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Wax apple (*Syzygium samarangense*) fruit extract ameliorates endothelial dysfunction and liver damage in high cholesterol diet-fed rats

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ABSTRACT

Background and aim: Wax apple fruit (*Syzygium samarangense*) is one of the most popular tropical fruit in Asia, and contains several essential nutrients. Therefore, this study explored the effects of the wax apple fruit extract on a high-cholesterol diet-induced vascular endothelial dysfunction and fatty liver in rats.
Experimental procedure: Male Sprague Dawley rats were fed a diet with 1.5% cholesterol (HCD) for 8 weeks, and were given wax apple fruit extract (50 and 100 mg/kg/day) orally for the last 4 weeks. After 8 weeks, blood sample, thoracic aorta, and liver were collected and processed for biochemical and histological analysis. Additionally, vascular endothelial function and the protein expression of oxidative stress markers in aortae were evaluated.
Results and conclusion: Wax apple reduced serum triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), aspartate aminotransferase (AST), and alanine aminotransferase (ALT), but increased high-density lipoprotein cholesterol (HDL-C) levels. Furthermore, the liver levels of TG and TC were reduced in wax apple-treated hypercholesterolemic rats. Histological studies revealed that wax apple ameliorated HCD-induced morphologic changes of aortic and liver tissues of rats. In aortic tissues, the impaired endothelium-dependent responses to acetylcholine, the reduced nitric oxide (NO) contents, the elevated endothelin (ET)-1 contents, and the increased expression of NADPH oxidase subunit $p47^{phox}$ and 4-hydroxynonenal in HCD-fed rats were reversed by wax apple treatment. These results suggest that oral administration of wax apple improves vascular dysfunction and damage in hypercholesterolemic rats possibly through increasing NO bioavailability, decreasing ET-1 levels and reducing oxidative stress. Furthermore, wax apple ameliorates the HCD-induced fatty liver in rats.

1. Introduction

Hypercholesterolemia is a lipid metabolism disorder characterized by elevated blood levels of total cholesterol and low-density lipoprotein-cholesterol (LDL-C), and is one of the major risk factors for cardiovascular diseases (CVD), which is the leading cause of death worldwide.[1] Moreover, hypercholesterolemia also causes other health problems, including liver diseases and obesity.[2,3] Epidemiological studies have demonstrated that the incidence of hypercholesterolemia is highly associated with poor dietary habits, such as the overconsumption of foods containing high amounts of cholesterol and saturated fats, which lead to the increased incidence of atherosclerosis.[4]

There is growing evidence that the primary cause of CVD is the development of atherosclerosis, which is a result of impaired

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List of abbreviations

CVD	cardiovascular diseases
NAFLD	nonalcoholic fatty liver disease
ET-1	endothelin-1
NO	nitric oxide
HCD	high-cholesterol diets
TC	total cholesterol
TG	triglyceride
LDL	low density lipoprotein
HDL	high density lipoprotein
AST	aspartate aminotransferase
ALT	alanine aminotransferase
AI	atherogenic index
ACh	acetylcholine
SNP	sodium nitroprusside
PE	phenylephrine
NADPH	nicotinamide adenine dinucleotide phosphate
4-HNE	4-hydroxynonenal

vascular endothelial function. Vascular endothelial cells that line the inner blood vessels play a crucial role in maintaining vascular homeostasis and regulating vascular tone by producing several vasoactive mediators, such as vasodilator nitric oxide (NO) and vasoconstrictor endothelin (ET)-1; endothelial dysfunction is one of the earliest events of atherosclerotic cardiovascular disease.[5–7] Impaired endothelium-dependent relaxation caused by a decreased endothelium-derived relaxing factor NO or an increased endothelium-derived contracting factor ET-1 is the main functional characteristic of endothelial dysfunction which has been demonstrated in animal models of hyperlipidemia.[8–10]

Oxidative stress is widely accepted to be responsible for endothelial dysfunction in numerous pathological conditions, including hypercholesterolemia.[7,11] Numerous experimental studies have reported that reactive oxygen species (ROS) generation during hypercholesterolemic conditions are related to the development of endothelial damage and atherosclerosis.[9,12] The increased availability of lipids in hypercholesterolemia facilitates the overproduction of ROS, with consequent oxidative stress, which damages the endothelium and promotes the initiation and progression of atherogenesis.[13] These reactive free radicals can quench NO bioavailability and impair endothelium-dependent relaxation, which consequently results in endothelial dysfunction and promotes vascular wall thickening and luminal narrowing.[14,15] This results in the occurrence and development of atherosclerosis.[14–16]

Another important pathological state involving hypercholesterolemia is non-alcoholic fatty liver disease (NAFLD), which is characterised by the accumulation of lipid droplets in the hepatocyte cytoplasm, and may progress to non-alcoholic steatohepatitis and possibly hepatocellular carcinoma.[2,17] Therefore, the prevention and treatment of hyperlipidemia may have a significant impact not only on the development of atherosclerosis but also on the development of NAFLD.

Epidemiological studies have demonstrated that the intake of fruits and vegetables has a strong protective effect against various chronic diseases including CVD and NAFLD.[18,19] *Syzygium samarangense* (Blume) Merrill and Perry, commonly known as wax apple, is an important tropical fruit and widely cultivated in Asia.[20,21] It is a plant species in the family Myrtaceae and contains numerous nutrients and bioactive components including phenols, flavonoids, flavonol glycosides, ascorbic acid, proanthocyanidins, anthocyanins, and ellagitannins.[20–22] The wax apple has been reported to exhibit various bioactivities such as antioxidant,[23,24] antiglycemic,[21] and anti-inflammatory[25] activities. Previous studies also reported that wax apples increased antioxidant enzyme activities, superoxide dismutase and catalase, and reduced nitrotyrosine in the pancreas of diabetic rats.[21] We hypothesised that the consumption of wax apples can improve a high cholesterol diet (HCD)-induced hypercholesterolemia and vascular oxidative stress, and lead to a decrease in vascular dysfunction and damage. To the best of our knowledge, the beneficial protection of wax apples on endothelial dysfunction and hepatic lipid accumulation caused by HCD intake, is still unreported. Therefore, the objectives of this study were to investigate the possible beneficial effects of wax apple on the structural and functional changes of vascular and liver tissues in rats fed a HCD, and to explore the mechanisms underlying these vascular benefits of wax apple.

2. Materials and methods

2.1. Reagents and chemicals

Acetylcholine chloride (ACh), phenylephrine (PE), and cholic acid were purchased from Sigma Aldrich Chemical Co (St Louis, MO, USA). Primary antibodies against p47 ^{phox}, 4-HNE, and β-actin were purchased from Abcam (Cambridge, MA, USA). Goat anti-rabbit IgG conjugate secondary antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). All other reagents and solvents were analytical grade.

2.2. Preparation of the powdered wax apple

The wax apple fruit species used in our experiments was authenticated and prepared by Dr Tatdao Paseephol at the Department of Food Technology and Nutrition, Faculty of Technology, Mahasarakham University, Thailand, as described previously.[21] Briefly, the fresh fruit (1 kg) was washed thoroughly, and cut into 0.5 × 0.5 cm^2 pieces and lyophilised using a laboratory scale freeze dryer (Heto Power Dry PL3000, Thermo Fisher Scientific) at −45 °C. The samples were then ground into powder using a grinder (MX AC 400, Panasonic), with a yield of ~16% (w/w), and the powdered wax apple was stored at −20 °C until required for further study.

2.3. Phytochemical and proximate analysis of wax apple

The contents of total phenolic and total flavonoid in the lyophilised powder of wax apple were determined by colorimetric methods, as previously reported[21 26]. The proximate composition and nutrient contents of powdered of wax apple including ash, carbohydrates, fat, moisture, protein, total dietary fibre, vitamin C, β-carotene, and total anthocyanin were analysed at the Central Lab Thai laboratory, Thailand, according to standard procedures, as previously described.[21,26]

2.4. Experimental protocol

All animal protocols described in this study were approved by the Institutional Animal Care Committee of Naresuan University (ethical approval number: 5801001). The male Sprague-Dawley rats that weighed 180–200 g were obtained from the National Laboratory Animal Centre at Salaya, Mahidol University, Thailand. All the rats were housed in the Centre for Animal Research at Naresuan University at 22 ± 1.0 °C, with a 12-h light/dark cycle, and were allowed free access to water and food during a 1-week acclimatisation period.

After acclimatisation, the rats were divided into four groups

(n = 6 in each group) as follows: group I: the control group (C) fed a normal diet; group II: the hypercholesterolemic group (HC) fed a high cholesterol diet (HCD); group III: the HC plus low dose of wax apple group (HC + WAL) fed HCD and 50 mg/kg of wax apple; group IV: the HC plus high dose of wax apple group (HC + WAH) fed HCD and 100 mg/kg of wax apple. The HCD was prepared according to the previous studies[9,26] with slight modifications consisting of 1.5% cholesterol, 0.37% cholic acid, and 20% palm oil and given to the rats for 8 weeks, and 50 and 100 mg/kg/day of wax apple was administrated orally for the last 4 weeks of the study (from week 5 to week 8 of dietary treatment). The dosage of wax apple used in this study was selected according to a previous study,[21] which reported that this dosage exerted beneficial health effects in animal model such as anti-oxidant, anti-inflammatory, and anti-diabetic properties.[21] The treatment doses of powdered wax apple at 50 and 100 mg/kg BW were converted to a human equivalent dose (HED),[27] which suggests that human consumption would be 484 and 968 mg, respectively, for adults weighing 60 kg. The initial and final body weights and daily food intake of the rats were also evaluated.

2.5. Blood and tissue collection

At the end of the experiment, the rats were fasted overnight and anesthetised with an intraperitoneal injection of 50 mg/kg/BW of sodium pentobarbital. Blood samples were then collected via cardiac puncture, and the serum samples were separated using a centrifuge at 1000 g for 10 min at 4 °C, and then stored at −80 °C until further analysis. Finally, all the rats were euthanised, and their thoracic and abdominal aortas were quickly isolated and cleaned of excess connective tissues and fat. One part of the isolated thoracic aortic ring was immediately placed in Krebs bicarbonate solution (composition (mM): NaCl, 118.0; KCl, 4.7; $MgSO4{\cdot}7H_2O$, 1.2; KH_2PO_4, 1.1; $NaHCO_3$, 25.0; D-glucose, 11.0; and $CaCl_2{\cdot}2H_2O$, 2.5) for vasorelaxation response experiments, while another part was fixed in 10% neutral buffered formalin solution for microscopic examination. The sections of abdominal aorta were collected and frozen for nitrate and nitrite assays and Western blot analysis. The livers were dissected, weighed, and divided into two parts, with the first being kept at −80 °C for biochemical analysis, while the second part was used for histopathological studies.

2.6. Biochemical assays

The serum concentrations of the total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL-C), high-density lipoprotein cholesterol (HDL-C), aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN) and creatinine were analysed using an automated biochemistry analyser (Cobas c 111 analyser). The atherogenic coefficient (AC) was calculated according to AC = (TC-HDL-C)/HDL-C and the atherogenic index of plasma (AIP) was calculated from Log (TG/HDL-C).[28]

2.7. Vascular function studies

The isolated thoracic aortas were cleared of connective tissue, then cut into 2–3 mm lengths. The aortic ring segments were attached to two stainless steel hooks that were connected to an isometric force transducer (AD Instruments Ltd, Australia) which was coupled to a Bridge Amplifier (PowerLab, AD Instruments Ltd., Australia) to measure their isometric tension. Each aortic ring was placed in a 20 mL organ bath chamber containing Krebs bicarbonate buffer at 37 °C and bubbled with 95% O_2 and 5% CO_2. The rings were maintained at an initial tension of 1 g, and allowed to equilibrate for 1 h. To induce maximum contraction ($KPSS_{max}$), the bath contents were replaced with an isotonic, 123 mM potassium physiological salt solution (KPSS), in which all the NaCl was replaced with 123 mmol/L of KCl. The aortic rings were then washed with fresh Krebs bicarbonate buffer, which allowed the presence of the endothelium to be determined. For these tests, the aortic rings were submaximally precontracted between 40% and 60% of the $KPSS_{max}$ response with phenylephrine (PE, 1 nM-10 μM), and then acetylcholine (ACh, 10 μM) was added to confirm the integrity of the endothelium. Cumulative concentration-response curves to the endothelium-dependent relaxant ACh (1 nM-10 μM) and the endothelium-independent relaxant sodium nitroprusside (SNP, 1 nM-10 μM) were determined in the aortic rings precontracted with PE (1 nM-10 μM). The relaxation responses were expressed as a percentage of the precontraction level obtained with PE.

2.8. Measurement of aortic nitrite/nitrate and serum ET-1 levels

The concentration of the final stable NO metabolites (nitrite/nitrate) were measured in aortic homogenates by the Griess method. Aortic tissues were prepared and homogenised in lysis buffer as previously described.[9] The supernatants were used to measure the total nitrite/nitrate levels using a colorimetric assay kit (Cayman Chemicals, Ann Arbor, MI, USA) according to the manufacturer's instructions, and the results are expressed as μM/mg protein. Serum ET-1 levels were quantified by enzyme-linked immunosorbent assay (ELISA) kit (BosterBio, USA) according to the manufacturer's instructions.

2.9. Measurement of hepatic lipid contents

The livers were excised and rinsed with cold phosphate-buffered saline, the total liver lipids were extracted using a lipid extraction kit (Abcam, UK). The concentrations of TG and TC were measured using appropriate commercial enzyme assay kits (Merck, Germany) according to the manufacturer's instructions, and the hepatic TG and TG levels were expressed as mg/g of tissue.

2.10. Western blot analysis

Western blotting was performed on the aortic homogenates in order to detect the protein expression of the $p47^{phox}$ and 4-HNE as previously described.[9] In brief, the aortic tissues were homogenised in ice-cold RIPA buffer containing protease inhibitor and centrifuged at 15,000×*g* for 20 min at 4 °C. The supernatant was collected, and its protein content was measured using a bicinchoninic acid protein assay kit (Merck, Germany). A total of 30 μg of protein per aortic homogenates were separated on 12.5% sodium dodecyl sulphate polyacrylamide gel by an electrophoresis system and then transferred to polyvinylidenedifluoride membranes which were blocked with 5% non-fat dried milk in Tris buffer saline with 0.1% Tween-20 (TBST) for 1 h and then incubated with *anti*-$p47^{phox}$ (1:500), anti-4-HNE (1:500), or *anti*-β actin (1:5000) at 4 °C overnight. The membranes were washed with TBST, then incubated for 1 h at room temperature with the anti-rabbit horseradish peroxidase conjugated secondary antibody (1:5000). The protein expression was visualised using Luminata forte HRP detection reagent (Merck, Germany), and the protein bands were quantified by densitometry using a Bio-Rad image analysis system (Bio-Rad Laboratories Inc., USA). The intensity of the bands were normalised to β-actin protein expression from the same sample, and the data are expressed as percentages of the control.

2.11. Histopathological analyses of aorta and liver

The thoracic aorta and liver tissue were fixed in 10% neutral buffered formalin solution, and processed routinely for paraffin embedding. The histological changes of the aortic and liver sections were studied using hematoxylin and eosin (H&E) staining. In addition the aortic sections were stained for determining elastin fibres in the aortic media layer using the Verhoeff-Van Gieson technique. All stained sections were photographed via a light microscopy and evaluated for histological changes. The aortic wall thickness and media to lumen ratio (M/L) were analysed using Image J (version 1.51j, National Institutes of Health, USA) as previously described.[29]

To examine hepatic fat accumulation, frozen liver samples embedded in optimum cutting temperature compound were sectioned on a cryostat microtome at 8 μm and stained with Oil-Red O as previously described.[30] Oil red O-strained area was quantified in four randomly selected fields per section, and the percentage of stained area were analysed using Image J (version 1.51j, National Institutes of Health, USA).

2.12. Statistical analysis

The results are expressed as the mean ± SEM. Concentration-response to ACh or SNP were fitted to a sigmoidal curve using GraphPad Prism, version 5 (GraphPad Software Inc., San Diego, CA, USA) to calculate the sensitivity of each agonist (pEC_{50}). Maximum relaxation (R_{max}) to ACh or SNP was calculated as a percentage of PE induced precontraction. The statistical significance of differences was determined using one-way analysis of variance (ANOVA), followed by the Tukey HSD multiple comparison test (GraphPad Software Inc.). $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Proximate composition and phytochemical screening

Proximate and nutritional compositions of the powdered wax apple fruits revealed high levels of carbohydrate, fibres, anthocyanin, and vitamin C (Table 1). In addition phytochemical screening revealed that the powdered wax apple had high total phenolic and total flavonoid contents at 862 ± 24 mg gallic acid equivalents (GAE)/100 g and 143.7 ± 6.9 mg quercetin equivalent (QE)/100 g, respectively.

3.2. Effect of wax apple on body weight and liver weight

Table 2 revealed that there was no significant difference in the initial BW and food intake during the feeding period among the four groups. At the end of the experiment, the final body weight, liver weight, and the liver to body weight ratio of the rats fed a HCD were significantly greater than the control group. However, treatment with wax apple reduced liver weight, and the liver to body weight ratio, but did not have any significant effect on the body weight of the HCD-fed rats.

Table 1
Compositions of the dried wax apple.

Component	Values
Energy (kcal/100 g DW)	348.44
Ash (g/100 g dried powder)	0.24
Carbohydrate (g/100 g dried powder)	79.85
Fat (g/100 g dried powder)	1.16
Moisture (g/100 g dried powder)	14.10
Protein (g/100 g dried powder)	4.65
Soluble Dietary Fiber(g/100 g dried powder)	0.40
Beta-carotene (μg/100 g dried powder)	194.80
Vitamin C (mg/100 g dried powder)	6.66
Anthocyanin (mg cyaniding-3-glucoside/100g dried power)	6.87

3.3. Effect of wax apple on serum biochemical parameters

The rats fed with a HCD for 8 weeks exhibited a significant increase in TC, TG, and LDL-C, and a significant decrease in HDL-C levels in the serum compared to the control rats. After 4 weeks of treatment, both high- and low-dose administration of the wax apple significantly reduced the serum levels of TC, TG, and LDL-C compared with the untreated hypercholesterolemic rats. In addition the serum HDL-C levels were significantly increased in the HC + WAH group, but not in the HC + WAL group, compared with the HC group. Additionally, the HC group had higher atherogenic indices, AC and AIP, than control group, and both dosages of wax apple significantly reduced the increased AC and AIP in HCD-fed rats (Table 2).

Liver enzymes, i.e. AST and ALT, are considered as effective biochemical markers of liver injury. As shown in Table 2, the serum AST and ALT levels were significantly increased after 8 weeks of HCD treatment compared to normal diet-fed rats. However, after wax apple administration to the HCD-fed rats for 4 weeks at both dosages (50 and 100 mg/kg/day), the serum AST and ALT levels were significantly reduced. These effects of decreased liver enzyme activities were more significant in the HC + WAH group than the HC + WAL group. These results demonstrated that the consumption of HCD caused hepatic injury in rats, while wax apple treatment could effectively attenuate the adverse effect of HCD-caused liver damage.

3.4. Effect of wax apple on aortic nitrite/nitrate and serum ET-1 contents

HCD feeding significantly decreased the contents of NO metabolites nitrite/nitrate in the aortic tissues by 44.2% compared to the control group. Treatment with low and high doses of wax apple increased the aortic total nitrate/nitrite levels by 44.8 and 62.1%, respectively, compared with the HCD group (Table 2). Conversely, serum ET-1 levels were significantly increased in the HC group compared to control group by 20%. Only high doses of wax apple significantly decreased serum ET-1 levels by 12% as compared to HC group (Table 2). These observations suggest that an improvement in these vasoactive substances may exert beneficial effects regarding endothelial function.

3.5. Effect of wax apple on vascular relaxation

Maximum relaxation (R_{max}), but not the sensitivity (pEC_{50}), to ACh was significantly decreased in the aortic rings of hypercholesterolaemic rats compared with those of the control rats, indicating that hypercholesterolemia impaired endothelium-dependent relaxation in the rat aorta. The treatment with low and high doses of wax apple increased the maximum relaxation to ACh in the aortic rings of hypercholesterolaemic rats, without affecting the sensitivity to ACh (Fig. 1A and Table 3). In addition, there was no significant difference in the sensitivity and maximum relaxation to the endothelium-independent agonist SNP in the aortae of all groups (Fig. 1B and Table 3). These results suggest that wax apple treatment was effective in improving endothelial dysfunction in HCD-induced hypercholesterolemia in the rats.

Table 2
Effect of wax apple on body weight, liver weight, and biochemical analysis in HCD-fed rats.

Parameter	C	HC	HC + WAL	HC + WAH
Initial BW (g)	204 ± 4	214 ± 9	206 ± 6	213 ± 7
Final BW (g)	445 ± 7	484 ± 3*	460 ± 5	477 ± 5
Food consumption (g)	28.6 ± 0.3	31.2 ± 0.6	28.8 ± 0.2	28.9 ± 0.2
LW (g)	13.2 ± 0.5	21.7 ± 0.6**	17.9 ± 0.4**,++	17.6 ± 0.4**,++
LW/BW ratio	0.03 ± 0.001	0.05 ± 0.001**	0.04 ± 0.001*,+	0.04 ± 0.001*,+
Serum TC (mg/dl)	76 ± 3	247 ± 13**	170 ± 9**,++	144 ± 14**,++
Serum TG (mg/dl)	50 ± 4	96 ± 3**	70 ± 3**,++	56 ± 5**,++
Serum LDL-C (mg/dl)	43 ± 4	192 ± 7**	136 ± 13**,++	114 ± 13**,++
Serum HDL-C (mg/dl)	52 ± 3	22 ± 2**	33 ± 3**	40 ± 4++
AC	1.12 ± 0.25	7.17 ± 0.65**	4.62 ± 0.12**,+	3.32 ± 0.83*,++
AIP	0.07 ± 0.01	0.29 ± 0.05**	0.08 ± 0.02++	0.09 ± 0.03++
Serum AST (U/L)	112 ± 6	265 ± 30**	189 ± 8*,+	170 ± 6++
Serum ALT (U/L)	46 ± 2	141 ± 6**	107 ± 11**,+	72 ± 10**,++,#
Serum ET-1 (pg/mL)	6.24 ± 0.08	7.48 ± 0.26**	6.88 ± 0.17	6.61 ± 0.14+
Aortic nitrate/nitrite (μM/mg protein)	5.2 ± 0.5	2.9 ± 0.2**	4.2 ± 0.3+	4.7 ± 0.3++

Results are shown as mean ± SEM for 5–6 rats. *$p < 0.05$, **$p < 0.01$ vs. C group, +$p < 0.05$, ++$p < 0.01$ vs. HC group, #$p < 0.05$ vs. HC + WAL group. BW, body weight; LW, liver weight; TC, total cholesterol; TG, triglyceride; LDL-C, low density lipoprotein-cholesterol; HDL-C, high density lipoprotein-cholesterol; AC, athrogenic coefficient; AIP, atherogenic index of plasma; AST, aspartate transaminase; ALT, aspartate aminotransferase; ET-1, endothelin-1.

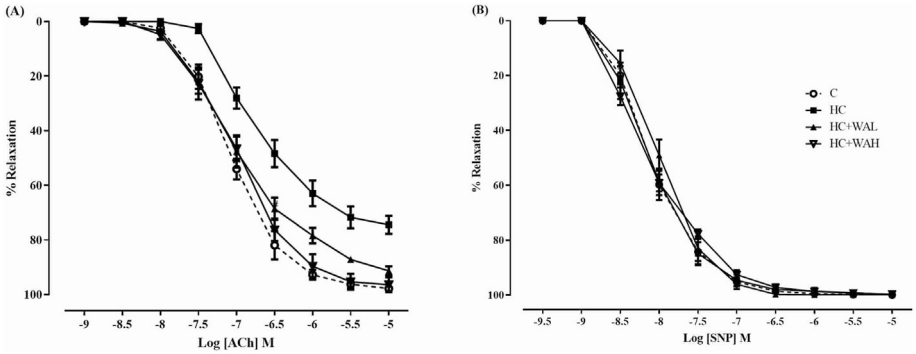

Fig. 1. Cumulative concentration-response curves to ACh (A) and SNP (B) in aortic rings of control (C), hypercholesterolemia (HC), HC + low-dose wax apple (50 mg/kg; HC + WAL) and HC + high-dose wax apple (100 mg/kg; HC + WAH) groups. The aortic rings were precontracted with PE. The pEC_{50} and R_{max} values presented in these graphs are shown in Table 3.

Table 3
A comparision of the sensitivity (pEC_{50}) and maximum relaxation (R_{max}) response to ACh and SNP in aortic rings from control rats, and hypercholesterolemia rats with or without wax apple treatment for 4 weeks.

	ACh		SNP	
	pEC_{50}	R_{max}	pEC_{50}	R_{max}
C	7.07 ± 0.07	98 ± 1	8.12 ± 0.07	100 ± 0
HC	6.77 ± 0.08*	74 ± 3**	8.23 ± 0.03	100 ± 0
HC + WAL	7.04 ± 0.10	91 ± 2++	8.12 ± 0.08	100 ± 0
HC + WAH	7.01 ± 0.08	96 ± 3++	8.20 ± 0.12	100 ± 0.1

Results are shown as the mean ± SEM (n = 5–6 per group). *$p < 0.05$, **$p < 0.01$ vs. C group, and ++$p < 0.01$ vs. HC group.

3.6. Histopathological finding of the aortic tissues

To further investigate the effects of wax apple against HCD-induced vascular injury, the histopathological changes of the aorta were observed by H&E and VVG staining. As shown in Fig. 2, the histological finding of the H&E-stained aortic section showed the normal structure of the aorta with intact endothelial cells in the control group, while the consumption of HCD caused alterations to the structure of the vascular wall, characterised as endothelial distortion, thickened intima, and disorganisation of the smooth muscle cells in the tunica media. These histopathological alterations were improved by both dosages of the wax apple, especially the high dosage. In addition, the VVG-stained aortic sections from all the rats, showed a normal structure of the elastic fibres with a thick continuous internal elastic lamina. The morphometric analysis of H&E-stained aortae revealed that both dosages of wax apple reduced the HCD-induced increased the aortic wall to lumen ratio and the medial thickness of the aorta (Fig. 2B and C).

3.7. Effect of wax apple on aortic oxidative stress markers

To evaluate the effects of wax apple treatment on vascular oxidative stress, the aortic expression of NADPH oxidase subunit $p47^{phox}$, the endogenous sources of ROS, as well as 4-HNE, a marker of lipid peroxidation, were determined. Western blot analysis revealed that the untreated hypercholesterolemic rats exhibited upregulated expression of $p47^{phox}$ and 4-HNE in aortic tissues, compared to the control group ($P < 0.05$). Notably, the administration of the two doses of wax apple significantly reduced the aortic expression of $p47^{phox}$ and 4-HNE compared to the HC group (Fig. 3A–C).

3.8. Effect of wax apple on hepatic steatosis

The gross appearance of rat liver tissue is shown in Fig. 4A. The livers of the control group were deep red, moist and glossy, while those of the HCD-fed group were pale yellow in colour. However,

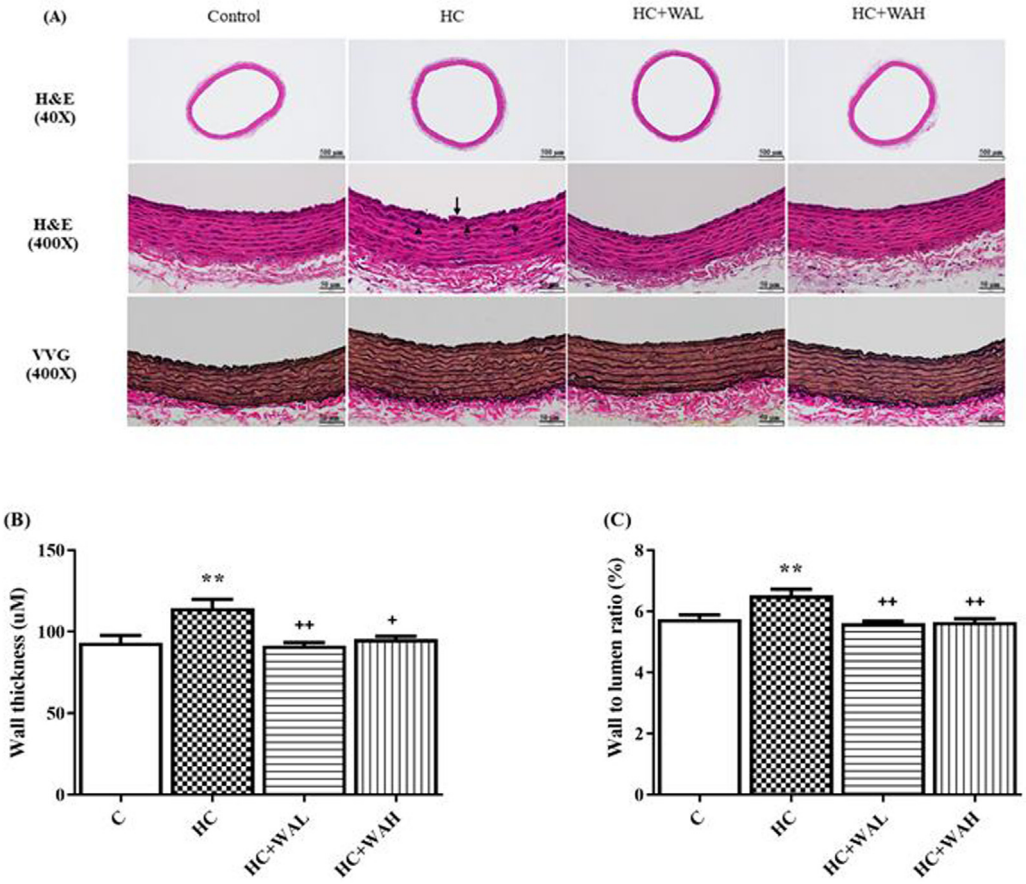

Fig. 2. Effect of wax apple on HCD-induced histopathological changes of rat aorta. (A) Representative photographs of aortic sections stained with H&E (objective magnification 4x and 40x, scale bar = 500 μm and 50 μm, top) and VVG (objective magnification 40x, scale bar = 50 μm, bottom). The black arrows indicate cellular oedema and partial exfoliation of endothelial cells, whereas the arrowheads demonstrate disorientation of smooth muscle cells. (B and C) Quantitative analysis of thickness of aortic wall, and ratio of wall thickness per lumen diameter of the thoracic aorta from each experimental group. Values are the mean ± SEM (n = 5 per group). **P < 0.01 compared with the control group; $^{+}$P < 0.05, $^{++}$P < 0.01 compared with the HC group.

the livers of the groups treated with both dosages of wax apple had changed from pale yellow to reddish brown. Concordant with the changes in morphology, H&E straining of liver tissues of the control rats showed a normal appearance and location of hepatocytes without intrahepatic lipid droplets, while the hepatocytes of the HCD-fed rats were enlarged and filled with large cytoplasmic lipid droplets, throughout the sections (Fig. 4B). Oil Red O staining also showed that the HCD increased the Oil Red O-positive area of the liver section, which was attenuated by the both dosages of wax apple treatment (Fig. 4B and C). In addition, the concentrations of TC and TG in the liver of the HCD-fed rats were significantly increased compared with the control group (Fig. 4D and E). The administration of both dosages of wax apple significantly reduced the liver contents of TC and TG by 44.2% and 50.9% in HC + WAL, respectively, and by 40.1% and 41.7% in HC + WAH, respectively, as compared to the untreated hypercholesterolemic rats (Fig. 4D and E). However, these levels were higher than those in the control group. These data indicate that wax apple exerts inhibitory effects on hepatic steatosis.

4. Discussion

This study demonstrated that wax apple treatment reduces serum lipid levels, and also improves endothelial dysfunction, assessed by increasing relaxant responses to ACh, and total nitrate/nitrite contents in the aortae, and reducing serum ET-1 contents of HCD-fed rats. The treatment of hypercholesterolemic rats with wax apple for 4 weeks also reduces vascular oxidative stress, as shown by the downregulation of $p47^{phox}$ and 4-HNE expressions. In addition, wax apple treatment reverses the HCD-induced hepatic steatosis and injuries.

Hypercholesterolemia is one of the major risk factors of cardiovascular disease,[1,13] accompanied by chronic liver disease.[17] Overconsumption of HCD causes a lipid homeostasis disturbance, which results in high lipid content, especially in the TC and TG, in both the blood and liver.[2,17] It is widely recognised that high blood cholesterol and LDL-C levels are the prime causes of vascular damage, and are used to estimate CVD and atherosclerosis process.[31] It is evident that LDL-C particles can not only enter and accumulate within the arterial wall easily, but also undergo modifications such as oxidation that caused the oxidized LDL to directly damage the vascular tissues, which subsequently progresses into atherosclerosis.[31] Therefore, lowering blood lipids is a primary step in the prevention of vascular disease. In agreement with other studies,[9,26,30] this study showed that rats fed with the standard diets supplemented with cholesterol and cholic acid induced significant hypercholesterolemia, as indicated by a significant increase in the serum levels of TC, TG, and LDL-C, and a decrease in serum levels of HDL-C. In addition, atherogenic coefficient/indices, a powerful marker of cardiovascular risk, were increased in the untreated hypercholesterolemia rats. However, after 4 weeks of wax apple consumption, the serum levels of TC, TG, and LDL-C, and the

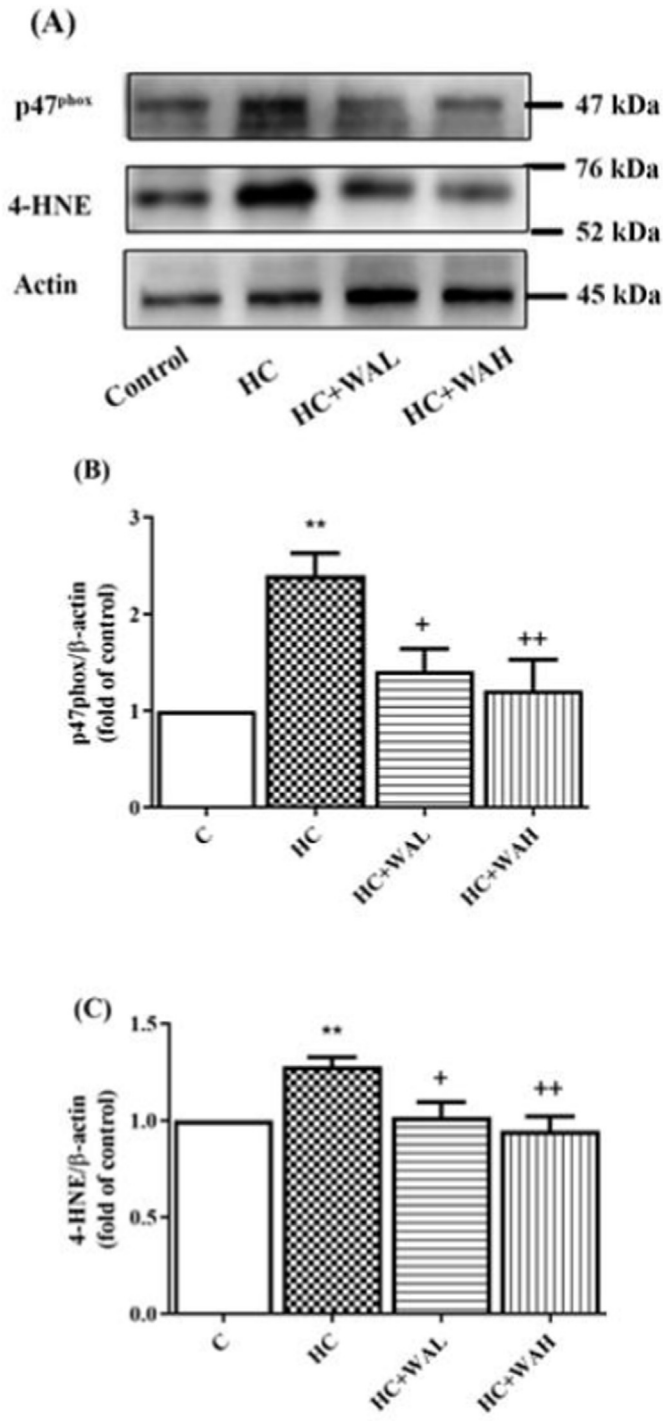

Fig. 3. Western blot analysis of protein expression in aortae. (A) Representative band of protein expression. (B and C) Western blot of $p47^{phox}$ and 4-HNE of control (C), hypercholesterolemia (HC), HC + low-dose wax apple (50 mg/kg; HC + WAL) and HC + high-dose wax apple (100 mg/kg; HC + WAH) groups. Results are shown as mean ± SEM (n = 5 per group). **P < 0.01 compared with the control group; $^{+}$P < 0.05, $^{++}$P < 0.01 compared with the HC group.

atherogenic indices were found to be effectively decreased in HCD-fed rats, while the HDL-C level was increased by only the high dosage. These results indicate that wax apple exhibited beneficial effects against dyslipidemia in HCD-fed rats, and may have the potential to prevent atherosclerosis and CVD.

Vascular endothelial dysfunction is considered to be an early detectable alteration of vascular diseases, which occurs before the appearance of typical evidence of an atherosclerosis.[31] The endothelium plays a crucial role in the regulation of vascular tone, by modulating the balance of endothelium-derived vasoactive substances such as vasodilator NO and vasoconstrictor ET-1.[6,11] The reduced NO bioavailability leads to the predominance of vasoconstrictors like ET-1, contributing to the decrease in endothelium-dependent relaxation, which is a crucial characteristic of endothelial dysfunction.[11,32,33] In addition to its effects on vascular tone, endothelium-derived NO exerts anti-atherogenic effect by inhibiting vascular smooth muscle cells proliferation and migration.[34] Numerous studies have shown that HCD consumption decreases endothelium-dependent relaxation and NO bioavailability,[9,10,35] and produces morphological abnormalities in vascular tissue.[10,35] This is in accordance with our findings, which showed that the vasorelaxation response to ACh but not SNP was decreased in the aortic tissues from HCD-fed rats, indicating an impairment of aortic endothelium-dependent relaxation in hypercholesterolemic groups. In addition both the reduced aortic nitrite/nitrate contents and the increased serum ET-1 levels confirmed vascular endothelial dysfunction in the untreated hypercholesterolemic rats. In this study, wax apple consumption for four weeks in HCD-fed rats produced a significant increase in ACh-induced vasorelaxation and aortic nitrite/nitrate contents, and decreased the serum ET-1 levels. Therefore, an improvement in the balance between NO and ET-1 contributed to the restoration of endothelium-dependent relaxation in wax apple-treated hypercholesterolemic rats.

Similar results were observed in histopathologic examination with endothelial damage and aortic wall thickening in HCD-fed rats. These alterations affect the structural integrity of the aortic tissues, which subsequently disrupts the balance between vasodilators and vasoconstrictors production.[6,8,36] Both dosages of wax apple consumption, however, ameliorated HCD-induced aortic structural changes. These findings suggest that daily intake of wax apple protects the vascular endothelium from the harmful effects of hypercholesterolemia, which leads to the improvement of its vascular endothelial function.

Increased oxidative stress is one of the underlying mechanisms in hypercholesterolemia-induced endothelial dysfunction and damage.[9,37] An increase in the generation of ROS causes oxidative stress injury in the form of lipid peroxidation, protein oxidation and DNA damage, which results in vascular disruption and damage. In addition, ROS, especially superoxide anions, can impair endothelium-dependent relaxation through the interaction of ROS with NO. This reaction produces a cytotoxic oxidant peroxynitrite and depletes the availability of NO for vasorelaxation.[9,16,37] It is well-known that the main source of vascular ROS production is NADPH oxidase, especially during hyperlipidemia-induced oxidative stress involving $p47^{phox}$, a regulatory subunits of NADPH oxidase.[9,37] In this study, we found increased expression of $p47^{phox}$, an NADPH oxidase subunit, and 4-HNE, a marker of lipid peroxidation, in the aortic tissues of the untreated hypercholesterolemic rats, which indicated the presence of oxidative stress in vascular tissues. These results were consistent with previous reports.[9,38] The elevation in the NADPH oxidase subunit $p47^{phox}$ increases ROS production, in particular superoxide anions, which leads to oxidative damage, as indicated by upregulated 4-HNE expression, and the inactivation of NO in hypercholesterolemic rats.

Accumulated evidence as indicated that a diet rich in fruits and vegetables has beneficial effects, principally via antioxidant actions, on various chronic diseases, including atherosclerosis.[18,19] Previous studies showed that wax apple extract has an antioxidant effect evidenced by an increase in antioxidant enzymes activities such as superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px). Additionally, wax apple extract decreased oxidative stress markers

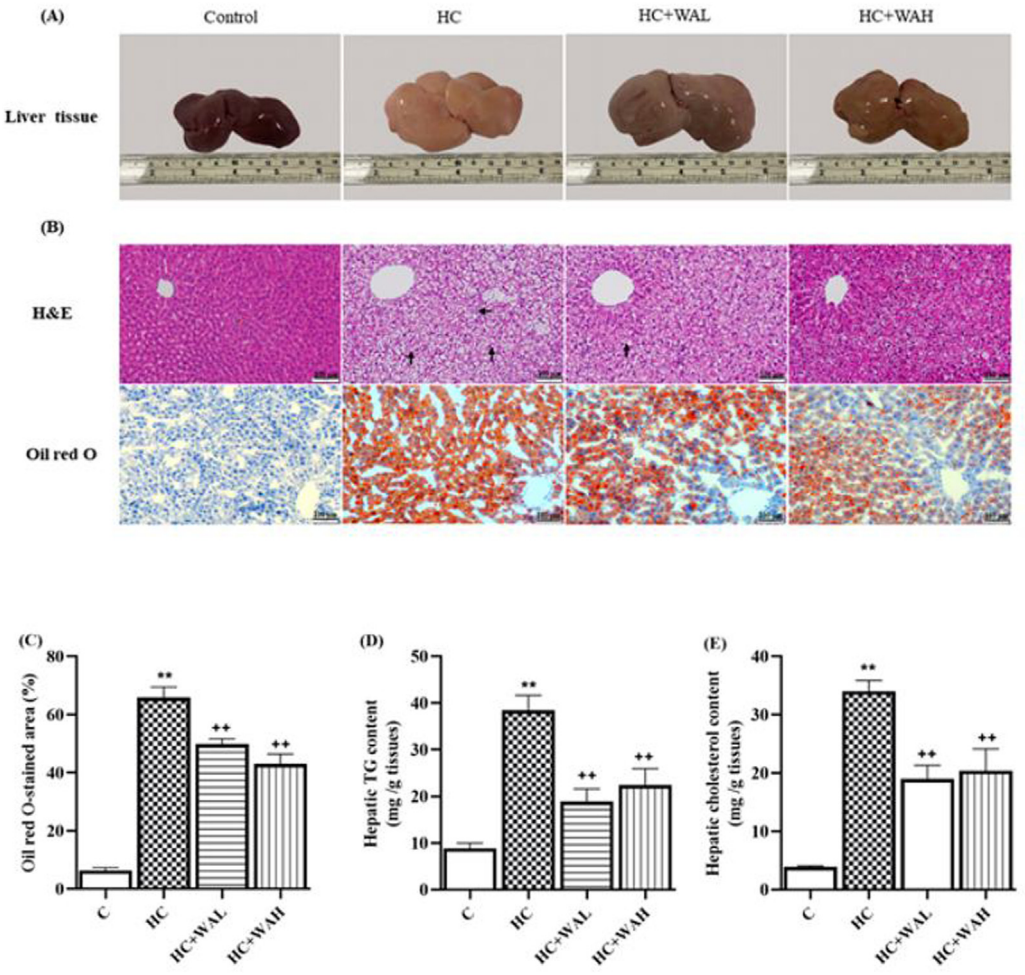

Fig. 4. Effect of wax apple on HCD-induced hepatic steatosis in rats. (A) Macroscopic pictures of the liver; (B) microscropic images of hematoxylin & eosin (H&E)- and Oil red O-stained liver tissues (objective magnification 20x, scale bar = 100 μm). The black arrows indicate fat droplets. (C) The percentage of Oil Red O-stain area; (D) hepatic triglyceride (TG) levels; (E) hepatic cholesterol (TC) levels. Values are the mean ± SEM (n = 5 per group). **P < 0.01 compared with the control group; $^{++}$P < 0.01 compared with the HC group.

in the liver of alcoholic mice,[39] and in the pancreases of diabetic rats.[21] Therefore, our study further examined the inhibitory effects of wax apple on oxidative stress markers in hypercholesterolemic rats. This study showed for the first time that wax apple treatment reduced oxidative stress markers, i.e. $p47^{phox}$ and 4-HNE, in the aortic tissues of hypercholesterolemia rats. This may contribute in part to the beneficial effect of wax apple against HCD-induced vascular dysfunction and damages.

Numerous studies have focused on the close association between hypercholesterolemia and the development of NAFLD.[2,17] The presence of hepatic steatosis and injury has previously been reported in HCD-induced hypercholesterolemic animals.[35,40,41] This is in accordance with our finding, which demonstrated that HCD consumption could induce hepatic steatosis in rats. However, daily administration of wax apple to hypercholesterolemic rats decreased hepatic lipid content, liver weight, and liver to body weight ratio. The results of the histopathological evaluation also confirmed that wax apple consumption reduced HCD-induced hepatic steatosis. To determine that the decreased liver fat accumulation in the wax apple-treated hypercholesterolemic rats was associated with decreased liver injury, important hepatic enzyme biomarkers (serum ALT and AST) were evaluated as markers of liver damage. Our results demonstrated that both AST and ALT activities were elevated in the rats fed the HCD. The observed increase in these aminotransferase activities of the liver of HCD-fed rats, is the result of changes in lipid metabolism and cellular damage caused by fat accumulation in the liver.[30] Interestingly, wax apple treatment lowered these parameters in HCD-fed rats, suggesting that it plays a protective role against lipid accumulation and liver damage induced by a HCD. However, additional experiments will be required to confirm these hepatoprotective effects.

Finally, although further studies are needed to characterise the compounds that support the vascular and hepatic protective properties of wax apple, previous phytochemical investigation of powdered wax apple indicated that it contains phenolic compounds, flavonoids, and anthocyanin.[21] The present study also confirmed that the powdered extract is rich in phenolic compounds and flavonoids. All these compounds have been previously described to have antioxidant, cardiovascular protective, lipid-lowering, anti-inflammatory, and *anti*-steatotic properties.[38,42,43] Therefore, the phenolic compounds and flavonoids present in powdered wax apple might be responsible for the vascular protective and *anti*-steatotic activities in HCD-induced hypercholesterolemia.

The limitation of this study is the absence of a positive control group that received lipid-lowering drugs, which have allowed us to carry out a comparison between the powdered wax apple fruit consumption, and the standard medicine treatment.

In conclusion, this is the first study to report that wax apple prevents endothelial dysfunction and damage, assessed by impaired endothelium-dependent vasorelaxation, in HCD-induced hypercholesterolemic rats. The protective actions of wax apple involve lowering circulating lipid levels, improving the balance between NO and ET-1, and reducing vascular oxidative stress; the 100 mg/kg dose of wax apple was more efficient than the 50 mg/kg dose. In addition, wax apple exerts beneficial effects against hepatic steatosis and injury in hypecholesterolemic rats. Therefore, this study supports the notion that it would be beneficial for hypercholesterolemic patients to consider the consumption of wax apple fruit in daily life to prevent vascular and hepatic complications.

Highlights of the findings and novelties

The short-term (4 weeks) oral gavage administration of wax apple fruit extracts restores vascular dysfunction and damage in aortic tissues of high cholesterol diet (HCD)-fed rats through the reduction of vascular oxidative stress. Additionally wax apple attenuated HCD-induced hepatic lipid accumulation and damage in rats.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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