel were separately mixed with 80 mL aliquots of absolute ethanol. Following this, the individual extracts were transferred to a shaker and shaken (150 rpm) at 30°C for 72 h. The extracts were then filtered through a cotton plug followed by a Whatman No. 1 filter. The crude extracts were then evaporated under reduced pressure (100 psi) at a controlled temperature (40°C) and were stored at -20°C until further analysis.

Phytochemical analysis

Estimation of phytoconstituents: The total polyphenol contents of pulp and peel *C. macroptera* extracts were estimated by spectrometric determination based on Folin-Ciocalteu's method (Amin *et al*., 2006) using a PD-303S spectrophotometer (APEL, Japan). The results are expressed as mg of Gallic Acid Equivalents (GAEs) per 100 g of sample. Total Flavonoid (TF) contents of the *C. macroptera* extracts were estimated using an aluminum chloride colorimetric assay (Chang *et al*., 2002). The TF was determined as Catechin Equivalents (CEs) and the results are expressed as mg of CEs per 100 g of sample.

The total tannin contents of the extracts were estimated using Folin-Ciocalteu's method (Folin and Ciocalteu, 1927) with tannic acid as a standard. The results are expressed as mg of Tannic Acid Equivalents (TEs) per 100 g of sample. The ascorbic acid contents of the extracts were estimated by a method that was established by Omaye *et al*. (1979) and are expressed as milligram of Ascorbate Equivalents (AEs) per 100 g of sample. The total protein contents of the *C. macroptera* extracts were estimated using Lowry's method of protein estimation (Lowry *et al*., 1951). Bovine Serum Albumin (BSA) was used as a standard and the results are expressed as g of BSA equivalents per 100 g.

Antioxidant activity analysis

DPPH free radical-scavenging activity: The percentage of antioxidant activity in each type of *C. macroptera* extracts was assessed using a DPPH free radical assay. The DPPH radical scavenging activity was measured based on an established method by Braca *et al*. (2002). Briefly, 1 mL of the extract was mixed with 1.2 mL of 0.003% DPPH in methanol at varying concentrations (2.5-80.0 μg mL^{-1}). The percentage of DPPH inhibition was calculated using the equation:

$$\text{Percentage of DPPH inhibition} = \frac{A_{DPPH} - A_S}{A_{DPPH}} \times 100$$

where, A_{DPPH} is the absorbance of DPPH in the absence of the extract and A_S is the absorbance of DPPH in the presence of either the extract or the standard.

The DPPH scavenging activity is expressed as the concentration of extract that is required to decrease DPPH absorbance by 50% (IC_{50}). The value can be graphically determined by plotting the absorbance (the percentage of inhibition of DPPH radicals) against the log concentration of DPPH and determining the slope of the nonlinear regression.

Ferric reducing antioxidant power assay: A Ferric Reducing Antioxidant Power (FRAP) assay was performed and measured according to a method established by Benzie and Strain (1999). The reduction of a ferric tripyridyltriazine complex into its ferrous form produces an intense blue color at low pH and can be monitored by measuring absorbance at 593 nm. The FRAP values are expressed as micromoles of ferrous equivalent (μM Fe (II) per 100 g of the extract).

Experimental animals: All of the experiments were conducted according to ethical guidelines that were approved by the Bio-safety, Bio-security and Ethical Committee of Jahangirnagar University [Approval No. BBEC, JU/M2015 (2)]. Adult, male Wistar rats (140-150 g) were used. The animals were bred and maintained in an animal house in the Department of Biochemistry and Molecular Biology, Jahangirnagar University at a constant temperature of 23±2°C and with humidity ranging from 40-70%. The rats were housed in sterile plastic cages with soft wood-chip bedding and received a natural 12 h day-night cycle. The rats were provided with a standard laboratory pellet diet and water *ad libitum*. To highlight the pharmacological value of CMPU, which is still considered a waste product, only CMPU was further investigated in animal studies.

Protocol for animal treatment: The rats were randomly divided into four groups that contained six animals each. Group I served as a control and received normal saline for 4 weeks (28 days). Groups II, III and IV were orally administered different doses of CMPU extracts (250, 500, 1000 mg kg^{-1}) that were suspended in water. Following the administration of the extracts at different concentrations, the rats were observed for 24 h, with special focus on the first 4 h post-administration, after this, they were observed once a day for 28 days.

At the conclusion of the experimental period, the rats in each group were sacrificed by deep anaesthetization with a ketamine hydrochloride injection followed by dissection. Blood samples (3 mL) were collected from inferior vena cava and liver and kidney tissue samples from individual rats and were stored at -20°C for biochemical experimentation, after which they were preserved in 10% formalin for histopathological examination. The blood samples were collected in dry test tubes and were allowed to coagulate at ambient temperature for 30 min before centrifugation at 2,000 rpm for 10 min to separate the serum. The serum was stored at -20°C for further biochemical analysis.

Tissue homogenate preparation: The liver and kidney samples were homogenized in phosphate-buffered saline (25 mM, pH 7.4) to produce an approximately 10% (w/v) homogenate. The homogenates were centrifuged at 1700 rpm for 10 min and the supernatant was collected prior to storage at -20°C until biochemical analysis.

Biochemical analysis of blood: Biochemical parameters in serum, such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), γ-glutamyl transferase (GGT), total protein (TP), albumin (ALB), total bilirubin (TB), total cholesterol (TC), triglyceride (TG), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), creatinine, urea, uric acid, sodium (Na^+), potassium (K^+), chloride (Cl^-), calcium (Ca^{2+}), magnesium (Mg^{2+}), phosphate (P^{3+}) ions, iron (Fe), total iron binding capacity (TIBC), glucose (GLU), amylase (AMY) and lipase (LPL), were estimated following standard protocols using an automated chemistry analyzer (Dimension EXL with LM Integrated Chemistry System, Siemens Medical Solutions Inc., USA).

The levels of very low density lipoprotein cholesterol (VLDL-C) in the serum were calculated based on the following Friedewald formula (Friedewald *et al*., 1972):

$$VLDL\text{-}C = TG/5$$

Oxidative stress parameters (lipid peroxidation) in tissue: The MDA levels were investigated for products of lipid peroxidation (LPO) in the liver and kidney tissues. The MDA, which is also referred to as Thiobarbituric Acid Reactive Substance (TBARS) was measured at 532 nm according to a method described by Ohkawa *et al*. (1979). The TBARS levels are expressed as nmol of MDA per mg of protein.

Histopathological examination: After each animal was sacrificed, histopathological observation was conducted on both its liver and kidney tissues. The tissue samples were washed in normal saline and were immediately fixed in 10% neutral formalin before being embedded in paraffin. Following this, the specimens were cut into slices that were 5 µm in thickness and were then stained with hematoxylin-eosin for examination under a light microscope (MZ3000 Micros, St Veit/Glan, Austria). The microscopic features of the liver and kidney samples were compared to those of the control group.

Statistical analysis: All analyses were executed in triplicate and the data are expressed as the Mean±Standard Deviation (SD). The data were analyzed using GRAPHPAD PRISM (version 6.05; GraphPad software Inc., San Diego, CA, USA), SPSS (Statistical Packages for Social Science, version 22.0, IBM Corporation, Armonk, New York) and Microsoft Excel 2013 (Redmond, Washington). Comparisons between groups were performed using one-way Analysis of Variance (ANOVA). Statistical analyses of biochemical data were conducted using Tukey's test. The minimum level of significance was fixed at <0.05.

RESULTS

Phytochemical analysis: The content of antioxidant phytochemicals of CMPU and CMPE as well as their protein content are listed in Table 1.

Analysis of antioxidant activity

DPPH free-radical scavenging activity: The antioxidant potentials of *C. macroptera* extracts were investigated by estimating the free radical-scavenging effects of DPPH radicals. The % inhibition values were plotted against the concentrations of the extracts. The IC_{50} values of CMPU, CMPE and an ascorbic acid standard were 1.75, 1.42 and 0.82 µg mL^{-1}, respectively (Fig. 1).

FRAP assay: The FRAP was performed to evaluate the antioxidant potentials of *C. macroptera* extracts. The FRAP values of CMPU and CMPE were 771±7.0 and 923±2.0 [µM Fe (II)] /100 g, respectively.

Effects of the extract on serum biochemical parameters: In the present study, the effects produced by the oral

Table 1: Polyphenols, flavonoids, tannins, ascorbic acid and total protein in 100 g samples of CMPU and CMPE

	Amount present in 100 g of sample	
Phytochemicals	CMPU	CMPE
Total polyphenols (mg/100 g GAEs)	291.06±10.14	620.91±7.75
Total flavonoids (mg/100 g CEs)	145.02±0.36	508.33±5.49
Total tannins (mg/100 g TEs)	526.08±3.32	585.99±4.46
Ascorbic acid (mg/100 g AEs)	120.83±0.0019	56.26±0.008
Total protein (g/100 g)	2.89±0.32	4.00±0.14

Data are presented as the Mean±SD, n = 3, GAE: Gallic acid equivalents, CE: Catechin equivalents, TE: Tannic acid equivalent, AE: Ascorbate equivalent

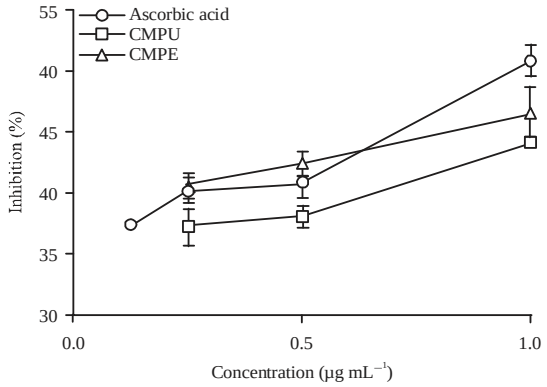

Fig. 1: IC_{50} values of CMPU and CMPE against an ascorbic acid standard showed dose-dependent inhibition potential

administration of a CMPU extract on biochemical parameters were analyzed. Treatment with three different doses of extract (250, 500, 1000 mg kg^{-1}) did not lead to any significant changes in hepatic enzyme biomarkers, except for in ALP and LDH enzymes, which exhibited significantly reduced levels compared to the control (Table 2). Conversely, the higher dose (1000 mg kg^{-1}) significantly decreased AST enzymatic activity. Similarly, the extract did not significantly affect hepatic metabolite biomarkers, such as TP, ALB and TB, at the varying doses by which it was administered.

The extract positively affected serum lipid profiles, in which significant reductions in the levels of TC, TG, VLDL-C and LDL-C and a significant increase in the level of HDL-C were observed. There were no significant differences in Fe and TIBC levels among the animals from the different groups. Although, creatinine levels were significantly decreased in the animals of group II versus group I, there were no significant changes in creatinine levels in the animals that received higher doses of the extract. There were also no significant differences in urea and uric acid levels, indicating that kidney functioning was not affected by the extract. Additionally, higher serum phosphate, which can also indicate kidney damage, was found not to be significantly affected.

Serum amylase was significantly reduced in all three of the treatment groups compared to the control, whereas lipase, another important biomarker of pancreatic function did not show any change at a 5% level of significance.

Electrolytes were included for measure in the study because their balance is important in maintaining the overall homeostasis of the body. Among the electrolytes that were evaluated, Na^+ and K^+ were significantly increased in the different groups when compared to the control. However, no significant changes were observed in Cl^- levels.

An assessment of glycemic status was performed by measuring serum glucose levels. Glucose levels were found to gradually decrease with increasing doses of extract (i.e., a dose-dependent response).

Table 2: Effects of CMPU on serum biochemical markers for liver, kidney and pancreatic tissues

Biochemical parameters	Group I	Group II	Group III	Group IV
Hepatocellular function biomarkers				
ALT (U L^{-1})	14.33±2.58	13.16±1.16	15.33±2.73	16.83±2.63
AST (U L^{-1})	120.16±7.49	116.50±5.00	110.00±9.32	104.00±8.01*
ALP (U L^{-1})	171.66±10.74	135.33±15.61*	137.17±15.66*	140.00±20.16*
GGT (U L^{-1})	13.50±0.83	13.16±0.40	13.16±0.40	12.66±0.51
LDH (U L^{-1})	376.33±20.81	252.66±33.54*	255.33±20.43*	250.50±25.64*
TB (mg dL^{-1})	0.11±0.01	0.12±0.01	0.11±0.00	0.11±0.00
Hepatic synthetic function biomarkers (g L^{-1})				
TP	60.66±4.32	56.33±2.33	60.33±4.63	59.16±1.83
ALB	11.83±1.16	11.66±1.03	12.83±1.16	11.33±0.81
Lipid profiles (mg dL^{-1})				
TC	51.66±3.01	48.33±2.06	48.00±2.28	46.66±4.17*
TG	47.33±2.25	40.00±5.40^C	34.83±4.49*	27.50±8.75*
VLDL-C	9.46±0.06	8.00±0.03^C	6.97±0.09*	5.50±1.20*
LDL-C	3.86±1.42	1.33±0.27*	1.20±0.40*	0.83±0.23*
HDL-C	38.33±2.16	39.00±2.36	39.83±2.28	40.33±4.41
Renal excretory function biomarkers (mmol L^{-1})				
CRE	50.33±3.88	39.66±5.75*^{C}	46.66±2.73	53.33±5.08
Urea	4.71±0.37	4.76±0.37	4.81±0.81	5.22±0.61
UA	44.50±7.36	43.66±5.16	42.83±5.26	37.33±6.74
Renal homeostasis maintenance biomarkers (electrolytes)(mmol L^{-1})				
Na^+	139.67±3.07*	153.16±3.97*^{C}	148.83±2.04*	146.50±2.34*
K^+	3.60±0.17	4.06±0.12*^{C}	4.03±0. 21*^{C}	4.06±0.37*
Ca^{2+}	2.27±0.19	2.18±0.04^C	2.33±0.03^C	2.56±0.04*
Mg^{2+}	0.66±0.05	0.63±0.10^C	0.76±0.08^C	1.10±0.08*
Cl^-	101.83±1.32	104.33±5.68	100.16±4.40	103.50±2.16
PO_4^-	2.09±0.03	2.06±0.04	2.11±0.06	2.15±0.06
Iron profiles (mg dL^{-1})				
Fe	173.66±44.63	180.33±5.98	193.00±11.33	172.50±17.75
TIBC	411.00±16.00	410.66±9.17	403.16±9.38	415.83±13.25
Pancreatic function markers (U L^{-1})				
AMY	637.66±3.77	479.33±31.08*	485.16±24.61*	501.50±3.20*
LPL	37.00±2.10	35.66±3.07	40.16±4.91	39.17±1.47
Glycemic status (mmol L^{-1})				
GLU	6.33±0.72	6.06±0.17^C	5.56±0.60	5.15±0.13*

Values are expressed as the Mean±SD, p values < 0.05 were considered significant and were determined using one-way ANOVA followed by Tukey's HSD multiple comparison tests, Asterisks and superscript "c" denote significant differences compared to groups I and IV, respectively, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, GGT: γ- glutamyl transferase, LDH: Lactate dehydrogenase, TB: Total bilirubin, TP: Total protein, ALB: Albumin, TC: Total cholesterol, TG: Triglyceride, VLDL-C: Very low density lipoprotein cholesterol, LDLC: Low density lipoprotein cholesterol, HDLC: High density lipoprotein cholesterol, CRE: Creatinine, UA: Uric Acid, TIBC: Total iron binding capacity, AMY: Amylase, LPL: Lipase, GLU: Glucose

Ca^{2+} and Mg^{2+} levels were investigated based on their involvement in different biochemical reactions. Their levels were significantly increased in groups III and IV compared to group I and a slight decrease was observed in group II.

Based on the investigation of an oxidative stress biomarker, there was a significant decrease in LPO levels in the liver and kidney tissues of the animals that were treated with CMPU compared to the control group (Fig. 2).

Histopathological findings: Microscopic examinations of liver (Fig. 3) and kidney (Fig. 4) tissues revealed that there were no significant changes in morphological or pathological lesions in the different treatment groups when compared with the control group. Examinations of liver and kidney histological photomicrograph preparations confirmed that the investigated organs had normal cellular architecture, even in animals receiving high doses of the extract, indicating that there was no toxicity to the major vital organs.

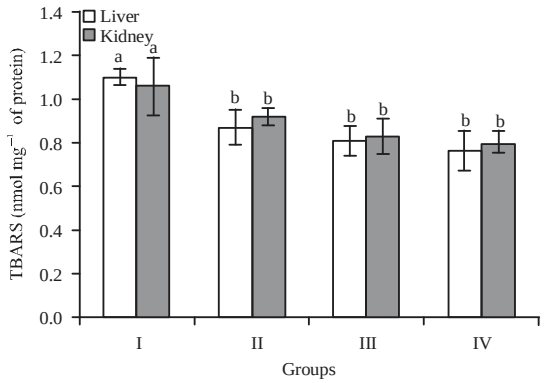

Fig. 2: Effects of CMPU extract on LPO levels in the liver and kidney tissues of rats from different groups, The bars represent the Mean±SD (n = 6), bars with different letters on top are significantly different at $p<0.05$

DISCUSSION

To the best of our knowledge, our study is the first to determine the antioxidant potentials of *C. macroptera* pulp and peel and to investigate the effects of a fruit pulp extract on major metabolic organs, such as the liver, kidney and pancreas by analyzing histopathological findings in a rat model. Phenolic compounds are secondary plant metabolites and primarily include polyphenol, flavonoid and tannin. They produce beneficial biological effects, such as antioxidant, antibacterial, anti-inflammatory and anti-allergic activities (Park *et al*., 2004). Polyphenols have been reported to contribute to the antioxidant capacities of fruits (Proteggente *et al*., 2003). Moreover, it has been suggested that the higher a total polyphenol content is the greater the antioxidant activity (Abu-Amsha *et al*., 1996). Our findings indicated that the total polyphenol contents of both CMPU and CMPE were high (291 and 620 mg GAE/100 g, respectively) with the peel having almost three-fold higher total polyphenol content than the pulp. The total polyphenol content levels were

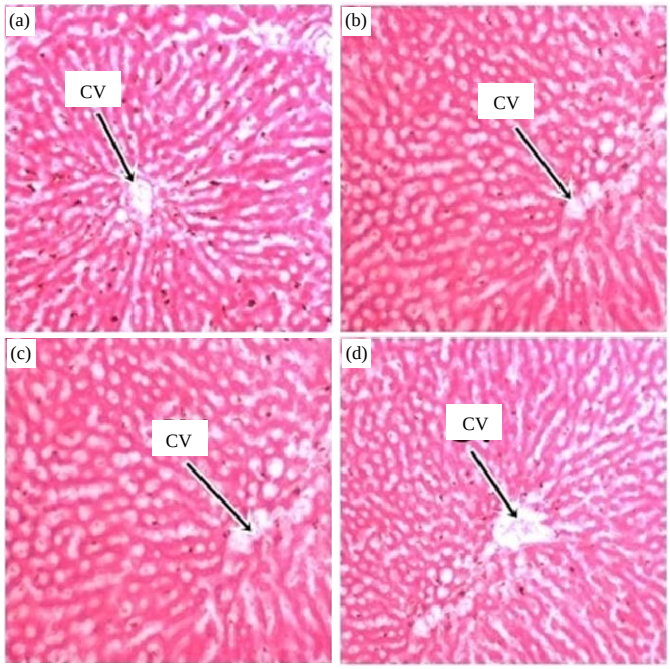

Fig. 3(a-d): Histopathological photomicrographs of liver sections, (a) Group I (Control), (b) Group II (250 mg kg^{-1}), (c) Group III (500 mg kg^{-1}) and (d) Group IV (1000 mg kg^{-1}), No morphological or pathological lesions were found among the groups (100X magnification, scale bar: 20 µm) CV: Central vein

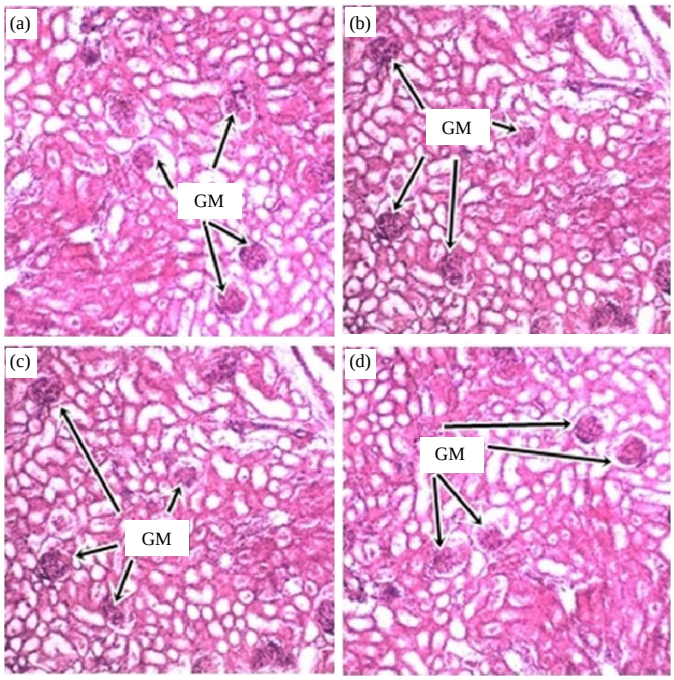

Fig. 4(a-d): Histopathological photomicrographs of kidney sections from different groups of treated rats, (a) Group I (control), (b) Group II (250 mg kg^{-1}), (c) Group III (500 mg kg^{-1}) and (d) Group IV (1000 mg kg^{-1}), No morphological or pathological lesions were found among the groups (100X magnification, scale bar: 20 μm), GM: Glomerulus

significantly higher than those of pomelo pulp and peel (61.72 and 406.65 mg GAE/100 g, respectively), which exhibited almost 5-fold higher total polyphenol content than the pulps (Toh *et al*., 2013) of other citrus fruits such as lemon, orange and grape, as reported by Gorinstein *et al.* (2001).

Furthermore, citrus fruits contain a wide range of flavonoid constituents that are made up of flavones, flavanones and flavanols sub-classes (Nogata *et al*., 2006). The flavonoid derivatives that were present in CMPU and CMPE were 145 and 508 mg/100 g, respectively, which were lower than the total polyphenol content; this is frequently observed with respect to flavonoid contents in the majority of plants. In comparison, lower levels of total phenols were found to be present in pomelo fruit (*Citrus maxima*) peels of Tambun White and Tambun Pink, which were measured to be 356.95 and 228.86 mg QE/100 g, respectively (Toh *et al*., 2013). The TF contents of both Tambun White and Tambun Pink pomelo pulps were also lower (13.06 and 13.6 mg QE/100 g, respectively) (Toh *et al*., 2013). The higher amount of TF that is present in CMPU compared to other varieties of pomelo pulp may be a result of variations in the harvest times of these fruits (Ramful *et al*., 2011).

Tannins are another important type of water-soluble secondary metabolites, the presence of which contribute to the bitter and sour taste of several citrus species (Okwu and Emenike, 2006). They also possess astringent, antimicrobial and antioxidant properties (Reddy *et al*., 2007). In our study, the peel contained a higher (585 mg TEs/100 g) level of tannin compared to the pulp (526 mg TEs/100 g).

Citrus fruits are popular sources of ascorbic acid. In our study, the pulp contained approximately two-fold higher ascorbic acid than the peel. In a previous study that was conducted by Toh *et al*. (2013), it was also reported that pomelo pulp extracts contained higher levels of ascorbic acid than the peel extracts by about four-to five-fold (Toh *et al*., 2013). With respect to TP content, the CMPU contained much higher levels (approximately 1-2 fold) than the peel, indicating that, in addition to providing a good source of protein, the fruit is a rich source of ascorbic acid.

The antioxidant activities of CMPU and CMPE were evaluated by FRAP and DPPH assays. The FRAP assays primarily measure the abilities of antioxidants to reduce ferric tripyridyltriazine (Fe^{3+}) into a ferrous form (Fe^{2+}), whereas DPPH assays measure percentages of radical scavenging activity (Pichaiyongvongdee and Haruenkit, 2009). The results of the FRAP and DPPH assays confirmed that both CMPU and CMPE have considerable antioxidant activities. Although both pulp and peel extracts were investigated in this study, the peel extract exhibited a higher level of antioxidant activity than the pulp, which supports the findings of previous studies (Abu-Amsha *et al*., 1996).

As an organ of detoxification, the liver is the first organ that encounters all materials that are absorbed from the gastrointestinal tract. It has been shown to react to toxicological insults in a number of ways, including undergoing cellular degeneration, necrosis, bile duct hyperplasia and fibrosis (Onu *et al*., 2013). In this study, liver function was evaluated by determining the activities of various serum enzymes (ALT, AST, ALP, GGT and LDH), metabolites (TB) and plasma proteins (TP and ALB). During hepatic injury, enzymes tend to leak out into the blood stream because of their cytoplasmic location, which facilitates their release into circulation in response to damage of liver structural integrity (Afroz *et al*., 2014). The extract tended to produce different effects on liver enzymes. There were no significant changes in plasma ALT and GGT levels, indicating that the extract did not exert any toxic effects in the liver. The highest dose (1000 mg kg^{-1}) was even found to significantly decrease AST enzymatic activity, indicating that the fruit may exert protective effects in the liver.

Because approximately 80% of AST is found in mitochondria, AST is more highly concentrated in a number of organs (liver, kidney, heart and pancreas) and is released more slowly than ALT, which is purely a cytoplasmic enzyme. Therefore, ALT is considered to be a more sensitive marker of hepatocellular damage than AST (Aniagu *et al*., 2005). However, all three of the different doses that were tested significantly reduced the levels of ALP and LDH, which were related to hepatic cell functioning. This may account for the protective effects that are imparted by the antioxidants in *C. macroptera* on liver disorders. The significant reduction in ALP levels that is caused by CMPU indicated that cholestasis might not have been occurring at the investigated doses because a rise in plasma ALP levels is usually a characteristic finding of cholestatic liver disease (Aniagu *et al*., 2005). Furthermore, the microscopic examinations of the livers of rats that were treated with various doses of CMPU did not reveal any evidence of centrilobular degenerative changes or necrosis and also did not reveal any changes in cell sizes and architecture compared with control group rat livers, further confirming the biochemical findings. Total bilirubin levels are used to determine hepatic dysfunction and are directly implicated in the extent of hepatic damage and toxicity (Tanvir *et al*., 2015b), they were unchanged in all of the groups of CMPU treated rats.

Again, the non-significant variations in the levels of serum TP and albumin lend support to the finding that the CMPU extract studied here was not toxic to the synthetic functioning of the liver. Furthermore, there was no evidence of a link with vital organ dysfunction, as albumin levels decrease in response to inflammation (Ruot *et al*., 2000) and serum TP levels provide information about the severity of necrosis and of synthetic protein capacity (Casillas *et al*., 1983).

Serum TC and TG levels are the most important aspects of a lipid profile. A cholesterol profile is often measured quantitatively to diagnose primary and secondary hyperlipoproteinemia, triglyceridemia and liver obstruction and high plasma triglyceride levels lead to a predisposition for cardiovascular disease (Zicha *et al*., 1999) and contribute to hypertension, obesity and diabetes mellitus (Shen, 2007). Dose-dependent reductions were observed in serum levels of TC, TG, VLDL-C and LDL-C in response to exposure to the fruit pulp, suggesting its lipid lowering activity and cardioprotective effect (Uddin *et al*., 2014a). Additionally, a dose-dependent incremental change in serum HDL-C was observed. Because a high level of HDL-C in plasma exerts a protective effect via reverse cholesterol transport because it scavenges excess cholesterol from the peripheral tissues of the body (Uddin *et al*., 2014a), the effects of CMPU would certainly be beneficial. Therefore, these findings provide strong support in favor of the cardioprotective effect of the fruit.

The CRE, Urea and UA are specific indicators and more sensitive biomarkers of kidney function and incremental changes in these molecules are only observed when marked damage to functional nephrons has occurred (Lameire *et al*., 2005). The low dose of the fruit extract reduced CRE levels; however, no significant changes were observed with the administration of higher doses, indicating that the fruit extract is not renal toxic. The extract also did not significantly affect other kidney parameters, such as urea and uric acid, even when it was administered at high doses. Therefore, this fruit extract was considered to be nontoxic and it exhibited no destructive effects on normal kidney functions. These findings were further confirmed by histopathological observations of kidney tissues.

Electrolytes are also responsible for maintaining a balance of fluids between intracellular and extracellular environments (Abbrecht, 1980). This balance is important for hydration, nerve impulses, muscle function and pH levels (Baer *et al*., 1970; Bohr and Goulet, 1961; Hess *et al*., 2001). In our study, the levels of Na^+ and K^+ were significantly increased, although there were no significant changes observed in Cl^- levels. In contrast, compared to group I, Ca^{2+} and Mg^{2+} were significantly increased only when the extract was administered at 500 and 1000 mg kg^{-1} doses, although the levels were slightly decreased in group II. The divalent cations Ca^{2+} and Mg^{2+} have been reported to play diversified roles in both intracellular and extracellular processes (Hebert *et al*., 1997). Our findings suggest that CMPU does not alter the homeostatic status of the body.

Serum levels of Fe and TIBC are important indicators of anemia (Yip and Dallman, 1988). Anemia most commonly affects young children, adolescents, women of childbearing age and elderly individuals (Toh *et al*., 2013; Uddin *et al*., 2014a, b). It has been assumed that Fe deficiency is the predominant cause of anemia in all of these groups, including in elderly individuals (Howe, 1983; McLennan *et al*., 1973). However, elevated levels of blood iron enhance the risk of cancer occurrence and death (Stevens *et al*., 1994). Our findings strongly suggest that CMPU did not significantly

affect the levels of iron at the investigated doses when compared to the control. These findings again ensure its lack of toxicity across a variety of doses.

Serum AMY and LPL activities are important parameters for indicating pancreatic damage because the diagnosis of acute pancreatitis can be further supported by increased serum levels of AMY and LPL (Banks *et al*., 2006). Diagnostic values of serum AMY and/or LPL in excess of three times the upper limit of normal are characteristic of acute pancreatitis and are usually not reported to occur in other conditions (Gumaste *et al*., 1993). In our study, although there was no significant change in LPL levels, serum AMY was significantly reduced in response to all three of the tested doses when compared to control, which is a positive attribute of CMPU.

Citrus macroptera fruit also had positive effects on glycemic status. The GLU levels gradually decreased with increasing levels of extract, which further supports its anti-diabetic properties (Uddin *et al*., 2014b).

Furthermore, significant decreases in lipid peroxidation levels in liver and kidney tissues were observed at different doses compared to the control group, which also indicates that CMPU has a potential effect against oxidative stress. *Citrus macroptera* is a natural source of antioxidants and are rich in flavonoids, ascorbic acid and phenolic compounds, all of which may work synergistically through free radical scavenging, hydrogen donation, singlet oxygen quenching and neutralization of free radical reactions.

Finally, the administration of CMPU did not lead to any significant histopathological alterations in liver or kidney tissues when administered at the three different doses that were tested, suggesting the safety of the extracts at the tissue level. These findings will be useful for guiding individuals to consume the rich antioxidant fruits of citrus varieties across the world. Further pharmacological and biochemical investigations should be conducted on *C. macroptera* peel and pulp extracts to elucidate the exact mechanism of their protective effects against various ailments.

CONCLUSION

Citrus macroptera is a promising source of natural antioxidants, as indicated by its high contents of polyphenols, flavonoids, tannins and proteins and by its considerable DPPH free radical scavenging activities and FRAP value. Here, an *in vivo* study confirmed that *C. macroptera* fruit does not produce any toxic effects to vital organ structures or serum biochemical parameters. Therefore, the use of this fruit as a potential source of natural antioxidants is an attractive option, particularly because it also has the potential to combat many diseases that are induced by oxidative stress.

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