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Protective effects of ethanolic peel and pulp extracts of *Citrus macroptera* fruit against isoproterenol-induced myocardial infarction in rats

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ABSTRACT

Increases in the incidence of cardiovascular disease (CVD) have aroused strong interest in identifying antioxidants from natural sources for use in preventive medicine. *Citrus macroptera* (*C. macroptera*), commonly known as "Satkara", is an important herbal and medicinal plant reputed for its antioxidant, nutritious and therapeutic uses. The aim of the present study was to investigate the cardio-protective effects of ethanol extracts of *C. macroptera* peel and pulp against isoproterenol (ISO)-induced myocardial infarction (MI) in rats. Male albino Wistar rats (n = 36) were pre-treated with peel and pulp extracts (500 mg/kg) for 45 days. They received a challenge with ISO (85 mg/kg) on the 44th and 45th days. Our findings indicated that subcutaneous injection of ISO induced severe myocardial injuries associated with oxidative stress, as confirmed by elevated lipid peroxidation (LPO) and decreased cellular reduced glutathione (GSH) and anti-peroxidative enzymes, including glutathione peroxidase, glutathione reductase and glutathione-S-transferase, compared with levels observed in control animals. Pre-treatment with *C. macroptera* peel and pulp extracts prior to ISO administration however, significantly improved many of the investigated biochemical parameters, i.e., cardiac troponin I, cardiac marker enzymes, lipid profile and oxidative stress markers. The fruit peel extract showed stronger cardio-protective effects than the pulp extract. The biochemical findings were further confirmed by histopathological examinations. Overall, the increased endogenous antioxidant enzyme activity against heightened oxidative stress in the myocardium is strongly suggestive of the cardio-protective potential of *C. macroptera*.

1. Introduction

Despite improved clinical care, the availability of modern medicines and greater health awareness [1], the World Health Organization (WHO) has predicted that cardiovascular disease (primarily myocardial infarction) will be a major cause of death worldwide by the year 2020 [2,3]. Myocardial infarction (MI), a common presentation of ischemic heart disease (IHD), occurs when cardiac ischemia surpasses a clinical threshold, resulting in irreversible myocardial damage. IHD is an acute condition leading to necrosis of the myocardium as a result of an imbalance between myocardial metabolic demands and the coronary supply of oxygen

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and nutrients [2,4,5]. MI leads to free radical generation in the heart, which contributes to further toxic reactions and eventually cardiac cell death [6].

Reactive oxygen species (ROS) such as free radicals, oxygen ions, and peroxides are generated during aerobic metabolism as by-products and are tightly controlled by antioxidants [7]. However, excess production of ROS or depletion of antioxidants can lead to a state of oxidative stress that can inflict damage to lipids, proteins, and DNA [8]. Following MI, ROS production is usually increased, which can lead to further damage to the myocardium. The first line of cellular defense against oxidative injury in the heart as well as most tissues includes antioxidant enzymes [9]. Dietary antioxidants can prevent the deleterious effects of ROS by restoring the balance between production and clearance of ROS by mechanisms such as scavenging ROS or enhancing endogenous antioxidant enzyme activity [10].

Natural products have high global demands because of their purported superiority in terms of both safety and efficacy against oxidative stress-induced cardiovascular disease, including MI [11]. The human body has myriad endogenous defense mechanisms that feature cellular antioxidants, such as glutathione, bilirubin and a free radical scavenging enzyme system that includes superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase, all of which work together against oxidative stress. The levels of these enzymes can be reduced because of enhanced lipid peroxidation. Therefore, the dynamic balance between ROS and antioxidants is important [12].

Citrus fruits have been a natural boon to mankind for years. Earlier studies have reported that almost all species of citrus fruits have significant antioxidant properties and are effective against stress-induced ulcer, cancer and other chronic diseases [13,14]. *C. macroptera* (Var. anamnesis) is a citrus fruit from the Rutaceae family. In Bengali, the fruit is known as “Satkara”, and in English, it is called “wild orange” (Fig. 1). This semi-wild fruit is widely distributed, particularly along the hill tracks of the Sylhet division of Bangladesh and in some regions of India. In Bangladesh, people usually use the rind of the fruit in chicken, meat dishes and pickle preparations for extra flavor [15,16]. Additionally, the fruit is widely used in the treatment of hypertension, to soothe stomach pain and in alimentary disorders in Assam, India [17,18]. In addition, *C. macroptera* is used as an herbal medicine for treating various fatal disorders in Northern India [19].

The phytochemical composition of *C. macroptera* was previously reported to contain water (90.40%), β-carotene (22.00 mg/100 g), thiamine (0.08 mg/100 g), riboflavin (0.01 mg/100 g) and (sodium 3.50, potassium 89.00, calcium 25.00, magnesium 10.00, iron 0.15, zinc 0.21, copper 0.07 and phosphorus 12.00 mg/100 g) [20]. Recently, the antioxidant properties of the fruit’s peel and pulp were analyzed and reported to contain 620.91 and 291.06 mg of polyphenols (gallic acid), 508.33 and 145.02 mg of flavonoids (catechin), 585.99 and 526.08 mg of tannin (tannic acid), 56.26 and 120.83 mg of ascorbic acids per 100 g, respectively [16]. *C. macroptera* also confers strong protection against lipid peroxidation in rat liver and kidney tissues because of its strong antioxidant properties [15].

Isoproterenol (ISO) is a synthetic catecholamine and β-adrenergic agonist that causes severe stress to the myocardium, resulting in an infarct-like necrosis of the heart muscle if administered in high doses [2]. It has been reported that pathophysiological and morphological changes of ISO-induced cardiac dysfunctions in laboratory animals are comparable to those in humans suffering from MI [4]. Studies have shown that hypoxia is the major cause of ISO-induced cardiac damage because of myocardial hyperactivity, coronary hypotension and excessive generation of highly cytotoxic free radicals resulting from the auto-oxidation of catecholamines [21]. Following oxidation, catecholamines form quinoid compounds, which stimulate the production of superoxide anions and subsequently hydrogen peroxide. Hydrogen peroxide becomes a highly reactive hydroxyl radical in the presence of iron, causing oxidative damage to preserved lipids, proteins and DNA, ultimately affecting the infarcted myocardium [22].

Although Uddin *et al.* [23] proposed the cardio-protective potential of *C. macroptera* fruit, this potential not been experimentally confirmed using an appropriate animal- or cell-based model. The present study was undertaken to investigate the cardio-protective effects of both fruit peel and pulp extracts of *C. macroptera* against experimentally induced MI in rats and to compare the effects of the two extracts. The possible mechanisms that may underlie the therapeutic efficacies were also investigated by studying the alterations in cardiac marker enzymes, troponin I, lipid metabolism, lipid peroxide reactions and the antioxidant defense system. Additionally, histopathological examinations of heart tissues were conducted to confirm the biochemical observations.

2. Materials and methods

2.1. Experimental animals

The experiment was carried out using adult male albino Wistar rats (n = 36) aged between 5 and 7 weeks and weighing between 110 and 140 g. The animals were bred and maintained in an institutional animal house facility under standard conditions of ventilation, temperature (28 ± 2 °C), humidity (40–70%) and light/dark conditions (12/12 h). The rats were individually housed in polypropylene cages with soft wood-chip bedding and access to standard food and water *ad libitum*. The experiments were

Fig. 1. *C. macroptera* (a) whole fruit, (b) peel, and (c) pulp.

conducted according to the ethical guidelines approved by the Bangladesh Association for Laboratory Animal Science. The experiment protocol was approved by the Bio-safety, Bio-security & Ethical Committee of Jahangirnagar University [Approval No. BBEC, JU/M2015 (3)].

2.2. Chemicals and drugs

ISO and 1, 1, 3, 3-tetraethoxy propane were purchased from Nacalai Tesque, Inc., Kyoto, Japan. All chemicals and reagents used in this study were of analytical grade.

2.3. Sample collection and extract preparation

Fresh, mature *C. macroptera* fruit samples were collected from the hill track region of Sylhet District, Bangladesh, in July 2015. Following collection, the fruits were packed into sterile polybags before immediate transportation to the Laboratory of Preventive and Integrative Biomedicine in the Biochemistry and Molecular Biology Department, Jahangirnagar University, Savar, Dhaka, Bangladesh. The fruit was authenticated to be of the correct specimen by Professor Nuhu Alam of the Botany Department, Jahangirnagar University. A voucher specimen (Acc. no. 38619) was deposited in the Bangladesh National Herbarium for future reference.

In the laboratory, the samples were rinsed thoroughly under cold running water. The fruit pulp was separated from the peel before being cut into small pieces using a sterile, smooth steel knife. They were then completely dried under direct sunlight for 28 h. The dried samples were subsequently ground into a fine powder using a household blender (Japan Commando, Mumbai-63, India). The blended peel and pulp samples (100 g) were separately soaked in 70% ethanol for 24 h. They were then shaken using a shaker (IKA400i, Germany) (150 rpm) at 30 °C for 72 h. Subsequently, the extracts were filtered through a cotton plug, followed by another filtration step using Whatman No. 1 filter paper. The crude extract was concentrated under reduced pressure (100 psi) at a controlled temperature (40 °C),followed by storage at −20 °C for subsequent studies.

2.4. Experimental design

Following a one-week acclimation period, the animals were randomly divided into six groups (with 6 rats in each group). Each group was treated as follows:

Normal control: Animals received a standard laboratory diet and drinking water *ad libitum*.

Peel: Animals received peel extract (500 mg/kg) dissolved in saline water (0.90% sodium chloride) by oral gavage needle for 45 days.

Pulp: Animals received only pulp extract (500 mg/kg) dissolved in saline water (0.90% sodium chloride) by oral gavage needle for 45 days.

ISO: Animals received a standard laboratory diet and drinking water *ad libitum*. On the 44th and 45th days, the animals were injected with ISO (85 mg/kg) subcutaneously (at an interval of 24 h). This group served as a negative control.

Peel + ISO: Animals were pretreated with peel extract (500 mg/kg) dissolved in saline water (0.90% sodium chloride) by oral gavage needle for 45 days. This was followed by subcutaneous injection with ISO (85 mg/kg) on the 44thand 45th days (at an interval of 24 h).

Pulp + ISO: Animals were pretreated with pulp extract (500 mg/kg) dissolved in saline water (0.90% sodium chloride) by oral gavage needle for 45 days, followed by subcutaneous injection with ISO (85 mg/kg) on the 44th and 45th days (at an interval of 24 h).

Throughout the experimental period, the rats body weights were recorded regularly and the doses modulated accordingly. The 500 mg/kg extract dose and the treatment period of 45 days were established based on our previous studies [15,16,24].

2.5. Experimental induction of MI

MI was induced by subcutaneous injection of ISO (85 mg/kg) dissolved in physiological saline at 24-h intervals for two consecutive days. The ISO dose was selected based on a pilot study on ISO dose fixation and the dose used in previous studies [2,4].The animals were sacrificed (as below) 48 h following the first ISO administration. The animals were anesthetized by the injection of ketamine hydrochloride (100 mg/kg) for sacrifice prior to dissection.

2.6. Serum preparation

Blood samples (4 mL) were collected from the inferior vena cava. The blood was placed in dry test tubes and allowed to coagulate at an ambient temperature for 30 min. Serum was separated by centrifugation at 2000 rpm for 10 min.

2.7. Heart tissue homogenate preparation

Immediately following blood collection, heart tissue was excised from the surrounding tissues and weighted. The tissue samples were then washed twice with ice-cold phosphate buffer saline (PBS) and homogenized in phosphate buffer (25 mM, pH 7.4) using a tissue homogenizer (F 12520121, Omni International, Kennesaw, USA) to generate approximately 10% w/v homogenates. The homogenates were then centrifuged at 1700 rpm for 10 min, and the supernatant was collected and stored at −20 °C until biochemical analysis. Some of the heart samples were stored in 10% formalin for histopathological examination.

2.8. Biochemical analysis in serum

Serum was used to estimate cardiac specific troponin I (cTn I) via standard enzyme immunoassay kits (JAJ International Inc., USA) using an ELISA microplate reader (Digital and analog system RS232, Das, Italy). The cardiac marker enzymes, including creatinine kinase-MB (CK-MB), aspartate transaminase (AST), lactate dehydrogenase (LDH), alanine transaminase (ALT), serum total cholesterol (TC), triglycerides (TG) and high-density lipoprotein-cholesterol (HDL-C), were estimated via commercially available standard assay kits (Stanbio Laboratory, USA) using a PD-303S spectrophotometer (APEL, Japan). Serum low-density lipoprotein-cholesterol (LDL-C) levels were calculated based on the following formula provided by Friedewald [25]:

$$3\ \text{LDL–C} = \text{TC} - [(\text{TG}/5) + \text{HDL–C}]$$

2.9. Biochemical analysis in heart tissue

Malondialdehyde (MDA) levels were assayed to determine the lipid peroxidation (LPO) products in heart tissues according to the method described by Ohkawa *et al.* [26]. Briefly, MDA, also referred to as thiobarbituric acid-reactive substance (TBARS), was measured at 532 nm; the levels of TBARS are expressed as nmol of TBARS per mg of protein. The total protein content in heart tissue homogenates was estimated by the Lowry method [27].

The levels of cellular antioxidant and anti-peroxidative enzymes such as reduced glutathione (GSH), glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Rx), and glutathione-S-transferase (GSTs) in the rats' heart tissues were estimated using standard assay kits (Abnova Corporation, Taiwan). To this end, the heart tissue homogenates were re-centrifuged at 12,000 rpm for 10 min at 4 °C using Eppendorf 5415D centrifuges (Hamburg, Germany). The resulting clean supernatants of tissue homogenates were fed into the assays. GSH level was expressed as μmol/mg of protein, while GSH-Px and GSH-Rx activities were expressed as nmol NADPH oxidized/min/mg of protein. On the other hand, GST activity was expressed as nmol CDNB conjugated/min/mg of protein. The total protein content in the re-centrifuged tissue homogenates was estimated by the method described by Lowry *et al.* [27].

2.10. Histopathological examination

The heart tissue was dissected and fixed in 10% formalin. The fixed tissues were embedded in paraffin before being cut into serial sections (5 μm thick) using a rotary microtome. Each section was stained with hematoxylin and eosin (H & E). Microscopic observation was performed using a fluorescence microscope with normal spectra (Olympus DP72, Tokyo, Japan). Photomicrographs were captured using a digital camera (Olympus DP72, Tokyo, Japan) attached to the microscope. The pathologist who performed the histopathological evaluation was blinded to the treatment assignments of the different study groups and graded the histopathological changes as 1, 2, 3 and 4 for none, mild, moderate and severe pathological changes, respectively [28].

2.11. Statistical analysis

The results are reported as mean values ± standard deviation (SD). Data were analyzed using SPSS (Statistical Packages for Social Science, version 20.0, IBM Corporation, New York, USA) and Microsoft Excel 2007 (Redmond, Washington, USA). The mean values of different groups were compared using one-way analysis of variance (ANOVA) and independent samples *t*-test. Statistical analyses of biochemical data were performed using Tukey's test; $p < 0.05$ was accepted as statistically significant.

3. Results

The effects of *C. macroptera* peel and pulp extracts and ISO treatment on the heart and body weights as well as the heart to body weight ratio are presented in Table 1. No deaths were observed in any of the treatment groups over the entire 45-day treatment period. No significant differences in the body weights were observed between the treatment groups. The absolute and relative heart weights were significantly increased in rats administered with ISO compared with those of the normal control rats. However, a significant reduction in heart weight was observed in rats pretreated with *C. macroptera* peel and pulp extracts prior to the ISO treatment compared with the heart weight measured for rats treated with only ISO. No significant differences in any parameters related to weight were observed in rats treated with peel and pulp extracts alone compared with the control rats.

The effects of *C. macroptera* peel and pulp extracts and ISO administration on heart function were indicated by serum cTn I levels and CK-MB, LDH, AST and ALT activities. ISO administration caused a significant increase in serum cTn I levels and CK-MB, LDH, AST and ALT activities compared with those of the normal controls. However, pretreatment with both *C. macroptera* peel and pulp extracts significantly decreased the cardiac enzymes and cTn I levels in rats challenged with ISO (Fig. 2 and 3). Fig. 4 presents the percentage protection observed in animals that received prior treatment with fruit peel and pulp extracts followed by the ISO challenge. The cardiac markers in rats pretreated with fruit peel extract were significantly more ameliorated compared with those in animals pretreated with fruit pulp extract, indicating that the fruit peel is superior in terms of antioxidant effects.

A significant increase in circulating levels of TC, TG and LDL-C and a corresponding non-significant decrease in HDL-C level were observed in ISO-treated rats relative to the levels measured in the normal controls. However, pretreatment with *C. macroptera* peel and pulp extracts significantly reduced the levels of TC, TG and LDL-C while restoring the HDL-C level compared with the levels measured for the ISO-challenged group (Table 2). Again, pretreatment with *C. macroptera* peel extract showed a stronger ameliorative effect in ISO-induced myocardial infarct rats compared with that observed in rats pretreated with fruit pulp (Fig. 4).

The level of the oxidative stress biomarker LPO was significantly higher in rats treated with ISO alone, as indicated by the increase in heart MDA levels compared with those of the normal control. It is plausible that *C. macroptera* extract pretreatment conferred a protective effect because animals pretreated with fruit peel and pulp extracts had significantly lower MDA levels compared with those of the ISO-induced infarcts rats. LPO level was significantly higher in the Peel + ISO group than in the Pulp + ISO group (Fig. 5 and 6).

The effects of *C. macroptera* extract administration on glutathione and antioxidant enzyme levels in rats' heart tissues are presented in Table 3. Again, the rats that received the ISO-alone treatment had a worse prognosis, with decreased levels of GSH-Px, GSH-Rx, GSTs and GSH compared with those of the normal control rats. Pretreatment with fruit peel and pulp extracts, however, significantly ameliorated the investigated parameters compared with those observed in the rats treated with ISO alone. This finding was supported by the higher levels of antioxidant enzymes observed in rats pretreated with fruit peel compared with those in the pulp group (Fig. 6).

Fig. 7 (a-f) shows the effect of *C. macroptera* peel and pulp and ISO treatments on the histoarchitecture of the rats' heart tissues.

Table 1
The effects of *C. macroptera* peel and pulp extracts and ISO on body, absolute and relative heart weights in the different animal groups.

Group	Initial body weight (g)	Final body weight (g)	Body weight gain (%)	Absolute heart weight (g)	Relative heart weight (g/100 g)
Control	137.00 ± 8.65^{a}	167.75 ± 6.98^{a}	17.16 ± 2.78^{a}	0.66 ± 0.05^{a}	0.39 ± 0.03^{a}
Peel	136.50 ± 5.74^{a}	173.57 ± 7.76^{a}	21.18 ± 2.96^{a}	0.63 ± 0.09^{a}	0.37 ± 0.19^{a}
Pulp	133.87 ± 6.80^{a}	172.00 ± 7.45^{a}	22.24 ± 1.40^{a}	0.66 ± 0.08^{a}	0.39 ± 0.0^{a}
ISO	108.70 ± 7.20^{a}	160.00 ± 7.50^{a}	31.71 ± 2.60^{a}	0.87 ± 0.03^{c}	0.57 ± 0.03^{c}
Peel + ISO	127.30 ± 8.80^{a}	165.60 ± 8.70^{a}	23.00 ± 1.20^{a}	0.75 ± 0.07^{b}	0.46 ± 0.02^{b}
Pulp + ISO	129.40 ± 5.04^{a}	157.00 ± 8.60^{a}	23.06 ± 1.20^{a}	0.75 ± 0.07^{b}	0.46 ± 0.02^{b}

[a]Data are presented as the means ± SD, (n = 6).
[a,b,c] Values in the same column that do not share superscript letters (a, b, c) indicate significant differences at $p < 0.05$.% body weight (bw) gain = [(final bw − initial bw)/final bw] × 100.
ISO: Isoproterenol

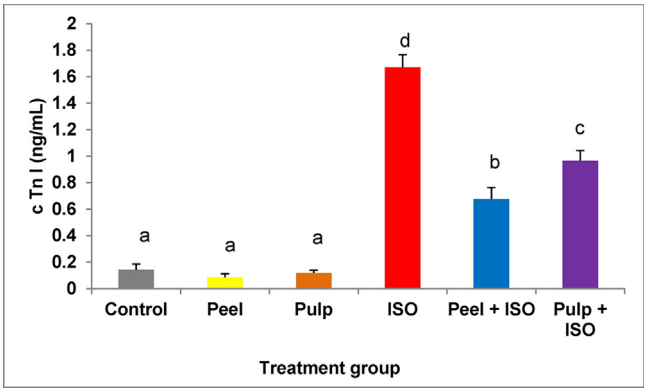

Fig. 2. The effects of *C. macroptera* peel and pulp extracts and ISO on serum cTn I levels in the different groups.
The bars represent means ± SD (n = 6); bars with different letters indicate significant differences at $p < 0.05$.
ISO: Isoproterenol, cTn I: Cardiac Troponin I

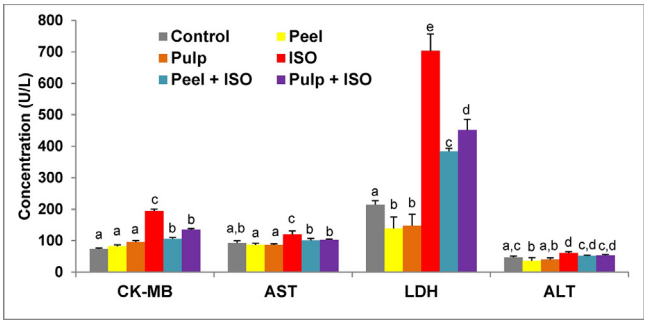

Fig. 3. The effects of *C. macroptera* peel and pulp extracts and ISO on serum CK-MB, AST, LDH and ALT activities in the different groups.
The bars represent means ± SD (n = 6); bars with different letters indicate significant differences at $p < 0.05$.
ISO: Isoproterenol, CK-MB: creatinine kinase-MB, AST: aspartate transaminase, ALT: alanine transaminase, LDH: lactate dehydrogenase

Table 2
The effects of *C. macroptera* peel and pulp extracts and ISO on serum lipid profiles.

Group	TC (mg/dL)	TG (mg/dL)	LDL-C (mg/dL)	HDL-C(mg/dL)
Control	49.17 ± 4.45^{a}	$58.33 \pm 12.08^{a,b}$	5.50 ± 1.79^{a}	32.00 ± 3.35^{a}
Peel	45.17 ± 2.56^{a}	51.50 ± 4.97^{a}	2.37 ± 1.28^{a}	32.50 ± 2.07^{a}
Pulp	46.67 ± 2.06^{a}	$56.16 \pm 3.06^{a,b}$	3.77 ± 2.37^{a}	32.17 ± 1.17^{a}
ISO	72.00 ± 5.37^{c}	70.16 ± 4.07^{c}	28.63 ± 5.95^{c}	29.33 ± 2.50^{a}
Peel + ISO	63.00 ± 3.58^{b}	$60.20 \pm 2.14^{a,b,c}$	19.36 ± 4.60^{b}	31.60 ± 2.15^{a}
Pulp + ISO	$66.67 \pm 3.83^{b,c}$	$62.83 \pm 3.60^{b,c}$	$23.43 \pm 5.84^{b,c}$	30.67 ± 3.08^{a}

[a]Data are presented as the means ± SD, (n = 6).
[a,b,c] Values in the same column that do not share superscript letters (a, b, c) indicate significant differences at $p < 0.05$.
ISO: Isoproterenol, TC: total cholesterol, TG: triglycerides, LDL-C: low-density lipoprotein-cholesterol, HDL-C: high-density lipoprotein-cholesterol

The normal control rats showed a regular arrangement of cardiac muscle fibers, with the integrity of the myocardial membrane clearly intact with no infarction. Histopathological findings confirmed the induction of MI by ISO based on the presence of widespread myocardial structural disorder, necrosis of myocytes, separation of cardiac muscle fibers and edematous intramuscular spaces, as well as the presence of inflammatory infiltrates. Pretreatment with fruit peel and pulp extracts (500 mg/kg) for 45 days, however, reduced the degree of infiltration of inflammatory cells where treated rats had relatively well-preserved cardiac muscle fiber morphology. In addition, all rats in the baseline group showed no changes in the histoarchitecture of the heart tissue compared with the normal control rats (Fig. 8).

4. Discussion

To our knowledge, this study is the first animal-based investigation that confirms the cardio-protective effect of *C. macroptera* fruit extracts against experimentally induced MI. In this study, a significant increase in heart weight with relatively unchanged body weight following ISO administration that contributed to an increased heart weight to body weight ratio was observed. The increased heart weight may be attributed to ISO-induced oxidative damage of cardiac tissues, resulting in cell swelling and increased fibrosis and water content, with the presence of edematous intramuscular space [4,29]. confirmed by

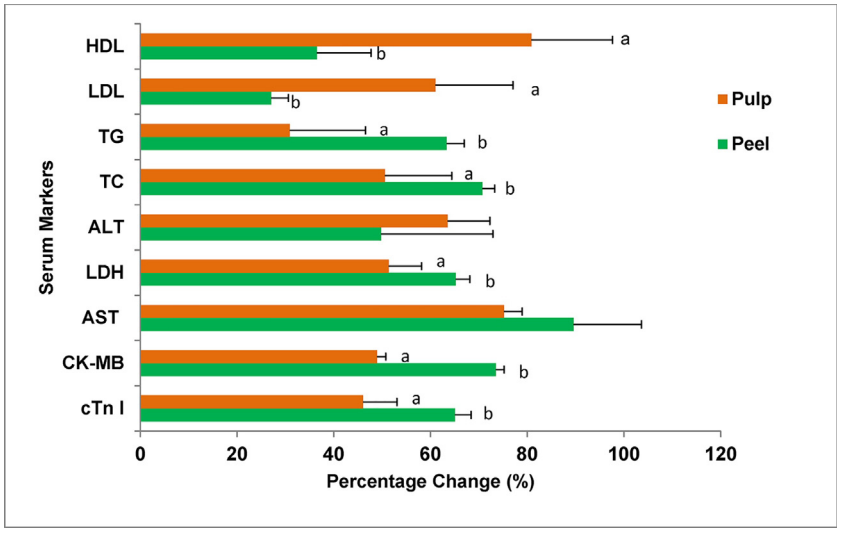

Fig. 4. Percentage change of cardiac biomarkers and lipid profile in serum for *C. macroptera* peel extract relative to pulp extract.
Percentage of protection conferred by fruit peel and pulp is calculated as 100 X[(value of ISO − value of Peel + ISO or Pulp + ISO)/(value of ISO − value of control)], bars with different letters indicate significant difference at $p < 0.05$.
HDL-C: high-density lipoprotein-cholesterol, LDL-C:low-density lipoprotein-cholesterol, TG: triglycerides, TC: total cholesterol, ALT: alanine transaminase, LDH: lactate dehydrogenase, AST: aspartate transaminase, CK-MB: creatinine kinase-MB and cTn I: cardiac troponin I.

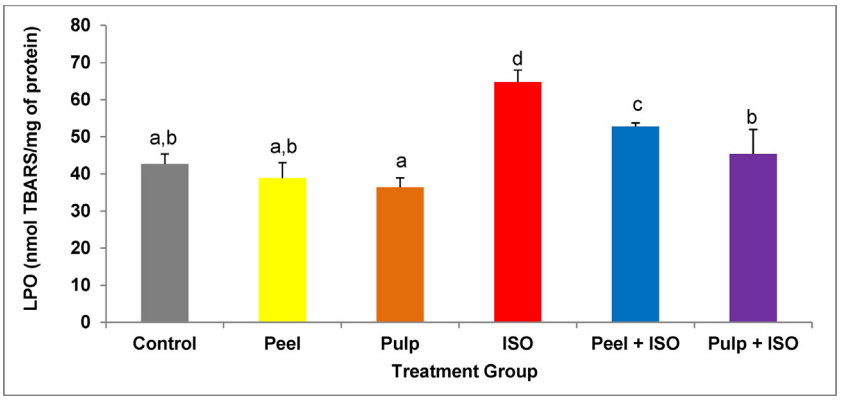

Fig. 5. The effects of *C. macroptera* peel and pulp extracts and ISO on cardiac LPO levels in the different groups.
The bars represent means ± SD (n = 6); bars with different letters indicate significant differences at $p < 0.05$. ISO: Isoproterenol, LPO: Lipid peroxidation

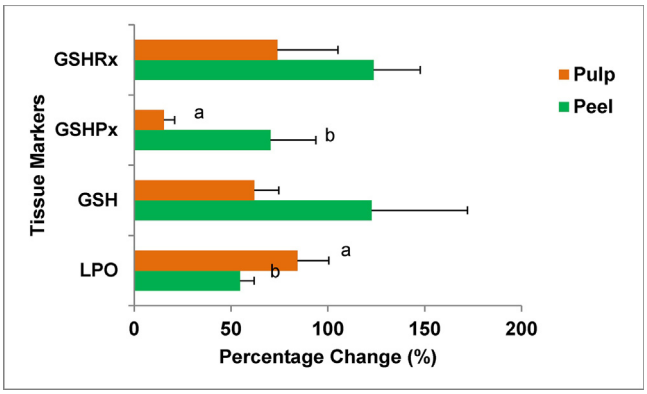

Fig. 6. Percentage change of LPO and antioxidant enzymes in heart tissue for *C. macroptera* peel extract relative to pulp extract.
Percentage of protection conferred by fruit peel and pulp is calculated as 100 X [(value of ISO − value of Peel + ISO or Pulp + ISO)/(value of ISO − value of control)], bars with different letters indicate significant difference at $p < 0.05$.
GSH-Rx: glutathione reductase, GSH-Px: glutathione peroxidase, GSH: reduced glutathione, LPO: lipid peroxidation.

biochemical and histopathological findings. Pretreatment with peel and pulp extracts, however, helped to maintain near-normal heart weights, indicating the therapeutic benefits of the fruit as an antioxidant.

Cardiac troponin I (cTn I) is a low-molecular-weight regulatory protein of the heart muscle that controls calcium ion (Ca^{2+})-mediated interactions between actin and myosin [30]. Normally, this protein is highly preserved in cardiac cells but is released upon myocardial injury, thus making this contractile protein a highly specific and sensitive diagnostic marker for MI [31,32]. As expected, an elevated level of cTn I was observed in rats treated with ISO alone compared with level measured for the normal controls. The results are consistent with those of previous studies [4,12]. Animals pretreated with peel and pulp extracts followed by ISO challenge, however, showed significantly lower cTn I levels compared with those measured for rats treated with ISO alone. This phenomenon may be attributed to the high amounts phenolics and other antioxidants in the *C. macroptera* peel and pulp, which may contribute to the preserved structural and functional integrity of the contractile apparatus in the rat's myocardium indicated by the prevention of oxidative injury of cardiac muscles.

In this study, ISO-treated rats showed high levels of diagnostic marker enzymes (CK-MB, LDH) and preserved enzymes (AST, ALT) in the bloodstream, possibly due to cardiac damages, corroborating the results of previous studies [2,4]. The cellular biomarker enzymes in the serum can indicate heart function and alterations in plasma membrane permeability [33]. Pretreatment with peel and pulp extracts was found to significantly ameliorate the activities of all marker enzymes in the serum, indicating that *C. macroptera* might help to maintain membrane integrity, thereby limiting the leakage of the enzymes. It is plausible that *C. macroptera* peel and pulp, which contain high concentrations of polyphenols, flavonoids, ascorbic acids, tannins and reducing sugars [16], may confer a protective effect against oxidative cardiac injury, thus limiting the leakage of these enzymes from the myocardium. Rats pretreated with the peel extract followed by ISO challenge showed significantly lower LDH levels than the pulp-extract-treated rats, which indicates that the peel extract conferred significantly higher early protection of the myocardium, as also confirmed by the stronger antioxidant properties of the peel [4].

Table 3
The effects of *C. macroptera* peel and pulp extracts and ISO on the levels of reduced glutathione and glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Rx), glutathione-S-transferase (GSTs) activities in the heart tissue of normal and treated rats.

Group	GSH (μmol/mg of protein)	GSH-Px (nmol NADPH oxidized/min/mg of protein)	GSH-Rx (nmol NADPH oxidized/min/mg of protein)	GSTs (nmol CDNB conjugated/min/mg of protein)
Control	5.05 ± 0.78^{a}	45.16 ± 10.69^{a}	$134.79 \pm 3.65^{a,b}$	3.34 ± 0.67^{a}
Peel	5.19 ± 1.15^{a}	$40.61 \pm 5.88^{a,b}$	142.81 ± 6.32^{b}	3.22 ± 1.45^{a}
Pulp	5.07 ± 0.91^{a}	$35.61 \pm 7.12^{a,b,c}$	$136.97 \pm 4.93^{a,b}$	2.12 ± 1.22^{a}
ISO	4.32 ± 0.48^{a}	21.01 ± 4.59^{c}	122.47 ± 8.27^{a}	1.42 ± 0.44^{b}
Peel + ISO	5.28 ± 0.64^{a}	$35.15 \pm 16.34^{a,b,c}$	138.88 ± 17.94^{b}	0.98 ± 0.70^{a}
Pulp + ISO	4.45 ± 0.23^{a}	$25.32 \pm 2.90^{b,c}$	$128.44 \pm 7.42^{a,b}$	1.86 ± 0.61^{a}

[a]Data are presented as the means ± SD(n = 6).
[a,b,c] Values in the same column that do not share superscript letters (a, b, c) indicate significant differences at $p < 0.05$.
ISO: Isoproterenol

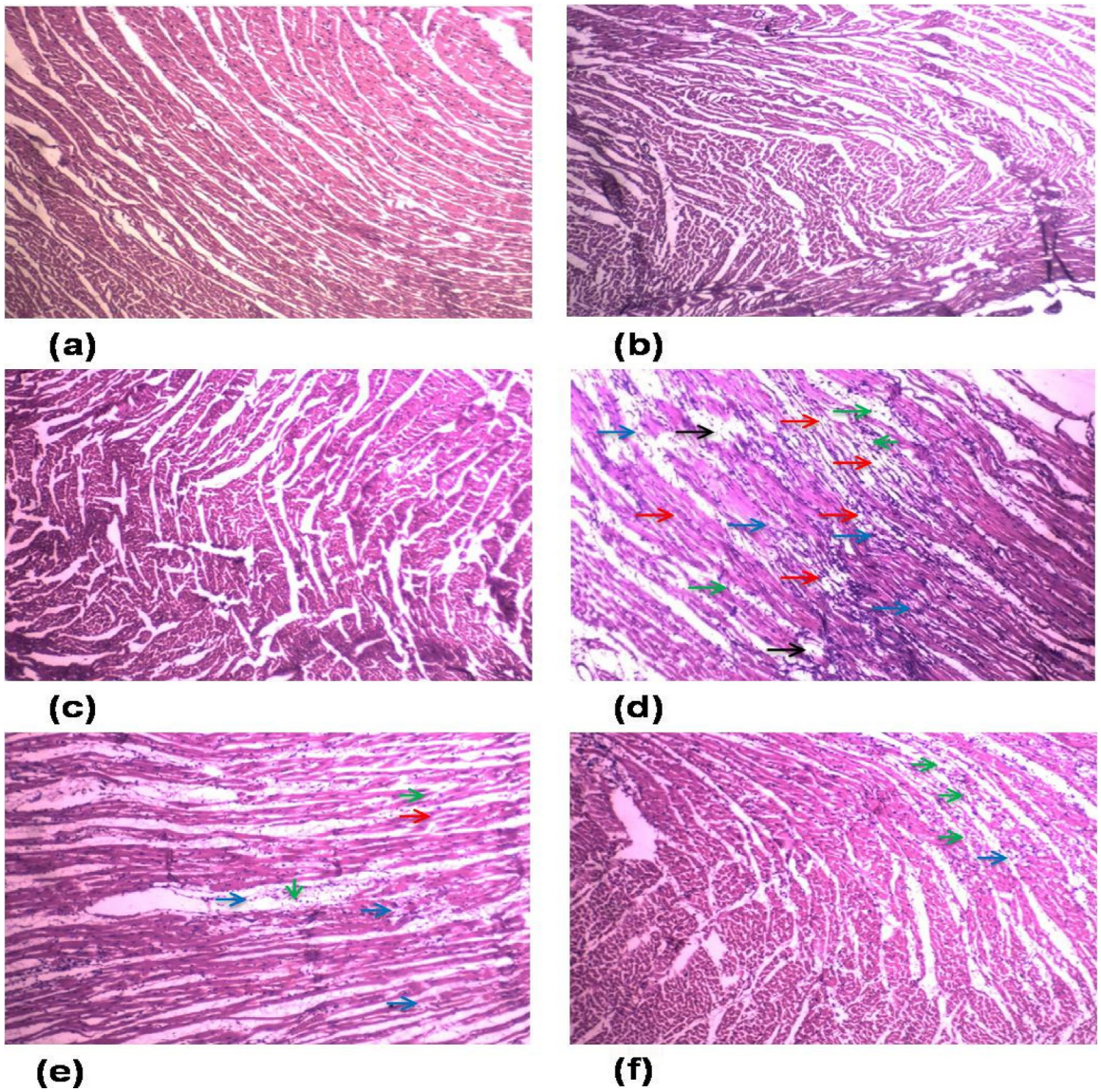

Fig. 7. The effects of *C. macroptera* peel and pulp extracts and ISO on the histopathological features of the heart tissues of experimental rats. a) The heart of a normal control rat showing normal cardiac muscle fibers; (b) pretreatment with *C. macroptera* peel extract (500 mg/kg) showing normal muscle fibers with the absence of any pathological changes. (c) *C. macroptera* pulp extract pretreated rat heart showing normal muscle fibers without any pathological changes. (d) ISO (85 mg/kg)-treated heart showing cardiac muscle fibers with muscle separation (red arrows), edematous intramuscular space (black arrows), cellular necrosis (green arrows) and infiltration of inflammatory cells (blue arrows). (e) *C. macroptera* peel (500 mg/kg) + ISO (85 mg/kg)-treated heart with reduced degree of muscular separation and reduced inflammatory cells (blue arrows), cell necrosis (green arrows) and muscle fibrous separation from the edematous intramuscular space. (f) Pulp (500 mg/kg) + ISO (85 mg/kg)-treated heart showing restored muscular assembly with markedly reduced cell necrosis (green arrows) and inflammatory cells (blue arrows). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Altered lipid metabolism is another conformational status indicator of MI. Cardiovascular injuries are usually associated with increased blood cholesterol and the build-up thereof in cardiac tissues [34]. Hyperlipidemia plays a key role in the pathogenesis of cardiovascular disease not only because of the development of atherosclerosis but also of because of the modification of cellular membranes' structure, composition and stability [2,35]. In this study, the ISO-induced rats showed significant elevations in serum TC, TG and LDL-C levels. The hyperlipidemia observed in ISO-treated rats might be attributed to either enhanced biogenesis of lipids via the cardiac cAMP cascade [36] or increased hydrolysis of stored TG via hormone-sensitive lipase (HSL) activity stimulated by cAMP-dependent protein kinase [12]. Our findings regarding the ISO-induced alteration of the lipid profile are also similar to those reported in previous studies [4,37]. However, pretreatment with fruit peel significantly reduced serum TC and LDL-C levels. Additionally, TG levels were reduced relative to those of rats receiving ISO alone, and administration of the pulp extract restored these parameters. However, these changes were non-significant. It has been reported that polyphenols from fruit can bind with bile acids to increase their excretion, which may be the mechanism by which fruit peel extracts lower cholesterol [38]. Nevertheless, serum HDL-C levels remained unchanged in the different treatment groups. Further investigation is required to clarify this observation.

An elevated level of LPO is a specific indicator of oxidative damage of the myocardium and is positively correlated with increased generation of ROS as a result of auto-oxidation due to ISO, which is responsible for altering membrane integrity and function and/or decreasing the activity of the antioxidant defense system [4,39]. The reduced antioxidant defense mechanism fails to combat free radical damage effectively [40]. In the present study,

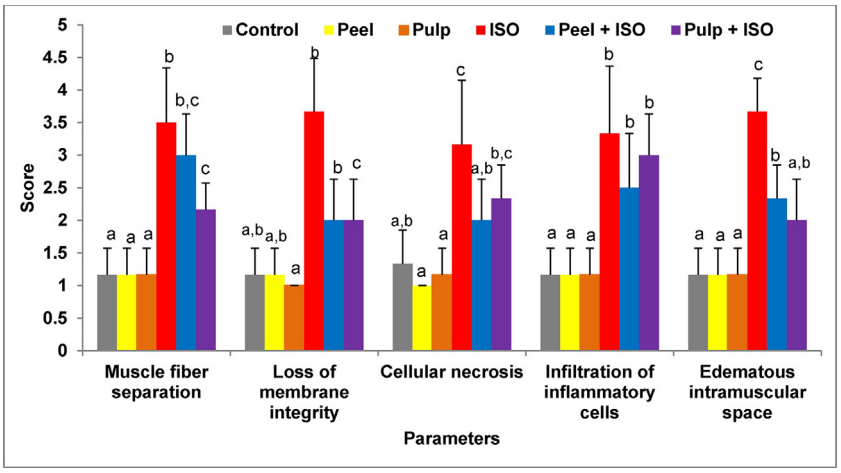

Fig. 8. Semi-quantitative scoring of the architectural changes as evidenced by histopathological examination of rat myocardial tissues. Scoring was performed as follows: none (1), mild (2), moderate (3) and severe (4) pathological changes.
The bars represent means ± SD (n = 6); bars with different letters indicate significant differences at $p < 0.05$. ISO: Isoproterenol.

animals treated with ISO alone showed significantly elevated levels of MDA, indicating LPO over-activity. However, pretreatment with *C. macroptera* extracts significantly decreased the levels of lipid peroxides in ISO-induced rats and conferred some protection to the cardiac tissue, possibly via the free radical scavenging activity of *C. macroptera*. Based on the LPO data, the pulp extract showed significantly higher activity than that of the peel, which might be attributed to a higher ascorbic acid concentration, as previously reported [16].

Oxidative stress induces cardiac damage, which was further confirmed by the decreased levels of the cellular antioxidant defense enzymes and low-molecular-mass antioxidants (reduced glutathione) [2,12]. Endogenous antioxidant enzymatic defense plays a key role in neutralizing oxygen free radical-mediated tissue injury [41]. SOD, catalase and GSH-Px are the primary free radical-scavenging enzymes involved in the first line of cellular defense against oxidative injury, removing both oxygen (O_2) and hydrogen peroxide (H_2O_2) before they can interact to form more reactive hydroxyl radicals [42,43]. In this study, decreased activities of GSH-Px, GSH-Rx and GST and reduced glutathione levels in the myocardium of ISO-treated rats were observed, which may be attributed to the increased generation of ROS and lipid peroxides. These changes lead to the inhibition of the aforementioned enzymes and result in decreased removal of superoxide radicals, H_2O_2 radicals and highly potent hydroxyl radicals [2]. *C. macroptera* extract pretreatment, however, ameliorated the levels of these enzymes in the heart tissue, suggesting that plant phenolics, flavonoids, tannins and other compounds may function as ROS scavenging compounds and form a cooperative network, inducing a series of redox reactions and interactions between ascorbic acid, phenolics and glutathione [44]. Thus, these compounds may act synergistically to confer some protection against the deleterious effects of ISO on the myocardium. It is also plausible that the phytoactive constituents of the peel and pulp extracts of *C. macroptera* may enhance the expression or activity of Nrf2 (transcription factor), released from its repressor (Keap1), which may occur during xenobiotic or oxidative stress. Nrf2 may then bind to the antioxidant response element (ARE) of cytoprotective genes, thus inducing their expression as well as the subsequent induction of free radical scavenging enzymes to neutralize and eliminate cytotoxic oxidants [12,45,46].

Overall, the peel extract showed a higher potential for restoring the antioxidant defense systems to normal than the pulp extract did. Interestingly, LPO level was significantly higher in the Peel + ISO group than in the Pulp + ISO group. However, all of the antioxidant defense system parameters of the Peel + ISO group were increased as well, indicating that despite the higher oxidative stress in the Peel + ISO group, the phytoconstituents of the peel extract may help boost the antioxidant defense mechanism, thus neutralizing the oxidative stress caused by ROS.

The histopathological findings obtained for the heart tissues are consistent with the biochemical observations. The cardiac tissues of the rats treated with normal control peel and pulp extracts alone showed normal muscle fibers with clear integrity of the myocardial cell membranes without any inflammatory cell infiltration. On the other hand, the heart tissues of rats treated with ISO alone showed severe necrosis of myocytes, inflammatory cell infiltration and massive separation of cardiac fibers. Animals that received *C. macroptera* extract pretreatment showed near-normal morphology of cardiac muscle with only mild necrosis of the myocytes. The animals also showed fewer inflammatory cells than those that received ISO alone. Similar histopathological findings have been obtained for rats treated with *C. medica* and *C. hystrix* followed by ISO challenge [47,48], indicating the protective potential of citrus fruits against oxidative injuries of the myocardium.

Overall, the histopathological results confirmed the biochemical findings. It is plausible that the antioxidant and anti-peroxidative compounds inherently present in the fruit might confer the observed cardio-protective effects. Identifying the exact mechanisms responsible for *C. macroptera*'s ability to attenuate disease pathogenesis will provide a clear pathway for the subsequent translation and commercialization of this new prophylactic therapy to reduce the growing burden of cardiovascular disease, particularly MI, on our health care system.

5. Conclusions

Both *C. macroptera* peel and pulp extracts conferred significant protection against ISO-induced MI in rats, although the peel extract was superior. The possible mechanisms underlying these effects include modulation of the levels of lipids and lipoprotein and improvement of the endogenous antioxidant enzyme system via inhibition of lipid peroxidation, thereby maintaining the levels of cardiac markers proteins and enzymes. The biochemical findings were further confirmed by histopathological examination.

Conflict of interests

The authors declare that there is no conflict of interest.

Acknowledgments

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