

Protective Effects of Defatted Dabai Peel Extracts in Hypercholesterolemic Rabbits Based on Histopathological Methods

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ABSTRACT

Defatted dabai peel contains a high amount of anthocyanin. Anthocyanins are known to prevent several types of disease, including cardiovascular-related complications. This study aimed to describe the effects of different doses of defatted dabai peel extract by histopathological analyses on lesions in the liver, kidney, heart and aorta. Histopathology methods were applied to determine the protective effects of defatted dabai peel extracts against hypercholesterolemia-induced oxidative damages to animal organs. Haematoxylin and eosin staining was applied for histopathology examination for liver, kidney, heart and aorta. Data showed that a high dose of defatted dabai extract (3000 mg per day) applied to hypercholesterolemic rabbits for eight weeks had mild protective effect, especially reducing the severity of hepatic fibrosis and steatosis of the renal medulla. The high dose of extract supplementation also reduced inflammation of aorta and formation of atherosclerosis plaque in the cell wall of right ventricle of the heart. The high dose of defatted dabai peel extract could be a protective agent against oxidative stress.

Keywords: Dabai, Extract, Histopathology, Hypercholesterolemic, Organ

INTRODUCTION

Dabai (*Canarium odontophyllum*) is a high-fat fruit that shares some similar characteristics to olive and palm fruits. The trees of dabai are farmed in Borneo Island, especially Sarawak for its fruit. Dabai fruit has 24% fat,¹ and the fat is rich in carotenoids and phenolic compounds. Dabai peel has high phenolic content, especially anthocyanins. Defatted dabai peel is a waste produced from dabai oil extraction. It has high total anthocyanins (80 mg/g extract) while the defatted dabai pulp is rich in phenolic compounds such as flavonoids and saponin derivatives.²

A previous study found that defatted dabai pulp powder significantly improved the lipid profile and atherosclerosis lesion area in hypercholesterolemic rabbits after eight weeks of supplementation.³ In vitro protective effects of defatted dabai extracts have been reported by Khoo et al.⁴ However, no documentation on toxicity effect of the anthocyanin-rich defatted dabai, except for liver function tests determined based on plasma ALT, AST and GGT. Besides, Fakeye et al.⁵ reported toxicity effect in male Charles Foster rats fed with a very high dose [2000 mg/kg body weight (BW)] of anthocyanin-rich extract of dried calyx of *Hibiscus sabdariffa*, but not at a lower dose (300 mg/kg BW). The toxic effect was preceded by severe weight loss with no significant histopathological changes. Moreover, Schini-Kerth et al.⁶ also reported that anthocyanin-rich extracts of grape and berries have vascular protective effects.

On the basis of the protective effects of anthocyanin-rich extract of defatted dabai against oxidative damage, especially defatted dabai peel,⁴ the anthocyanin-rich extract of defatted dabai peel of different dosages should be further examined for protective effects against oxidative damages in an animal model using histopathological method. Histopathological examination is one of the important tools that can be applied to determine protective effects of plant extracts in liver and kidney tissues.

MATERIALS AND METHODS

Experimental design

A total of 36 male New Zealand white rabbits at age of 8–10 weeks were acclimatised for two weeks in the ambient temperature of 28°C. The initial body weights of the rabbits (1.5–1.7 kg) were recorded and caged individually.

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After two weeks, the rabbits were randomly distributed into six groups (n=6 per group) on basic diet in individual cages. Rabbits in normal diet group (NC) were fed on normal chow while rabbits in hypercholesterolemic group (PC) were given normal basal diet containing 0.5% cholesterol.⁷ The other rabbits in hypercholesterolemic groups were supplemented with statin (HS), low (HL), moderate (HM), and high (HH) doses of defatted dabai peel extract.

All rabbits received 20 g of the respective diets and supplemented *ad libitum*. HS group received 10 mg of simvastatin per kg BW daily. HL, HM and HH groups were supplemented with 1000, 2000 and 3000 mg of defatted dabai peel extract per day (80 mg of total anthocyanin equivalent in 1000 mg extract) throughout eight weeks of the experimental period. Preparation of high cholesterol diet was adapted based on the procedure described by Shimizu et al.⁸ The experimental protocol was approved by the Animal Care and Use Committee of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (Approval no.: UPM/FPSK/PADS/BR-UUH/00385).

At the end of week 8, all survived rabbits (3-5 rabbits per group) were sacrificed. Livers, kidneys, hearts and aortas of all the rabbits were collected. Whole organs were dissected except for the aorta that was dissected from the region between its origin and bifurcation into the iliac arteries. Rabbit organs including aortas were washed with normal saline, and fat residue on the outer surface was removed. All organs and aortas of three rabbits in each group (n=3) were fixed in 10% formalin and stored at 4°C before histopathology study. The organs of other rabbits were kept at -40°C for other analyses.

Histopathology

Formalin-fixed organs and aortas of the experimental rabbits (n=3 for each group) were rinsed with 70% ethanol to remove the formalin. The organs were trimmed into pieces of ~4 mm thick, inserted into cassettes and processed in a tissue processor. Aortas were sectioned from the ascending part of arch and liver was sectioned from right lobe. After 24 h of tissue processing, the pieces of tissues were embedded in paraffin. Paraffin blocks of tissues were then cut at 4 µm using a microtome and stained with haematoxylin and eosin (H&E).

All the slides were observed under magnification of 10, and the cross-sectional areas were digitised. Severity of hepatic fibrosis and kidney steatosis were scored using a 4-scale scoring system adapted from Liu et al.⁹ The aorta and heart were observed for the occurrence of atherosclerotic plaque at the inner cell walls of aortas and right ventricle of the rabbit hearts. A higher score indicates a more severe condition.

Statistical analysis

Experimental data were expressed as mean ± standard deviation. Comparison of the severity scores for the experimental groups were assessed using analysis of variance (ANOVA) coupled with Tukey's multiple comparisons. The results were analysed using Minitab statistical software (version 19) with a significant value of $p < 0.05$.

RESULTS AND DISCUSSION

Severities of hepatic fibrosis and renal steatosis

Micrographs obtained from H&E staining of liver and kidney tissues are shown in Figures 1 and 2, respectively. Microscopic examination of the liver showed that rabbits in PC group had markedly expanded hepatocyte cytoplasm. Such observation has been reported by Brunt¹⁰ in fatty liver diseases, and has been named as microvesicular steatosis. The parenchyma was diffusely involved, and slight necrosis was indistinctly seen. Inflammation that coupled with fibre extension and accumulation of collagen signified fibrosis of liver tissues in the hypercholesterolemic rabbits. This observation is in line with a study by Arhan et al.¹¹ that typical lesions of steatohepatitis were observed in rabbits supplemented with 0.5% cholesterol.

Figure 1 shows the normal morphology of hepatocytes histological specimen of the rabbit (Figure 1A), hepatic fibrosis (Figure 1B), lesser in the degree of hepatic fibrosis in the specimens of the defatted dabai peel extract (three doses) (Figures 1C–E), and statin (Figure 1F) supplemented to hypercholesterolemic rabbits. The clear hepatic fibrosis was observed, but there was no heavy fat infiltration observed as the biomarker of fatty liver in all hypercholesterolemic groups especially PC group. Minor improvement in hepatic fibrosis was clearly seen in HL and HS, and a remarkable improvement in the hepatic fibrosis was observed in HM and HH. In this study, the used of statin showed no great improvement in liver fibrosis. Besides, Choe et al.¹² reported the possible limitation in the use of statin and it is hepatotoxic.

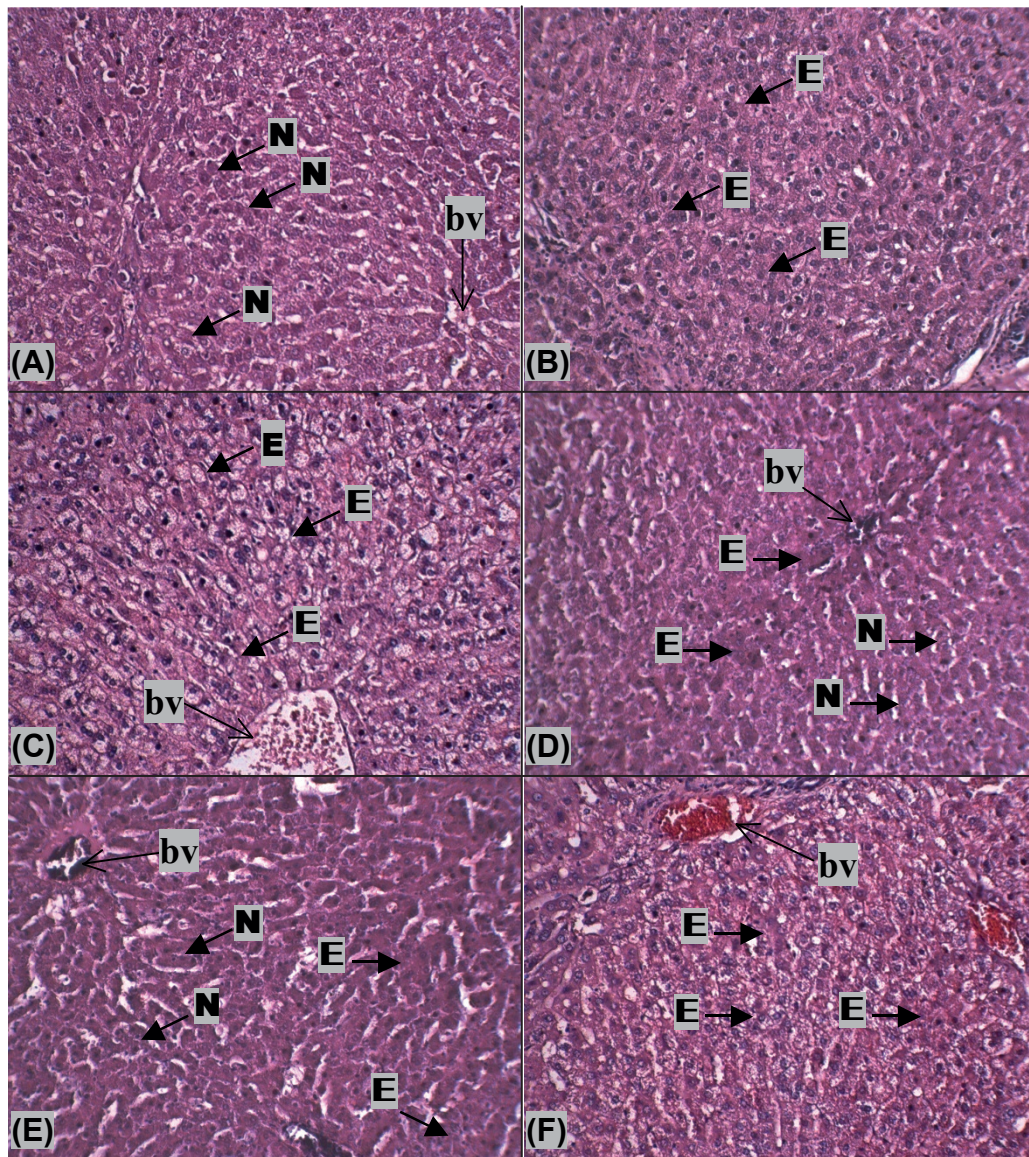


Figure 1. Pathology of liver tissue at week 8 of defatted dabai peel extract supplementation in hypercholesterolemic rabbits. Sections of liver tissue were stained with H&E (10× magnification). Five fields were randomly selected from the liver section of each slide for scoring. Descriptions of (A–F) refer to the text. As shown in Table 2, the scoring system for liver fibrosis is as follows: picture shown as in (A) scored as 0-point (no fibrosis), (D) scored as 1-point (mild fibrosis), (C) scored as 2-point (moderate fibrosis), and (B) scored as 3-point (severe fibrosis). N: typical morphology of hepatocyte with no hepatic fibrosis; E: fibrosis characterises by markedly fibre extension and accumulation of collagen; bv: blood vessel.

Micrograph of renal medulla in normal diet group is seen with no morphological changes (Figure 2A), and Figure 2B shows severe steatosis in positive control group with high accumulation of fat cells inside cell wall of collecting ducts in renal medulla. The sections of the renal medulla from hypercholesterolemic rabbits treated with three doses of defatted dabai peel extract (Figures 2C–E) and statin (Figure 2F) showed mild steatosis couple with interstitial nephritis of the collecting ducts with diffuse interstitial. It also lacked nuclei and granular eosinophilic cytoplasm. H&E staining of the renal samples from the hypercholesterolemic groups showed a lighter stained effect of tissue that contain lesser or no nuclei in the collecting ducts of the renal medulla. It is often called ‘ghost cells’ and characterised by fat necrosis.¹³

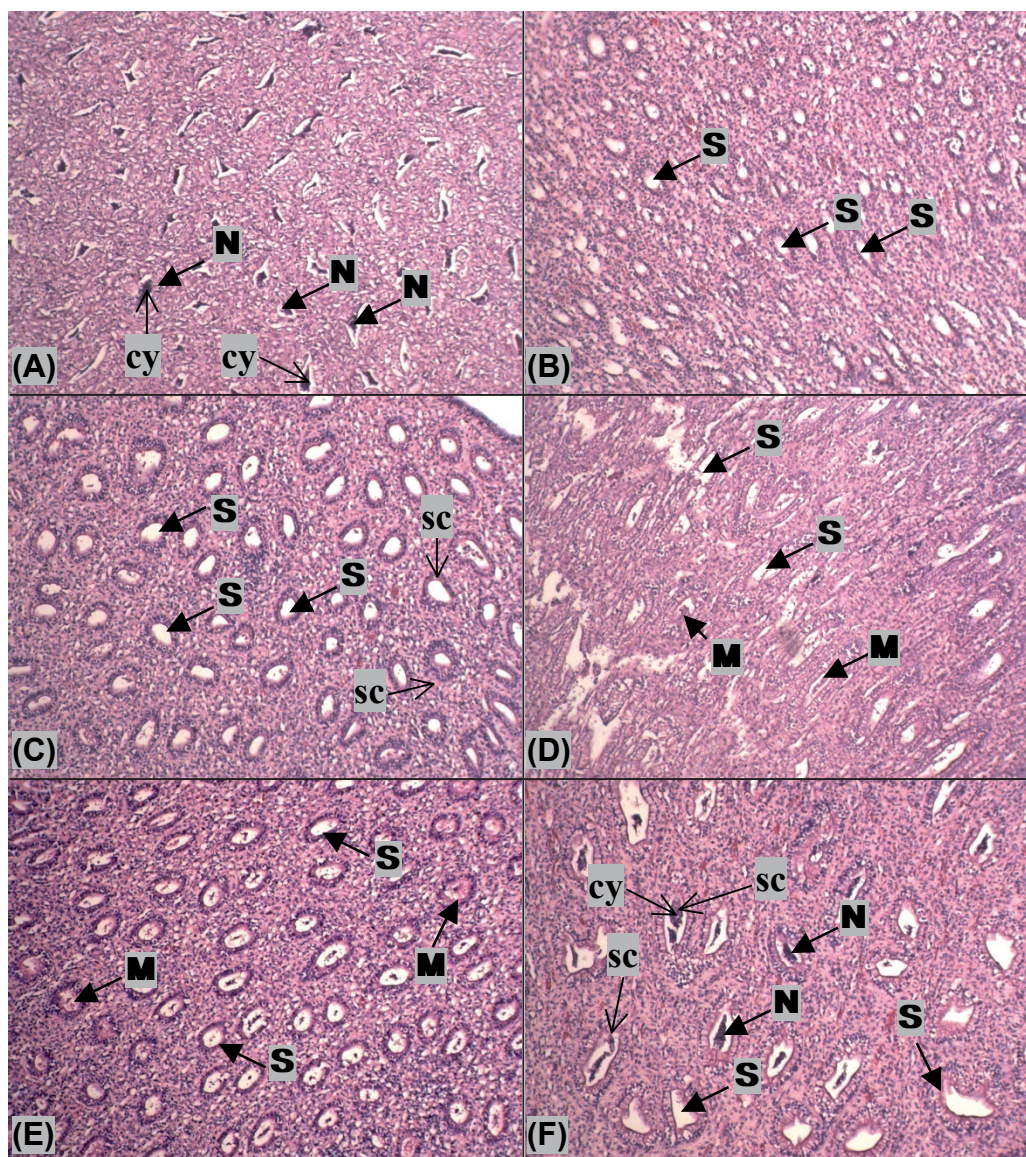


Figure 2. Kidney pathology of renal tissue at week 8 of defatted dabai peel extracts supplementation in hypercholesterolemic rabbits. Sections of renal tissue were stained with H&E (10× magnification). Five fields were randomly selected from the renal medulla section of each slide for scoring. Descriptions of (A–F) refer to the text. As shown in Table 3, the scoring system for steatosis of the renal medulla is as follows: picture shown as in (A) scored as 0-point (no steatosis), (F) scored as 1-point (mild steatosis), (D) scored as 2-point (moderate steatosis), (B) scored as 3-point (severe steatosis). N: typical morphology of collecting ducts with no steatosis; M: mild steatosis; S: severe steatosis; sc: stromal cells; cy: cytoplasm.

Severity scores for the severity of hepatic fibrosis and renal steatosis are tabulated in Table 1 and Table 2, respectively. As shown in Table 1, rabbits from NC group had zero scores for hepatic fibrosis, whereas the PC and HL groups had the highest score of 2.7 (Table 1). As discussed earlier, a more severe lesion of steatohepatitis was observed for PC and HL group (Figure 1). Besides, severity score of rabbits from HH group (0.7) was significantly lower than the score for PC group (2.7) ($p < 0.05$), but not significantly lower than the scores for HS (1.3) and HM (2.0) groups ($p \geq 0.05$).

Table 1. Severity scores of liver fibrosis for the experimental rabbits

Group	Severity score of hepatic fibrosis (incidence) ^a					Mean score ^b
	n	0	1	2	3	
NC [#]	3	3	0	0	0	0.0±0.0 ^a
PC*	3	0	0	1	2	2.7±0.6 ^b
HS*	3	0	2	1	0	1.3±0.6 ^{a,b}
HH [#]	3	1	2	0	0	0.7±0.6 ^{a,c}
HM*	3	0	1	1	1	2.0±1.0 ^{b,c}
HL*	3	0	0	1	2	2.7±0.6 ^b

^a Data represent the number of rabbits rated with a given level of hepatic fibrosis.

^b The scores for individual severity in rabbits were expressed as mean ± standard deviation.

^c NC: normal diet group, PC: hypercholesterolemic group, HS: statin group, HH: high dose group, HM: moderate dose group, HL: low dose group.

^d Different superscript lower case letters denote a significant difference between two groups. *p<0.05 as compared to NC group; [#]p<0.05 as compared to PC group, based on one-way ANOVA result.

As shown in Table 2, the severity score of steatosis in renal medulla for the rabbits from HL group (2.7) was higher than PC group (2.3). However, no significant difference was found ($p \geq 0.05$). Although HS group had a score of 1.3, it was not significantly different from NC group (0). It shows that statin helped to reduce steatosis in the collecting ducts of renal medulla. Both HH and HM groups had a high score of 2.0, and the score was significantly higher than NC group ($p < 0.05$). Both moderate and high concentrations of defatted dabai peel extract showed lesser protective effect with a mild reduction in severity than the statin group.

Table 2. Severity scores of steatosis in renal medulla for the experimental rabbits

Group	Severity score of steatosis (incidence) ^a					Mean score ^b
	n	0	1	2	3	
NC [#]	3	3	0	0	0	0.0±0.0 ^a
PC*	3	0	0	2	1	2.3±0.6 ^b
HS*, [#]	3	0	2	1	0	1.3±0.6 ^{a,b}
HH*	3	0	1	1	1	2.0±1.0 ^b
HM*	3	0	1	1	1	2.0±1.0 ^b
HL*	3	0	0	1	2	2.7±0.6 ^b

^a Data represent the number of rabbits rated with a given level of steatosis in renal medulla.

^b The scores for individual severity in rabbits were expressed as mean ± standard deviation.

^c NC: normal diet group, PC: hypercholesterolemic group, HS: statin group, HH: high dose group, HM: moderate dose group, HL: low dose group.

^d Different superscript lower case letters denote a significant difference between two groups. *p<0.05 as compared to NC group; [#]p<0.05 as compared to PC group, based on one-way ANOVA result.

In general, the high cholesterol-induced rabbits have morphological changes in their liver and kidney. Steatosis is the main changes that can be seen in the high cholesterol-induced animals. However, in this study, steatosis is not clearly seen in the hepatic samples obtained from the hypercholesterolemic rabbits. It might due to 0.5% dietary cholesterol alone (without high-fat diet) is not enough to trigger liver steatosis in adult rabbits. Hence, the high cholesterol induced duration is just eight weeks, which was not long enough to induce fat accumulation in hepatocytes that lead to either acute liver fibrosis or mild steatosis. In comparison to a previous study,¹¹ 0.5% cholesterol given to rabbits for four months clearly resulted in liver steatosis.

In the sections of renal medulla, steatosis was observed with focal rupture. Observation from the H&E samples showed that the steatosis of renal medulla was due to the hypercholesterolemic effect. High cholesterol diet had also caused a lack of nuclei and granular eosinophilic cytoplasm in the collecting ducts of renal medulla.¹⁴ As observed, steatosis in the collecting ducts was more severe than the hepatocytes. The reason is unknown because no study has been performed to identify the possible effect and mechanism of action in 0.5% cholesterol supplementation alone to normal diet of rabbits.

Incidences of atherosclerosis plaque in aorta and heart

Depositions of atherosclerosis plaque in aorta and right ventricle of the hypercholesterolemic rabbits were determined based on H&E staining. Micrograph of right ventricle wall (Figure 3A) and aorta (Figure 3B) in NC group was seen with no depositions of atherosclerosis plaque while Figures 3C–F show depositions of atherosclerosis plaque and inflammation in the hypercholesterolemic group. Figures 3C and 3E represent mild severity for the left ventricle and aorta, respectively while Figures 3D and 3F show severe plaque formation for the left ventricle and aorta, respectively. The plaque formation was scored as “yes” or “no”, and severity of the formation is shown in Table 3. In NC group, no deposition of atherosclerosis plaque observed in aorta, except one rabbit with less than 2 mm of plaque deposition. Thick layers of the plaques were measured 5–7 mm as seen in the micrograph under magnification of 10 from one of the hypercholesterolemic rabbits in PC group.

Table 3. Severity scores of deposition of atherosclerosis plaque on right ventricle wall and inflammation of aorta for the experimental rabbits

Group	Deposition of atherosclerosis plaque ^a				Severity	
	n	Right ventricle wall		Aorta wall		
		Yes	No	Yes		No
NC	3	1	2	1	2	Mild
PC	3	3	0	3	0	Severe
HS	3	1	2	1	2	Mild
HH	3	2	1	2	1	Mild
HM	3	2	1	3	0	Severe
HL	3	3	0	3	0	Severe

^aData represent the number of rabbits rated with a given level of atherosclerosis plaque deposition on right ventricle wall and aorta.

^bNC: normal diet group, PC: hypercholesterolemic group, HS: statin group, HH: high dose group, HM: moderate dose group, HL: low dose group.

Supplementation of 1000 mg of defatted dabai peel extract per day to the hypercholesterolemic rabbits resulted in improvement of the plaque deposition (reduced to 5 mm or less) in two out of three rabbits. The 3000 mg per day of extract supplementation had also further reduced the thickness of atherosclerosis plaque with one of the rabbits without deposition of atherosclerosis plaque. Similarly, defatted dabai peel extract had a mild reduction in the incidence of atherosclerosis plaque formation in the right ventricle of the hypercholesterolemic rabbits. The thickness of atherosclerosis plaques was not measured due to only partial deposition of the plaque in certain areas of the heart ventricle. The increment in the dose of defatted dabai peel extract had further decreased the thickness of the atherosclerosis plaque. Supplementation of a high dose of the defatted dabai peel extract (3000 mg per day) and 1.2 mg simvastatin per kg BW per day had inhibited the formation of atherosclerosis plaque in the inner cell wall of aortas in one and two of the hypercholesterolemic rabbits, respectively.

Oxidative stress mainly causes atherosclerosis plaque formation. In the high oxidative condition, reactive oxygen and nitrogen species cause inflammation to the inner cell wall of aorta. Inflammatory plaque of the aorta observed in this study is shown in Figure 3F, where the inflammatory plaque can be seen as purplish-blue colour. The severity of the plaque formation was categorised into mild and severe, where the PC, HM and HL groups fall into the severe category. Deposition of a fatty streak was also observed in the right ventricle of the rabbit's heart (Figure 3D). The supplemented anthocyanin-rich extract played an important role in scavenging free radicals, thus reduced oxidative stress.

Anthocyanin compounds have no direct role in the reduction of atherosclerosis plaque formation, except acted as antioxidant to inhibit inflammation and further decrease the formation of inflammatory plaque in aortas of the experimental rabbits. Xia et al.¹⁵ reported that anthocyanins extracted from black rice enhanced atherosclerosis plaque stabilisation in experimental mice through the anti-inflammatory mechanism. The anti-inflammatory activity of anthocyanins from black rice was due to the suppression of COX-2, INOS and ICAM-1 expression that might be modulated by NF- κ B.¹⁶

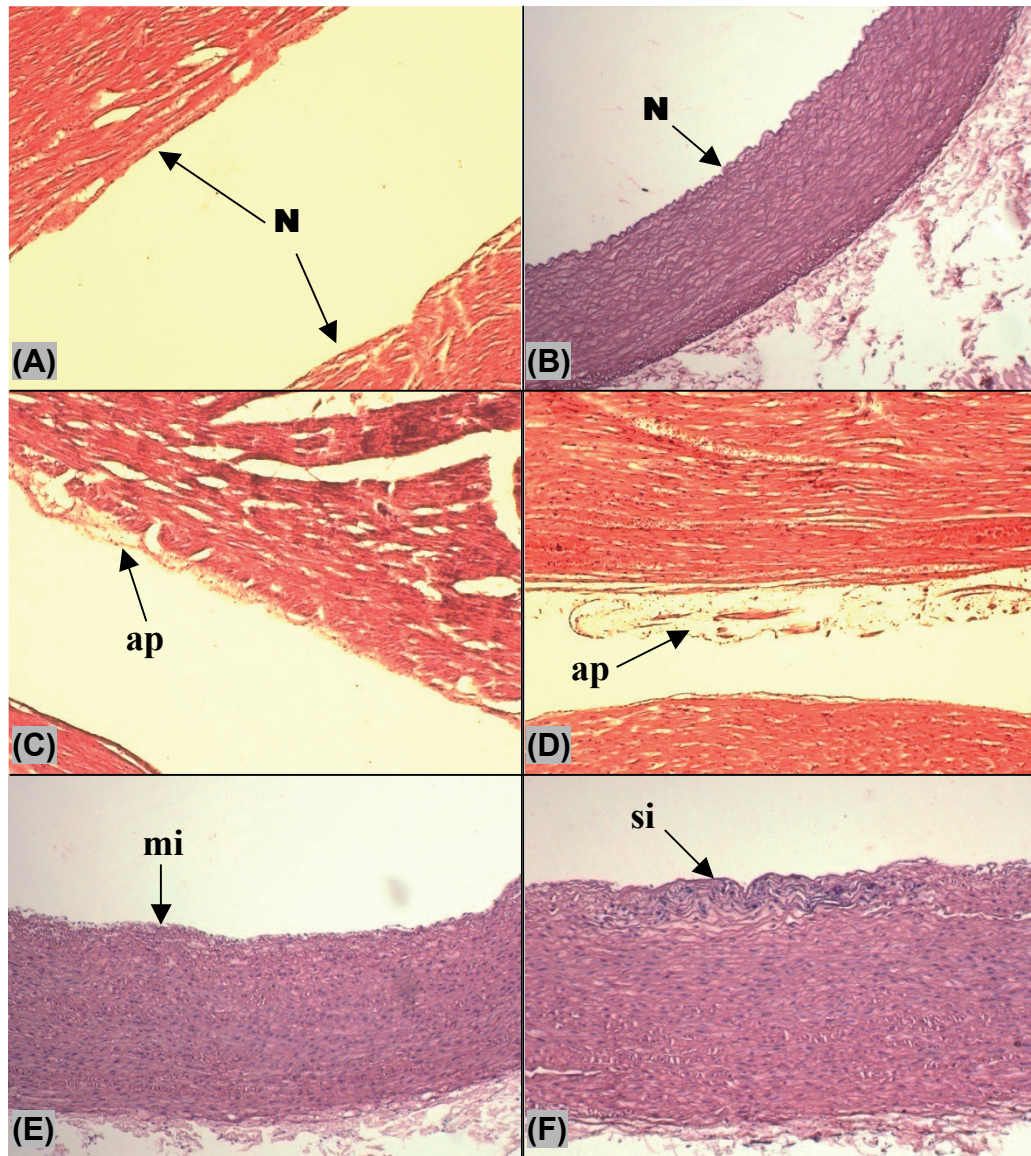


Figure 3. Pathology of heart and aorta tissues at week 8 of defatted dabai peel extract supplementation in hypercholesterolemic rabbits. Sections of cardiac and aorta tissues were stained with H&E (10 \times magnification). For scoring, the sections of heart and aorta were observed as whole, and no particular fields were selected. Descriptions of (A–F) refer to the text. As shown in Table 4, deposition of atherosclerosis plaque is scored based on 2-scale system as follows: picture shown as in (A) or (B) scored as “No” (no deposition or inflammation) and (C–F) scored as “Yes”. **N**: no atherosclerosis plaque formation on the ventricle wall of heart or no inflammation to the aorta; **ap**: atherosclerosis plaque; **mi**: mild inflammation; **si**: severe inflammation.

CONCLUSION

Protective effects of the anthocyanin-rich extract of defatted dabai peel at three different doses supplemented to hypercholesterolemic rabbits were observed. High dose (3000 mg per day) of the extract supplementation showed better protective effects than the other doses in reducing high cholesterol-induced liver fibrosis, but not for steatosis of the renal medulla. Minor improvements for the lesions of inner cell wall of the aorta and the right ventricle of the heart of the hypercholesterolemic rabbits were also observed. Although minor improvement in the lesions was observed for the treatment groups, the protective effects of defatted dabai peel extracts against diseases are not conclusive. Due to the protective effects were tested based on a single assay, the use of a few types of staining is recommended for future studies to confirm further the protective effect of anthocyanin-rich extract in the prevention of diseases. Among different doses of the extract studied, we suggest the use of a high dose of anthocyanin-rich extract (3000 mg per day) for supplementation.

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REFERENCES

1. Chew LY, Prasad KN, Amin I, et al. Nutritional composition and antioxidant properties of *Canarium odontophyllum* Miq. (dabai) fruits. *J Food Compos Anal* 2011;24(4):670-77.
2. Khoo HE, Azrina A, Ismail A, et al. Solid phase extraction of antioxidative properties in the waste of dabai oil extraction. *Molecules* 2012;17(8):9754-73.
3. Shakirin FH, Azlan A, Ismail A, et al. Anti-atherosclerotic effect of *Canarium odontophyllum* Miq. fruit parts in rabbits fed high cholesterol diet. *Evid Based Complement Alternat Med* 2012;2012:Art ID 838604.
4. Khoo HE, Azlan A, Ismail A, et al. Inhibition of oxidative stress and lipid peroxidation by anthocyanins from defatted *Canarium odontophyllum* pericarp and peel using in vitro bioassays. *PloS One* 2014;9(1):e81447.
5. Fakeye TO, Pal A, Bawankule DU, et al. Toxic effects of oral administration of extracts of dried calyx of *Hibiscus sabdariffa* Linn. (Malvaceae). *Phytother Res* 2009;23(3):412-16.
6. Schini-Kerth VB, Étienne-Selloum N, Chataigneau T, et al. Vascular protection by natural product-derived polyphenols: *in vitro* and *in vivo* evidence. *Planta Med* 2011;77(11):1161-67.
7. Nurulhuda MH, Azlan A, Ismail A, et al. Cholesterol-lowering and atherosclerosis inhibitory effect of Sibu olive in cholesterol fed-rabbit. *Asian J Biochem* 2012;7(2):80-9.
8. Shimizu T, Nakai K, Morimoto Y, et al. Simple rabbit model of vulnerable atherosclerotic plaque. *Neurol Med Chir* 2009;49(8):327-32.
9. Liu JY, Chen CC, Wang WH, et al. The protective effects of *Hibiscus sabdariffa* extract on CCl₄-induced liver fibrosis in rats. *Food Chem Toxicol* 2006;44(3):336-43.
10. Brunt EM. Pathology of fatty liver disease. *Modern Pathol* 2007;20:S40-S48.
11. Arhan M, Öztürk HS, Turhan N, et al. Hepatic oxidant/antioxidant status in cholesterol-fed rabbits: effects of garlic extract. *Hepatol Res* 2009;39(1):70-7.
12. Choe CC, Kim HS, Jeong TS, et al. Naringin has an antiatherogenic effect with the inhibition of intercellular adhesion molecule-1 in hypercholesterolemic rabbits. *J Cardiovasc Pharmacol* 2001;38(6):947-55.

13. Goyal S, Huhn KM, Provost TT. Calciphylaxis in a patient without renal failure or elevated parathyroid hormone: possible aetiological role of chemotherapy. *Bri J Dermatol* 2000;143(5):1087-90.
14. Butin-Israeli V, Uzi D, Abd-El-Latif M, et al. DNA-free recombinant SV40 capsids protect mice from acute renal failure by inducing stress response, survival pathway and apoptotic arrest. *Plos One* 2008;3(8):e2998.
15. Xia X, Ling W, Ma J, et al. An anthocyanin-rich extract from black rice enhances atherosclerotic plaque stabilization in apolipoprotein E-deficient mice. *J Nutr* 2006;136(8):2220-25.
16. Zhang YM, Tang ZH, Xia M, et al. Effect of anthocyanins on inflammatory signal transduction in ApoE knock-out mice. *Acta Nutr Sin* 2005;27(3):249-52.