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Cholesterol-lowering and Artherosclerosis Inhibitory Effect of Sibu Olive in Cholesterol Fed-rabbit

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ABSTRACT

The present study was designed to explore the possible cholesterol lowering effect of Sibu Olive (SO) supplementation on cholesterol level and atherosclerosis inhibitory effect in hypercholesterolemic rabbits. A total of five groups (n = 7); NC (normal diet), PC (normal diet+0.5% cholesterol), HS (hypercholestrolemic diet+10 mg kg day^{-1} simvastatin), HF (hypercholsterolemic diet+5% fullfat SO) and HD group (hypercholesterolemic diet+5% defatted SO) were established. Body weight and lipid profile analysis [total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C)] were compared to PC. There was rise in body weight changes (1.75-2.24 kg) of the animals. The HD group showed significant reduction in total cholesterol (96.3%) and LDL-C (26.5%) together with increment in HDL-C (46.8%) relative to PC. Furthermore, the atherosclerotic plaque formation in HD group diminished by 79.4% compared to PC. The possible cholesterol lowering effect was contributed by the presence of polyphenols such as phenolic acids, flavonoids, anthocyanidins and anthocyanin of the SO fruit. Hence, these findings are beneficial as it provide alternative means to treat hypercholesterolemia and atherosclerosis.

Key words: Sibu olive, hypercholesterolemia, rabbit, artherosclerosis, polyphenols

INTRODUCTION

The physical appearance of 'Sibu olive' and olive were resembles to each other and have smooth texture and rich with flavor (Chew *et al*., 2011a). Despite of physical similarity, both Sibu olive oil and olive oil were comparable in term of physicochemical properties in certain aspect such as total fat content and total phenolic content but differ in flavonoid content (Azrina *et al*., 2010a). Sibu Olive (SO) is botanically known as *Canarium odontophyllum* Miq, classified in subfamily of *Burseraceae*. The peel of the fruit is dark purple, while the pulp is yellow with a single seed in the centre (Azrina *et al*., 2010a). The nutritional composition of the SO was reported by previous researches which consist of essential minerals such as potassium (352 mg/100 g), phosphorus (65 mg/100 g), calcium (200 mg/100 g) and magnesium (106 mg/100 g) (Chew *et al*., 2011b). Apart from that, the strong antioxidant capacity of the SO was reported in different parts of the fruit thus confer potential health benefits when consumed (Azrina *et al*., 2010b).

Natural antioxidants have been shown to offer vast array of health effects including lowering the cholesterol level. The antioxidant is directly or indirectly affecting the enzymes which responsible in lipid metabolism and atherogenesis (Yang *et al.*, 2006). Initially, the development of atherosclerosis was due to accumulation of cells with excessive lipids within the arterial wall. Hyperlipidemia or high levels of serum triacylglycerol (TG) and cholesterol are risk factors for premature atherosclerosis (Chisolm and Stinberg, 2000). Thus, in present study; the *in vivo* experiment on hypercholesterolemic rabbits was performed to investigate the cholesterol-lowering effect of SO fruits and the potential atherosclerosis inhibitory effect.

MATERIALS AND METHODS

Preparation of fruit: Fresh SO fruits (40 kg) were obtained from Agriculture Research Centre, Semongok, Sarawak, Malaysia. The pulp and kernel were separated and freezed-dried (35XL, Virtis Co. Inc. New York, United States) at Forestry Research Institute of Malaysia (FRIM). After freeze-drying, the dried-samples were ground using a dry grinder (Braun Multiquick ZK100, Germany). The resulting powder is known as full-fat pulp. The defatted pulp was prepared by extracting the oil from the dried powder sample.The fruit powder was mixed with chloroform: methanol (2:1 v/v) at a ratio of 1:5 (w/v). The mixtures were soaked overnight at room temperature in a container covered with aluminium foil. Then, the mixtures were filtered using filter paper (Whatman No. 1, Maidstone United Kingdom). The organic solvent was completely evaporated using an evaporator (Buchi Rotorvapor R-200, Berlin, Germany) at 40°C. The residue is known as defatted pulp and was dried in oven at 40°C.

Experimental design: Male New Zealand white rabbits at age of 8-10 weeks were purchased from East Asia Company, Malaysia. A total of thirty five rabbits with initial body weights of 1.5-1.7 kg were placed in individual cages. After acclimatization for about two weeks, the animals were randomly distributed into five groups (n = 7) of normal and hypercholesterolemic groups. Normal group was named NC and received normal basal diet while the hypercholesterolemic groups (PC, HS, HF and HD) received hypercholesterolemic diet (normal basal diet containing 0.5% cholesterol). The hypercholesterolemic groups: PC received hypercholesterolemic diet; HS received hypercholesterolemic diet+10 mg kg^{-1} day simvastatin; HF received hypercholesterolemic diet+full-fat pulp of SO and HD received hypercholesterolemic diet+defatted pulp of SO. Body weight and food intake, were recorded throughout the study. All experimental protocols involving animals were approved by the Animal Care and Use Committee of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM), Serdang, Selangor (Approval No: UPM/PADS/BR-UUH/00238).

Preparation of animal diets: Commercialized rabbit chow and food ingredients (soybean, corn, palm kernel cake, tapioca starch, molasses, corn oil and mineral mix, vitamin mix) were purchased from East Asia Company, Malaysia. DL-methionine, calcium carbonate and calcium hydrogen phosphate were purchased from Merck (Darmstadt, Germany). In the present study, two types of diets were used: Commercialized rabbit chow and experimental diet. The commercialized rabbit chow was given during the two weeks of acclimatization while experimental diet was given during eight week of the treatment period. The experimental diets were prepared based on the NC diet which is a normal basal diet. The normal basal diet contain soybean (15%), corn (30%), starch (10%), molasses (2%), corn oil (2%), vitamin mixture (0.3%), mineral mixture (3.5%), DL-methionine (0.2%), calcium carbonate (0.5%) and calcium hydrogen phosphate (0.5%). Similarly, the experimental diets named as HS, HF and HD were prepared using the same method as described for the NC diet with some modifications and the addition of other food ingredients

in the particular diet. In the HS group, a hypercholesterol diet was given, with simvastatin (10 mg kg^{-1} per day) given orally by force-feeding. The simvastatin was prepared by dissolving simvastatin with distilled water. Meanwhile, for the HF diet, 5% of full-fat pulp SO was added to represent the replacement of total fat and 8% of carbohydrate required. For the HD diet, 5% of SO defatted pulp was added. Defatted pulp is a rich source of dietary fiber, thus representing 14% replacement of the daily requirement of dietary fiber in a HD diet (Hainida *et al*., 2008).

Preparation of 0.5% cholesterol diet: The cholesterol diets were prepared initially by dissolving the cholesterol in chloroform. Briefly, 0.6 g of cholesterol was suspended in 2 mL chloroform. The mixture was stirred with the aid of a magnetic stirrer at room temperature. Then, the dissolved cholesterol (representing 0.5% of the cholesterol in a daily diet) was sprayed on 20 g of the diet for each animal's diet. The pellets were dried in an oven (Memmert GmbH and Co. KG, Schwabach, Germany) at 40°C overnight to allow evaporation of chloroform (Shimizu *et al*., 2009).

Lipid profile analysis

Total Cholesterol (TC): Plasma total cholesterol was automatedly determined on a chemistry analyzer (Hitachi 902, Japan). Cleavage of the cholesterol ester by cholesterol esterase produced free cholesterol. The free cholesterol was made oxidised by cholesterol oxidase to produce cholest-4-en-3-one and H_2O_2. The H_2O_2, later reacts with peroxidase to produce a red coloured solution which was determined photometrically on the analyzer. The colour intensity was directly proportional to the concentration of cholesterol and was determined photometrically.

Low density lipoprotein cholesterol (LDL-C): Plasma LDL was determined based on the cleavage of the cholesterol ester through the action of cholesterol esterase. Free cholesterol was later oxidized by cholesterol oxidase to hydrogen peroxide. Hydrogen peroxide then reacts with 4-amino-antipyrine and HSDA under a catalytic reaction by peroxidase to form purple red pigment. The colour of the purple-blue pigment was directly proportional to the cholesterol concentration and measured photometrically.

High density lipoprotein cholesterol (HDL-C): The HDL-cholesterol was determined enzymatically by cholesterol esterase and cholesterol oxidase coupled with PEG-cholesterol. The cholesterol was initially cleaved through the action of PEG-cholesterol esterase. The free cholesterol was reacted with oxygen under the catalytic action of PEG-cholesterol to produce cholestenone. In the presence of peroxidase, the hydrogen peroxide formed a colour solution with 4-aminophenazone. The colour intensity is directly proportional to the cholesterol concentration and measured photometrically as described in TC determination.

Atheroma plaques scoring

Aorta preparation and Sudan IV staining: At the end of week 8, the rabbits were sacrificed. The aorta between its origin and its bifurcation into the iliac arteries was dissected, opened longitudinally and prepared for accurate detection and estimation of lipid deposits in the intima by the macroscopic method (Prasad, 2008). The aortas that were taken out were washed out with normal saline and used for Sudan IV staining. After that, the aortas were dissected and the fat residue on the outer surface was properly immersed in Herxheimers solution (Sudan IV in ethanol and acetate) for 2-3 min at room temperature. Sudan IV stain is lipophilic, so it will stain the lipid components on the surface of the tissue. After 15 min, the aortas were consecutively washed in

running tap water for 1 h. Water flow was ensured to be not too heavy to avoid tissue damage. This staining allowed a clear illustration of the plaques due to their deep red colour.

Macroscopy study: Image of the aortas were captured using a digital camera (EOS Canon, Japan). The total atherosclerotic areas of the intimal surface of the aortas were measured in mm^2 using graph paper. The extent of atherosclerosis was expressed as percentage of the luminal surface that covered by atherosclerotic changes (Bocan *et al.*, 1993). The lesion was estimated by using the following formula:

$$\text{Lesion area} = \frac{\text{Lesion area on intimal surface of the aorta}}{\text{Whole area of the aorta}}$$

Statistical analysis: Data were analyzed using SPSS 16.0 (SPSS Corporation, Chicago, IL) and presented as group Means±SEM. Differences in group means were determined using one way Analysis of Variance (ANOVA). Turkey pos hoc test was used for multiple group comparison. The significance value was set at $p<0.05$.

RESULTS

Body weight and food intake: The highest total body weight gain was recorded in PC group which manifested the hypercholesterolemic condition and was significantly different ($p<0.05$) with other treatment groups. The total food intakes among the groups were not significantly difference.

Lipid profile: In this study, the incorporation of 0.5% cholesterol (PC) resulted to 94.3% greater in total cholesterol level in plasma compared to normal diet. Significant reductions ($p<0.05$) of TC levels were observed in the group receiving statin (HS) and defatted pulp (HD) at week 8. The HD group showed significant reduction in total cholesterol by 96.3% compared to PC. Generally, remarkable changes in TC levels were observed after 8 weeks of the study (Fig. 1). The treatment with defatted pulp in HD group exhibited significant reduction in LDL-C (26.5%) concentration in plasma compared to control group. However, the highest reduction in LDL-c was observed in HS group with significant different relative to other groups (Fig. 2). In the group with high cholesterol diets (HS, HF and HD), the general trend is an increase in HDL-C levels from 0 to 8 weeks with the highest increment observed in the HF group (73.3%). The least increment in HDL-C levels at 8 week was observed in the HS group (26%). Despite of that, there was significant different in HDL-C increment (46.8%) in HD group relative to PC (Fig. 3).

Atheroma plaques scoring: The anti-atherosclerotic effect of CO fruit parts can be observed by staining atherosclerotic deposit in aortas of rabbits with Sudan IV for 8 weeks. No visible atherosclerotic plaques were found in the aorta of animals fed on a normal diet and rabbits

Table 1: Initial and Final body weight, total body weight gain and total food intake

Parameter	NC	PC	HS	HF	HD
Initial body weight (kg)	1.87±0.03^a	1.82±0.09^a	1.86±0.02^a	1.84±0.08^a	1.75±0.00^a
Final weight (kg)	2.09±0.06^a	2.34±0.04^b	2.22±0.09^a	2.02±0.01^a	2.24±0.01^a
Total body weight gain (g)	220±0.05^a	520±0.07^b	360±0.05^a	180±0.07^a	490±0.01^a
Total food intake (g)	5038.04±0.06^a	5009.2±0.06^a	4580.8±0.06^a	5601.40±0.06^a	5446.00±0.06^a

* Values within group followed by the same superscript letter are not significantly different at $p<0.05$

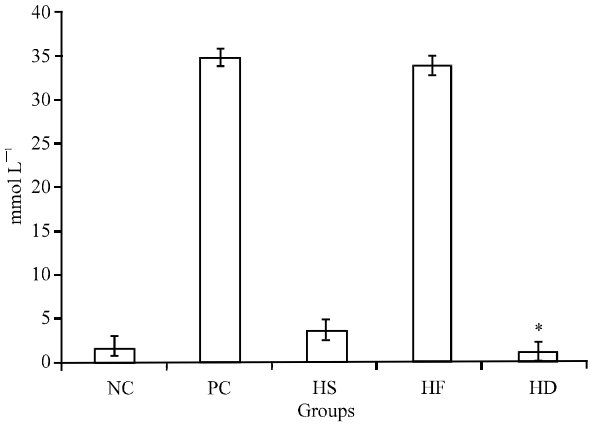

Fig. 1: Effect of SO treatment on concentration of total cholesterol in high-cholesterol-fed rabbits. Values represent Means±SEM (n = 7), *p<0.05 compared to PC group

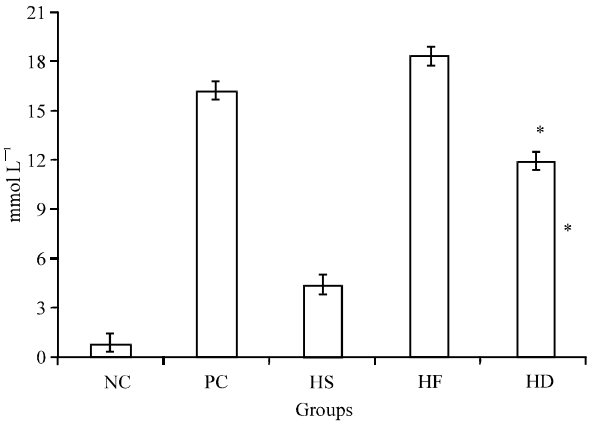

Fig. 2: Effect of SO treatment on concentration of LDL-c in high-cholesterol-fed rabbits. Values represent Means±SEM (n = 7), *p<0.05 compared to PC group

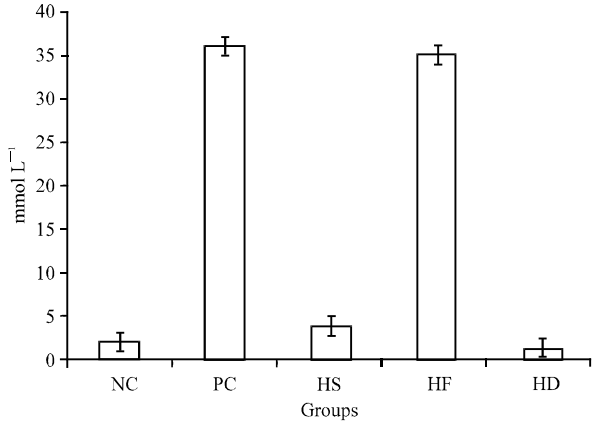

Fig. 3: Effect of SO treatment on concentration of HDL-c in high-cholesterol-fed rabbits. Values represent Means±SEM (n = 7)

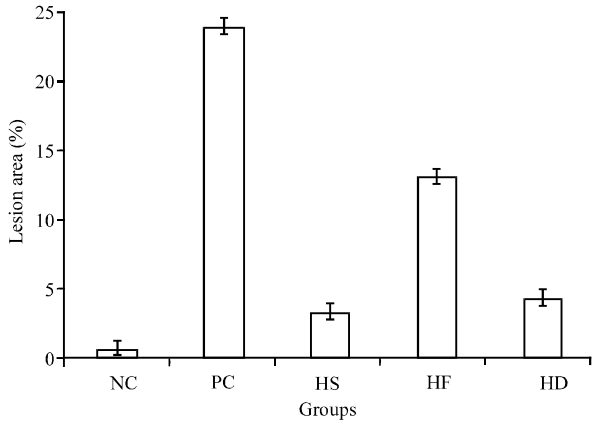

Fig. 4: Effect of SO treatment on atheroma plaque formation in high-cholesterol-fed rabbits. Values represent Means±SEM (n = 7)

supplemented with simvastatin. No significant different was observed between these two groups. The plaques were more severe in animal fed with a high cholesterol diet (PC) (22.08%) than in animals supplemented with full-fat pulp (HF) and defatted pulp (HD). The plaque areas of the aortas in HF and HD were significantly lower ($p<0.05$) by 43.39% and 79.4%, respectively compared to PC. Thus, among the treated groups, HD exhibited the greatest reduction in atherosclerotic plaque formation (Fig. 4).

DISCUSSION

The aim of this study was to explore the cholesterol-lowering potential of Sibu Olive in an animal model of hypercholesterolemia. Hypercholesterolemia refers to increased levels of total cholesterol (TC), triglyceride (TG), low density lipoprotein cholesterol (LDL-C) and decreased level of high density lipoprotein cholesterol (HDL-C) in the blood (Chen *et al.,* 2005). In this experiment, 0.5% cholesterol load to the animals could generate hypercholesterolemia. Studies both in animals and human have demonstrated that prolonged high cholesterol concentration in the circulating blood have a positive correlation on developing atherosclerosis (Pratico, 2001; Kurosawa *et al.,* 2005). The remarkable physical changes were shown on the weight gain among the normal diet and hypercholesterolemic diet. Though there was a significant difference in body weight between the high cholesterol diet (PC) and normal diet (NC) groups at week 8, no significant difference in daily food intake between these groups were observed during this period. . In addition, the increment of body weight of animals receiving a high cholesterol diet (PC) might be due to lipid deposition in the animal's body (Cheong *et al.*, 2010). The significant increase in body weight of animals fed with a high cholesterol diet in the present study was in accordance with a previous study showed by (Lee *et al.,* 2007). The increment in food intake might be due to the high satiety effect induced by the diets. The diets might support the normal growth of the rabbits as the increment of animal body weight were found not to be statistically different from the animals a fed normal diet (NC). Generally, the body weight of all animals increased concomitantly with the increased of food intake. By daily observation, all animals remained healthy and active throughout the experiments.

Based on lipid parameters, animals fed on high cholesterol diet (PC) showed increased on TC, LDL-C and HDL-C as compared to normal animals. This difference was statistically significant

($p<0.05$). The result showed that the hypercholesterol model was successfully established by means of feeding a high cholesterol diet over 8 weeks. In the present study, simvastatin was used as positive control because it is a potent hypolidemic drug with a known mechanism of action as HMG-CoA reductase inhibitor in cholesterol biosynthesis. This well accepted cholesterol-lowering drug was administered in the HS group to compare the effectiveness of the fruit parts in reducing plasma cholesterol levels (Shin *et al*., 2007). Based on the present data, the simvastatin treated group had significantly reduced TC and LDL-C concentrations in blood similarly as reported by previous studies (Rauch *et al*., 2002; Rosenson, 2004).

The present study showed that the supplementation of defatted pulp of SO exhibited a positive hypocholesterolemic effect which manifested by remarkable reduction in TC and LDL-C levels ($p<0.05$) (Fig. 1 and 2).The absorption of cholesterol is mainly related to LDL-R which combines with LDL to modulate homeostasis. Liver cells have LDL-R, whose main function is taking cholesterol into cells to proliferate cells as well as synthesize steroid hormone and bile salt. This metabolic process is called LDL-R (Chen *et al*., 2011). LDL is the uppermost carrier of transportation of cholesterol. Therefore, LDL-R plays a significant role in modulating homeostasis of cholesterol and total concentration of cholesterol in plasma. Dysfunctional LDL-R is one of the main factors that cause hypercholesterolemia (Gorinstein *et al*., 2002). Results from feeding hypercholesterolemic diet, indicate that plasma total cholesterol of the PC group showed 96% increment compared to normal diet. These changes are associated with a phenomenon that excessive load of cholesterol to the liver above the acceptable level of its normal process causes the system to be unable in metabolising the lipids resulting in high cholesterol return in the circulating blood (Gorinstein *et al*., 2002). Treatment of high cholesterol fed animals concomitantly with SO defatted pulp however showed a remarkable reduction of lipid profiles towards the level of the control group. The reduction of plasma cholesterol levels could be due to the action of polyphenol compounds in the defatted pulp, especially the skin part. The polyphenol content was found at 68±1.2 mg GAE g^{-1} in skin extracts which was found to be the highest compared to other parts (Shakirin *et al*., 2010). Previous study has revealed the existence of polyphenols in SO such as phenolic acids (ellagic and vanillic acids), flavonoids (catechin, epicatechin,epicatechingallate, epigallocatechingallate, apigenin), anthocyanidins (cyanidin, pelargonidin and delphinidin) and anthocyanins (malvidin-3,5-di-O-glucoside, cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside and peonidin-3-O-glucoside) (Chew *et al*., 2011a). Moreover, polyphenol compounds are believed to play a significant role in lowering plasma cholesterol by binding to bile acids which in turn increased faecal loss (Zunft *et al*., 2003).

Moreover, the reduction of LDL-C may be attributed to the increasing level of LDL receptors which can enhance the clearance of LDL-C which assist rapid catabolism of LDL-C, thereby enhance the depletion of LDL-C (Goldstein and Brown, 2009; Lemhadri *et al*., 2006). HDL-C plays an important role in the protection against cardiovascular disease. HDL-C is responsible for transporting cholesterol from cells and from the arteries to the liver for catabolism (Barter *et al*., 2003). The higher the plasma HDL-C, the better effects on cholesterol metabolism. However, the increased of HDL-C in the PC group did not mean that feeding a high cholesterol diet was favourable to plasma lipid profile, because the increase of HDL-C may not cause the vanishing of 'bad' cholesterol (LDL-C) which has negative effect on lipid lowering properties (Xing *et al*., 2009). Apart from that, cholesterol-lowering properties of defatted SO may be attributed to the presence of dietary fiber. The SO defatted pulp consisted of more than 50% Total Dietary Fiber (TDF), characterized by 73% and 27% of insoluble dietary fiber (IDF) and Soluble Dietary Fiber (SDF),

respectively (Kang *et al.*, 1999). The capacity of dietary fiber to lower serum cholesterol by hindering the digestion and absorption of dietary fat, modifying bile acid absorption and metabolism, forming a short chain fatty acid which can inhibit cholesterol and fatty acid synthesis in the liver and altering the concentration of insulin and hormones (Lecumberri *et al.*, 2007). The reduction of plasma cholesterol levels in this study are likely attributed to the binding of bile acids and dietary fat to the fiber compound as demonstrated by previous studies (Ramos *et al.*, 2008; Anderson *et al.*, 2009). Moreover, tremendous animal and human studies have shown that consumption of food rich in dietary fiber, with the presence of antioxidant compounds has positive effects on the different parameters associated with cardiovascular disease, such as endothelial function, platelet activation and biomarkers of lipid peroxidation (Lecumberri *et al.*, 2007).

In the present study, we also found that supplementation of SO defatted pulp in high cholesterol fed animals could inhibit the progression of atherosclerosis. The formation of atheromatous plaque in the HD group was found to be significantly lower compared to that of the non-treated group (PC). The inhibition effect by defatted SO in atherogenesis might be attributable partly to its hypocholesterolemic property, since decrease of TC level is capable to improve endothelial function. Therefore, lowering cholesterol and protecting vascular endothelia play significant roles in preventing the occurence of athresclerosis and reducing the morbidity of cardiovascular diseases (Valgimigli *et al.*, 2003). Furthermore, previous study has shown the purplish powder in grape powder have the potential to reduce atherosclerotic lesion development. The reduction in atherosclerotic lesion development could be related to a reduction in serum lipid oxidative stress. In addition, grape polyphenols exert an antioxidative protective effect not only on serum lipids but also on macrophages. This demonstrates that polyphenols are the main constituents in grape powder which confer their anti-atherosclerotic effects, probably due to their antioxidant capacity (Fuhrman *et al.*, 2005). Therefore this study was parallel to previous study that indicates the purplish powder of defatted SO exhibit atherosclerosis inhibitory effect.

CONCLUSION

Among the fruit parts, the defatted pulp of SO exhibited the most remarkable reduction in lipid profile and greatest reduction of atherosclerotic plaque formation. The reduction of atherosclerotic plaque was induced by significant reduction of plasma LDL-C and TC. Therefore, defatted pulp may be beneficial in preventing hypercholesterolemic, atherosclerosis and reduce the risk factors for coronary artery disease.

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