Research

Ficus deltoidea var. *kunstleri* Extract Administration in Hypercholesterolaemic, Atherosclerotic Rabbits: Effects on Organ Function, Morphology, and Atherosclerosis Development

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ABSTRACT

Ficus deltoidea (FD) is used in traditional Malay medicine to treat various ailments and has been shown to be safe in toxicity studies. However, the information on the safety and efficacy of FD in the atherosclerosis-induced animal model is limited. This study aims to investigate the safety of FD var. *kunstleri* (FDK) extract on high cholesterol diet (HCD)-induced atherosclerotic rabbits and its efficacy in treating atherosclerosis. New Zealand White rabbits were randomly divided into two groups: G1 (1% HCD for 4 weeks) and G2 (1% HCD for 8 weeks). Each group was randomised into FDK700 (700 mg FDK/kg/day for G1 and G2), FDK800 (800 mg FDK/kg/day for G2), simvastatin (5 mg/kg/day) and placebo. The body weight, blood pressure, serum biochemistry and histopathological examination were obtained to assess any toxicity signs. Fasting lipid profile, soluble c-reactive protein (sCRP) level and atherosclerotic plaque formation were compared between treated and placebo groups to evaluate treatment efficacy. Results: No significant differences were observed in all safety parameters between the treated and placebo groups ($p<0.05$). FDK treatment did not show significant differences in all parameters evaluated in both treatment arms. In conclusion, FDK extract up to 800 mg/kg is safe for use in atherosclerotic rabbits. It has neutral effects on lipid profile, inflammation and atherosclerosis formation.

Key words: Atherosclerosis, efficacy, *Ficus deltoidea* var. *kunstleri*, New Zealand White rabbits, safety

INTRODUCTION

Hypercholesterolaemia is a major risk factor for atherosclerosis formation, the main pathology underlying cardiovascular disease, attributed to excessive plasma low-density lipoprotein (LDL) (Gil-Pulido & Zernecke, 2017). Lipid lowering is an important treatment strategy; with dietary restrictions as well as drug therapy used to achieve favourable lipid control in patients with atherosclerosis (Bergheanu *et al.*, 2017). Statin (3-hydro-3-methylglutaryl-coenzyme A reductase inhibitor) is a drug class used to explicitly target HMG-CoA reductase, an enzyme in the rate-limiting step of cholesterol biosynthesis. Despite being frequently used as a first-line treatment, statins can have dose-limiting adverse effects such as rhabdomyolysis and deranged liver enzymes (Ramkumar *et al.*, 2016; Wolf & Hunziker, 2020). Thus, natural products could serve as a complement or an alternative to statin therapy, especially for patients with statin intolerance. In the search for an effective, safe and inexpensive alternative, *Ficus deltoidea* has recently enticed attention.

Ficus deltoidea (FD) Jack (Moraceae) or locally known as 'Mas Cotek' is one of the most versatile medicinal plants in Malaysia with traditionally acclaimed health benefits. It is used in folk medicine to relieve headaches, fever and toothache (Bunawan *et al.*, 2014). The plant is listed as a priority herb under Entry Point Project 1 (EPP1) of the National Key Economic Area (NKEA) Agriculture. FD is traditionally taken as a tea by steeping 5 to 10 pieces of dried leaves in hot water (Choo *et al.*, 2012). Nowadays, FD is sold commercially as herbal tea, juices, capsules and ointments (Bunawan *et al.*, 2014). Studies have reported numerous pharmacological attributes of FD, including anti-inflammatory (Zakaria *et al.*, 2012), anti-oxidant (Misbah *et*

_al._, 2013; Omar _et al._, 2011) and antidiabetic (Adam _et al._, 2012; Bakar _et al._, 2017; Choo _et al._, 2012; Misbah _et al._, 2013). More importantly, _in vivo_ studies have shown that an improvement in cholesterol regulation can be achieved by FD (Abdel-Rahman _et al._, 2020; Ham _et al._, 2020; Kalman _et al._, 2013) and its bioactive compounds (Lei & Yang, 2020). Considering that inflammation and oxidative plays a pivotal role in the pathogenesis of atherosclerosis, and the risk factors include diabetes mellitus and hyperlipidemia, FD is a suitable candidate to evaluate as an anti-atherosclerotic agent.

Our recent study showed that FD extracts attenuate endothelial activation, inflammation, monocyte-endothelial cell binding and oxidative stress in lipopolysaccharide (LPS)-stimulated human coronary artery endothelial cells (Ariff _et al._, 2020). Therefore, dietary consumption of FD extract may reduce atherosclerosis, through these anti-atherogenic mechanisms. This hypothesis needed to be assessed _in vivo_. To the best of our knowledge, this study is the first to report on the anti-atherosclerotic effect of FD _in vivo_. Toxicity studies reported that FD did not induce acute and subacute toxicity in rats (Farsi _et al._, 2014; Farsi _et al._, 2013; Ilyanie _et al._, 2011; Noor _et al._, 2016; Nugroho _et al._, 2020), however, data on the safety of FD in higher vertebrates, such as rabbits, are limited. The results of toxicity tests may vary significantly between different species. This is important data to have, especially for this present study since rabbits are commonly used as experimental models in atherosclerosis studies. This is due to their sensitivity to a high cholesterol diet (HCD), inducing hypercholesterolaemia and subsequently atherosclerosis formation (Duff, 1935; Niimi _et al._, 2020). Thus, the present study aimed to investigate the safety and efficacy of FD var. _kunstleri_ (FDK) administration in HCD-induced rabbits.

MATERIALS AND METHODS
Materials
Chemicals and reagents used in the present work are as follows: Ethanol (HmbG Chemicals, Hamburg, Germany), 10% buffered neutral formalin (R&M, Essex, UK); Xylene (Sigma-Aldrich, St. Louis, MO, USA); Paraplast plus (Sigma-Aldrich, St. Louis, MO, USA); Eosin 515 LT (Surgipath, Leica Biosystems, Richmond, IL, USA); Hematoxylin 560 MX (Surgipath, Leica Biosystems, Richmond, IL, USA).

Plant material and extract preparation
The FDK leaves were collected from Kuala Terengganu, Terengganu, Malaysia. The specimens were then deposited at Universiti Sultan Zainal Abidin (UniSZA) herbarium and identified by Professor Nashriyah Mat, School of Agricultural Science and Biotechnology with voucher specimen number 00048. The aqueous ethanolic FDK extracts were prepared by our team from Atta-ur-Rahman Institute for Natural Product Discovery (AuRIns), Universiti Teknologi MARA (UiTM), Puncak Alam Campus, Selangor, Malaysia. The samples were extracted according to the ultrasonic-assisted extraction method (Noor _et al._, 2016). Optimization of the aqueous ethanolic FDK extracts (Mohammad Noor _et al._, 2020; Noor _et al._, 2016) and the fingerprinting data of the same extract (Afzan _et al._, 2019) have been published by our team from AuRIns. The leaves were dried at 35 °C, soaked in 50% ethanol (50:50; ethanol: water (v/v)) and sonicated at 40 °C for 30 min. Then, the mixture was filtered using a vacuum filter and the residual solvents were removed using a rotary evaporator. The extracts were then subjected to freeze-drying and stored at -20 °C until further use. The percentage yield of crude extract (10.8%) was calculated according to equation as follows:

$$Yield\ (\%) = \frac{weight\ of\ crude\ (g)}{weight\ of\ plant\ sample\ (g)} \times 100$$

Animals and diets
The animal use and experimental protocols involved in this study were reviewed and approved by the Committee on Animal Research and Ethics (UiTM CARE: 155/2016). A total of 35 healthy male New Zealand White rabbits aged 2 months old, weighing between 2.0-3.0 kg (A Sapphire Enterprise, Seri Kembangan, Malaysia) were placed singly in a stainless-steel cage. All rabbits were acclimatised upon arrival for two weeks and were maintained in a temperature-controlled room (25 ± 2 °C, relative humidity (70-80%) and a 12 h light/dark cycle). They had access to tap water _ad libitum_. These rabbits were then randomly assigned into treatment subgroups. The rabbits were initially fed with 1% high cholesterol diet (HCD) (SF00-221, Specialty Feeds, Western Australia) before converting to a normal diet (ND) (Perternakan Hong Lee Sdn. Bhd., Malaysia) with interventions. The standard laboratory ND contained crude protein 18.0%, crude fat 4.0%, carbohydrates 26%, vitamins and minerals. Both ND and HCD were identical in their nutritional content except that 1% cholesterol was added to the HCD.

Experimental design and procedure
The experimental design was adopted from a previous study using the same animal model with a slight modification (Rahman _et al._ 2016). The rabbits were randomly divided into two groups: G1 (n=15) were fed with HCD for 4 weeks to induce early atherosclerosis; G2 (n=20) were fed HCD for 8 weeks to induce established lesions. Subsequently, the animals in G1 were randomised into three subgroups: FDK700 (n=5), simvastatin (n=5) and placebo (n=5), while those in G2 were divided into four subgroups: FDK700 (n=5), FDK800 (n=5), simvastatin (n=5), placebo (n=5). The animals were given daily treatment by force-feeding. The placebo groups received distilled water. Simvastatin groups received 5 mg simvastatin/kg/day, while FDK700 and FDK800 groups received 700 mg FDK/kg/day and 800 mg FDK/kg/day, respectively. Two doses were

used only for G2 group due to the limited amount of FDK crude extracts available from the Atta-ur-Rahman Institute for Natural Product Discovery (AuRIns), Universiti Teknologi MARA (UiTM). The treatments were given for 8 weeks. The research design and treatment protocol are represented in Figure 1.

The FDK dosage of 700 mg/kg/day and 800 mg/kg/day were chosen based on calculated effective FD extract dose from a previous study (Ab Azis *et al.*, 2019), using the animal equivalent dose (AED) calculation (Nair & Jacob, 2016). The active constituents targeted is vitexin and isovitexin. These substance have been shown to reduce total cholesterol, LDL, inflammatory markers (such as ICAM-1, IL-6 and TNF-α) in an animal study (Lei & Yang, 2020). The extraction method used was congruent with the active substance intended. Considering dose conversion between species, the concentration of vitexin in 700 and 800 mg/kg/day of FDK was within the acceptable range to ensure optimal activity in the experiments.
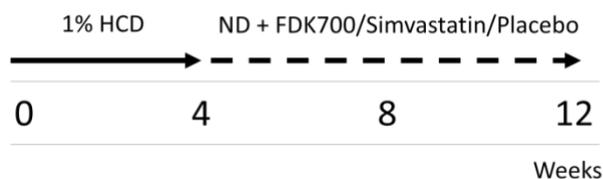
Fig. 1. Research design and treatment protocol timelines for the HCD-fed rabbits in early and established atherosclerosis models. The upper timeline shows the early atherosclerosis group where rabbits were initially fed 1% HCD for 4 weeks before switching to a normal diet (ND) with treatment intervention for 8 weeks. The lower timeline shows the established atherosclerosis group where rabbits were initially fed 1% HCD for 8 weeks before switching to ND with treatment intervention for 8 weeks.

Determination of body weight and blood pressure
Body weight was recorded at baseline (B0), pre-intervention (week 4 or 8) and post-intervention (week 12 or 16). Blood pressures were measured at similar time points using CONTEC08A-VET Digital Veterinary Blood Pressure Monitor (Contec Medical Systems Co., Ltd., Qinhuangdao, China).

Biochemical analysis
All rabbits were fasted overnight, and blood samples were collected from marginal ear veins at baseline (at the beginning of the experiment), pre- and post-intervention. The blood samples were centrifuged at 4000 rpm for 10 min and the resultant sera were carefully collected. Spot urine samples were collected at similar time points, centrifuged at 1500 rpm for 5 minutes and the supernatants produced were separated. All samples were stored at -20 °C until further analysis.

The serum was used to measure lipid profile, consisting of total cholesterol (TC), triglyceride (TG), High-density lipoprotein (HDL-c) and Low-density lipoprotein (LDL-c), biochemical markers of liver injury including alkaline phosphatase (ALP), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), and synthetic function of the liver (albumin (ALB), and total protein (TP)). Serum and urine creatinine (CREAT), calcium (Ca) and phosphate (PHOS) were also measured. All the tests were performed by enzymatic reference methods using an automated analyser (Cobas C501 Analyzer, Roche Diagnostics, Indianapolis, USA) according to manufacturer specifications and employing proprietary reagents.

Soluble C-reactive protein (sCRP) measurement
Serum sCRP in the rabbits was assessed using Rabbit CRP ELISA Kit (Fine Test, Wuhan, China), according to the protocol provided by the manufacturer. The concentration of sCRP in test sample was measured with absorbance at 450 nm using Victor™ X5 plate reader (PerkinElmer, Waltham, MA, USA).

Sudan IV staining
At the end of the experiment, the rabbits were euthanised with 5 mL sodium pentobarbital /3kg body weight (Dolethal, Vetoquinol, Lure, France). The aorta was dissected from its origin to the bifurcation of the

iliac arteries and incised longitudinally. It was then fixed overnight in 10% neutral buffered formalin. The endothelial surface was then rinsed with 70% ethanol and immersed in Sudan IV (Santa Cruz Biotechnology, Santa Cruz, USA) solution (5 g Sudan IV dissolved in a mixture of 500 mL 70% ethanol and 500 mL acetone) for 15 min at room temperature. The aorta was then washed under running tap water for 1 hour. The inner surface of the aorta was photographed and the atherosclerotic lesions were measured using image analysis software (analySIS® FIVE, Olympus, USA). Atherosclerotic lesions were calculated as a percent of the entire aortic area.

Hematoxylin & Eosin (H&E) staining

The heart, spleen, brain, liver, and kidney were dissected and placed in 10% buffered neutral formalin. They were subsequently processed to produce paraffin-embedded tissue blocks, sectioned at 3 to 5 μm thickness (Microm HM360; Microm, Walldorf, Germany), deparaffinized and stained with Haematoxylin & Eosin (H&E) for histopathological examination. Photomicrographs were captured (BX53, Olympus, Germany) with a DP72 digital camera under a magnification of ×10 and ×40.

Statistical analysis

All parameters were expressed as mean ± SEM. Data distribution was tested with Shapiro-Wilk normality test. Comparison between groups was evaluated using One-way ANOVA and post-hoc Bonferroni test. To examine the difference between treatment interval and baseline in each group (within-group), Paired T-test was used. $p < 0.05$ was considered statistically significant. All calculations were performed with statistical analysis system software, SPSS version 25.0. Histological findings were reported as descriptive data under the guidance of a histopathologist (EO).

RESULTS
Safety of FDK supplementation
Effect of FDK on body weight and blood pressure

In G1, the body weight of animals in all groups increases after HCD induction, intervention and treatment ($p < 0.05$). However, there is no significant difference in the amount of weight gain among the groups. Similar findings were seen in G2 (Figure 2).
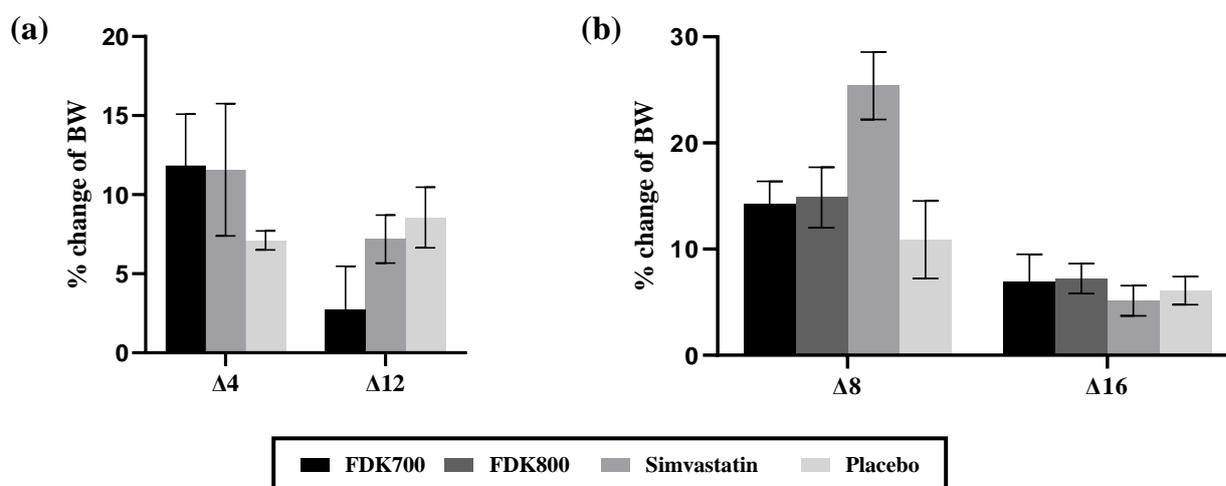
Fig. 2. The change in body weights of the subjects in **(a)** G1 and **(b)** G2. The data are presented as means ± SEM. $p < 0.05$ was considered statistically significant. No significant difference was seen in the body weight changes in any of the groups.

No significant difference was evident in systolic BP in both G1 and G2, between all treatment groups throughout the treatment period. There was a significant increase in diastolic BP of the placebo group in G1 at W4 compared to B0 ($p < 0.05$). Receiving daily treatments maintained diastolic BP in all groups in G1 compared to W4. Similar trends showed by all treatment groups in G2, except that of the placebo which showed a significant reduction of diastolic BP at W16 compared to W8 ($p < 0.05$). Changes in systolic and diastolic BP were shown in Figure 3.
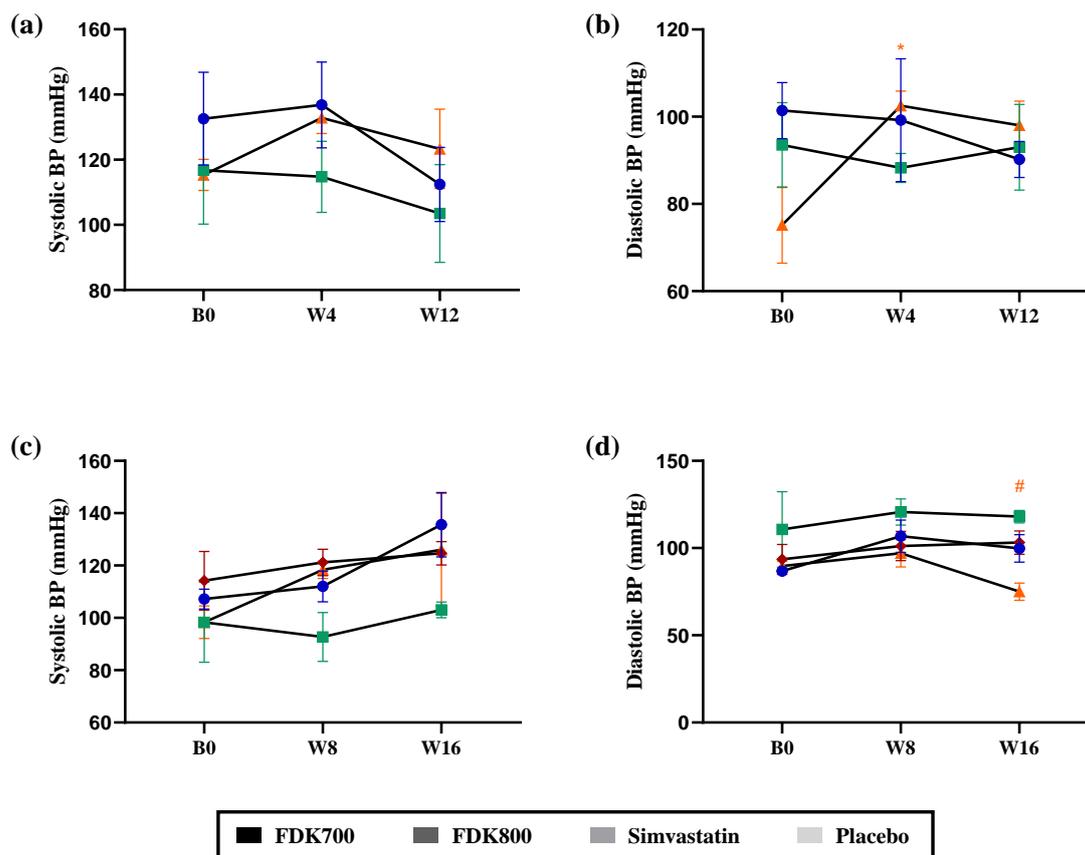
Fig. 3. Systolic and diastolic BP of different treated groups **(a, b)** G1 and **(c, d)** G2. The data are presented as means ± SEM. *P* < 0.05 was considered statistically significant. * compared with B0; # compared with W4/W8.

Effects of FDK on biochemical parameters

There was no significant difference in liver enzyme levels in the FDK treated groups. Similarly, there was no reduction in albumin and total protein levels in these groups within the early and established atherosclerosis experiments.

There was no significant change in serum calcium in all the FDK treated groups compared to the beginning of the treatment in established atherosclerosis (G2). However, in the early atherosclerosis group, the FDK700 group showed a significant decrease in serum phosphate levels. Otherwise, all the other parameters are comparable (Supplementary Data 1).

Effects of FDK on histological changes

Histopathological examination of the liver showed changes due to prolonged HCD in the established atherosclerosis group (G2). These include a mild portal, periportal and pericentral chronic inflammatory cell infiltration, mild parenchymal inflammatory infiltrates, mild parenchymal destruction, micro-steatosis, hepatocyte oedema, mild portal-central spindle cell proliferation and hepatocyte regeneration (Figure 4(a)). These changes were seen in all the subjects of the G2 group. The spleen also showed changes due to the HCD represented by an increase in foam cells in the red pulp of some of the animals, again without clustering in any treatment group, Figure 4(b) and (c).

Examination of the heart in the established atherosclerosis group revealed changes related to cardiovascular disease, ranging from hyaline thickening of the wall of the arterioles (hyaline atherosclerosis), intimal accumulation of foam cells to myocardial infarction (Figure 4(d) and (e)). These changes were seen in both FDK (800) and placebo groups within the established atherosclerosis model.

The kidneys were within normal limits. Mild perivascular lymphocytic cuffing was seen in the brain, Figure 4(f), with no clustering of such findings in any of the groups. The significance of this change is uncertain as the animals did not show any sickness or behavioural changes. The pancreas was normal in all the animals.
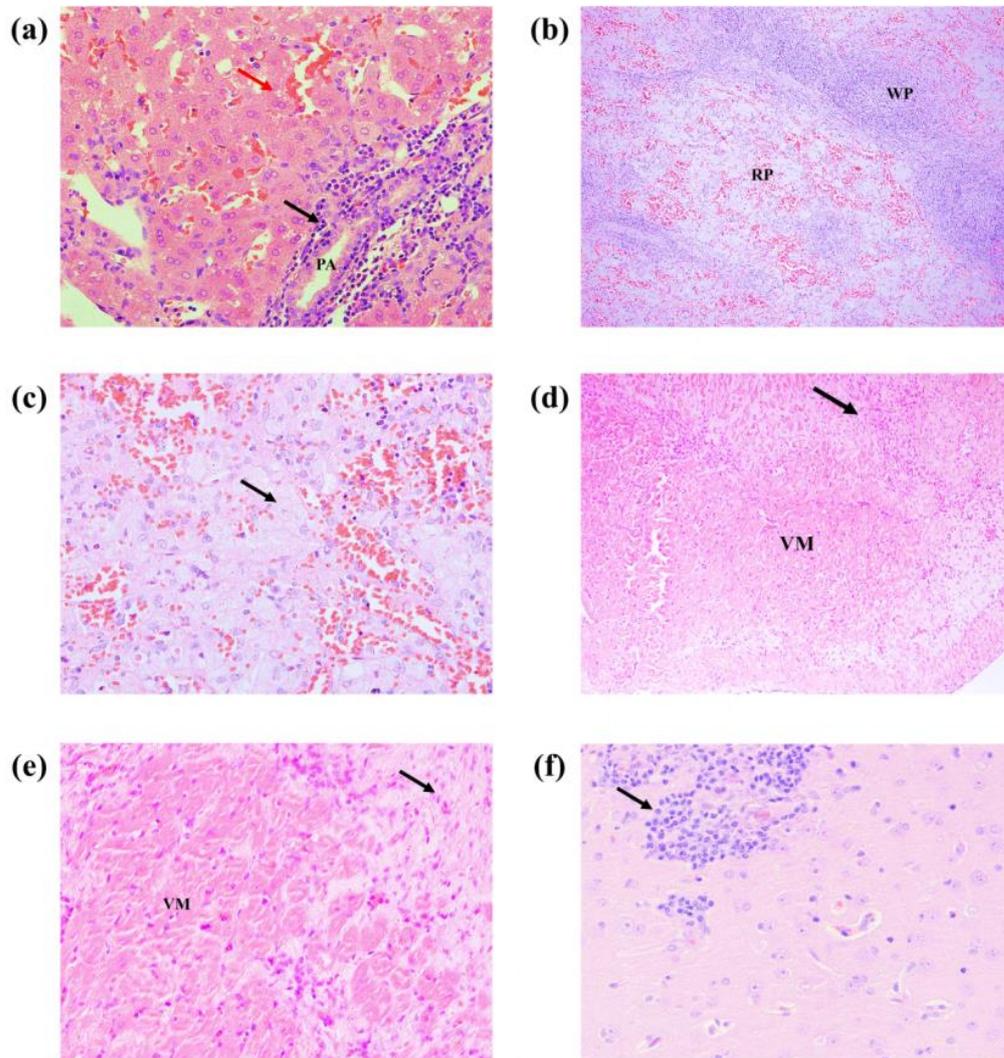
Fig. 4. Representative sections from **(a)** the liver showing mild micro-steatosis (red arrow) and mild portal/central lymphocytic infiltration (black arrow) (×40) **(b, c)** the spleen showing numerous foam cells in the red pulp (RP) (black arrow) leading to its expansion and having a pale colour at low power (**(b)**,×10)), while on high power, the foam cell accumulation is seen clearly (**(c)**,×40), **(d,e)** the heart showing viable myocardium (VM) and myocardial infarction (black arrow) (×10, ×40), **(f)** the brain showing mild focal perivascular white blood cell cuffing (×40), PA= portal area, RP = red pulp, WP = white pulp VM = viable myocardium. H&E staining.

Efficacy of FDK treatment
Effect of FDK on fasting lipid profile

All subgroups in G1 showed a significant increase ($p<0.05$) in TC, HDL and LDL levels after 4 weeks of cholesterol feeding. Daily administration of 700 mg/kg/day FDK, simvastatin and placebo for 8 weeks lead to a significant reduction of TC, HDL and LDL levels compared to B0 ($p<0.05$). There was a significant decrease in TG levels in the placebo group at W4 and all treatment groups at W12 compared to B0 ($p<0.05$). No significant changes in TG levels were observed in all groups after FDK, simvastatin or placebo interventions at W12. Alteration in TC, HDL, LDL and TG values in all treatment groups across the treatment durations is illustrated in Figure 5.
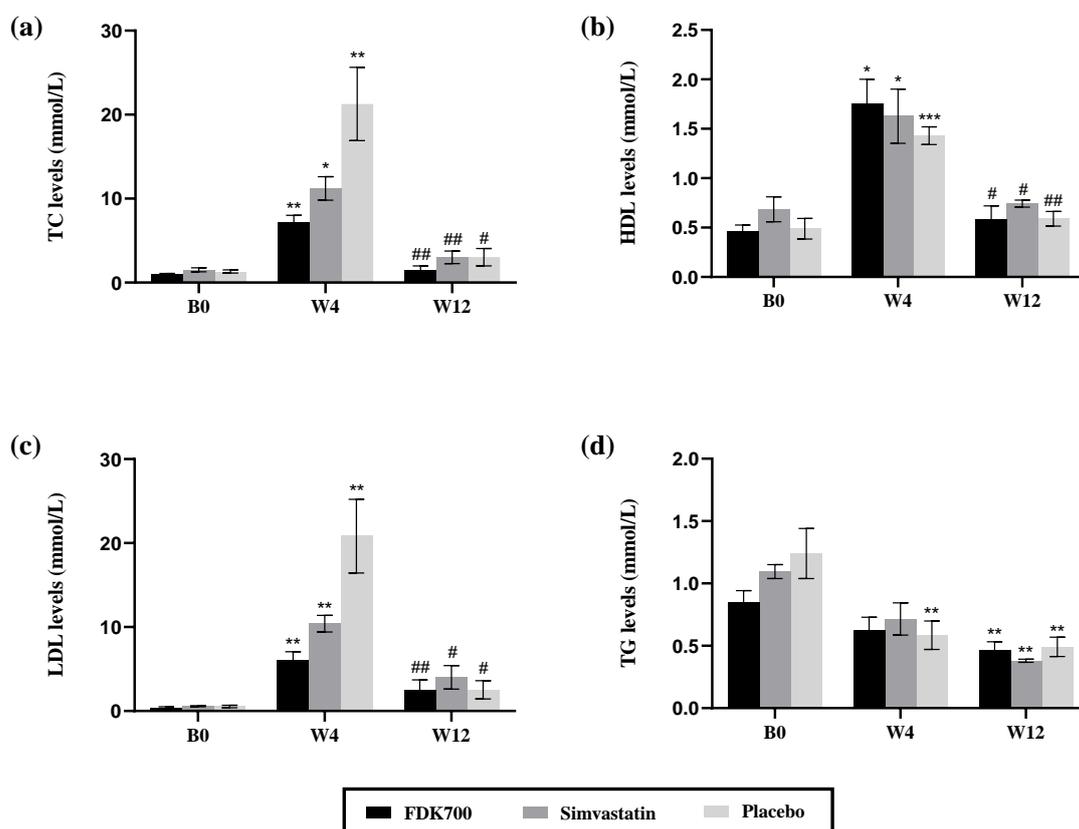
Fig. 5. The lipid profile of HCD fed rabbits in all subgroups in G1. **(a)** total cholesterol, **(b)** HDL, **(c)** LDL and **(d)** Triglyceride levels, at baseline (B0), week 4 (pre-intervention) and week 12 (post-intervention). The data are presented as means ± SEM. $P < 0.05$ was considered as statistically significant. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared with B0; #$p< 0.05$, ##$p<0.01$ compared with W4.

All groups in G2 showed a significant increment of TC levels following HCD inducement ($p<0.05$). The increases in TC levels were maintained after both doses of FDK interventions compared to W8. Only simvastatin treatment showed a significant reduction of TC levels compared to W8 ($p<0.05$). HDL levels significantly increased in all groups except simvastatin after 8 weeks of HCD consumption ($p<0.05$). The increases in HDL levels were maintained following FDK interventions compared to W8. There were no significant changes in simvastatin intervention across all treatment durations. All groups showed a remarkable increment of LDL levels compared to B0 ($p<0.05$). FDK700 showed decreasing trend compared to W8, although not reaching statistical significance. Simvastatin significantly reduced LDL levels compared to W8 ($p<0.05$). The placebo group showed a significant rise in LDL compared to W8 ($p<0.05$). No significant changes in TG levels in all groups across all treatment durations, except FDK800, which significantly increased after HCD inducement ($p<0.05$) and further increased following the treatment. Alteration in TC, HDL, LDL and TG values in all treatment groups across the treatment durations is shown in Figure 6.
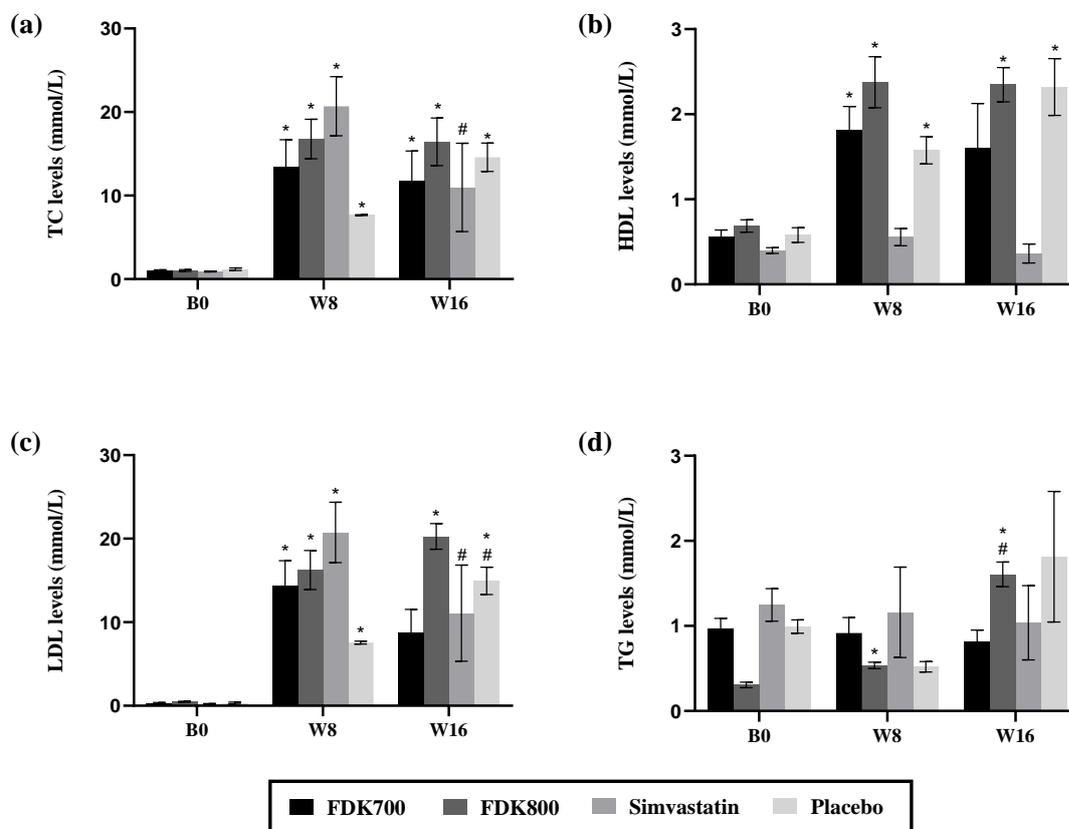
Fig. 6. The fasting blood parameters of HCD fed rabbits in all groups in G2. **(a)** total cholesterol, **(b)** HDL, **(c)** LDL and **(d)** Triglyceride levels, at baseline (B0), week 8 (pre-intervention) and week 16 (post-intervention). The data are presented as means ± SEM. $p < 0.05$ was considered as statistically significant. *$p < 0.05$ compared with B0; #$p < 0.05$ compared with W8.

Effect of FDK on soluble sCRP levels

Figure 7(a) shows that in G1, the sCRP levels of FDK700 at W12 and placebo at W4 and W12 were significantly increased when compared to B0 ($p < 0.05$). In contrast, simvastatin at W12 significantly reduced sCRP levels compared to B0 and W4 ($p < 0.05$).

Figure 7(b) depicts that in G2, FDK700 and placebo groups significantly increased sCRP levels at W8 compared to B0 ($p < 0.05$) and significantly reduced at W16 compared to W8 ($p < 0.05$). However, the placebo significantly decreased sCRP levels at W16 compared to B0 ($p < 0.05$).

Effect of FDK on atherosclerotic lesions

Sudan IV-stained lipid within the macrophage and atherosclerotic lesion was detected as a red colour in the aortic intima. Atherosclerotic lesions were observed in all groups following 4 weeks and 8 weeks of atherosclerosis induction. In G1, the area of lesions of placebo was significantly higher compared to control B0 and control G1. However, there was no reduction in atherosclerotic lesions in FDK700 and simvastatin compared to the placebo.

In G2, the area of lesions of control G2 was significantly higher compared to control B0. All groups showed no difference in atherosclerosis area compared to the placebo. The quantitative analyses of the atherosclerotic lesions were shown in Figure 8.
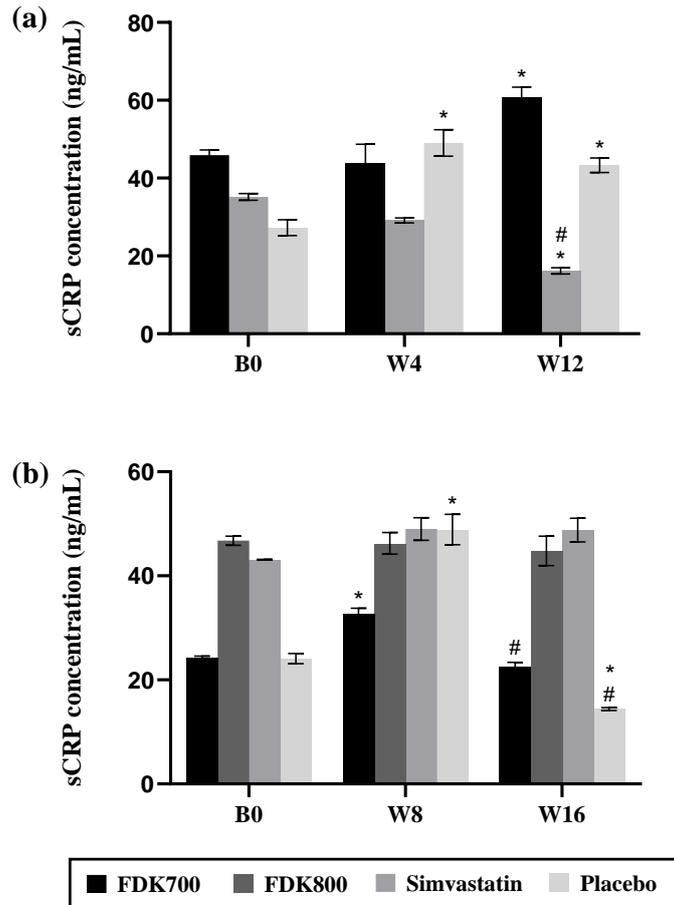
Fig. 7. Comparison of sCRP levels between different treated groups **(a)** G1 and **(b)** G2 at B0, W4/W8 (pre-intervention) and W12/W16 (post-intervention). Data are expressed as Mean ± SEM. * indicates significant difference compared to B0 ($p<0.05$). # indicates significant difference compared to W4/W8 ($p<0.05$).
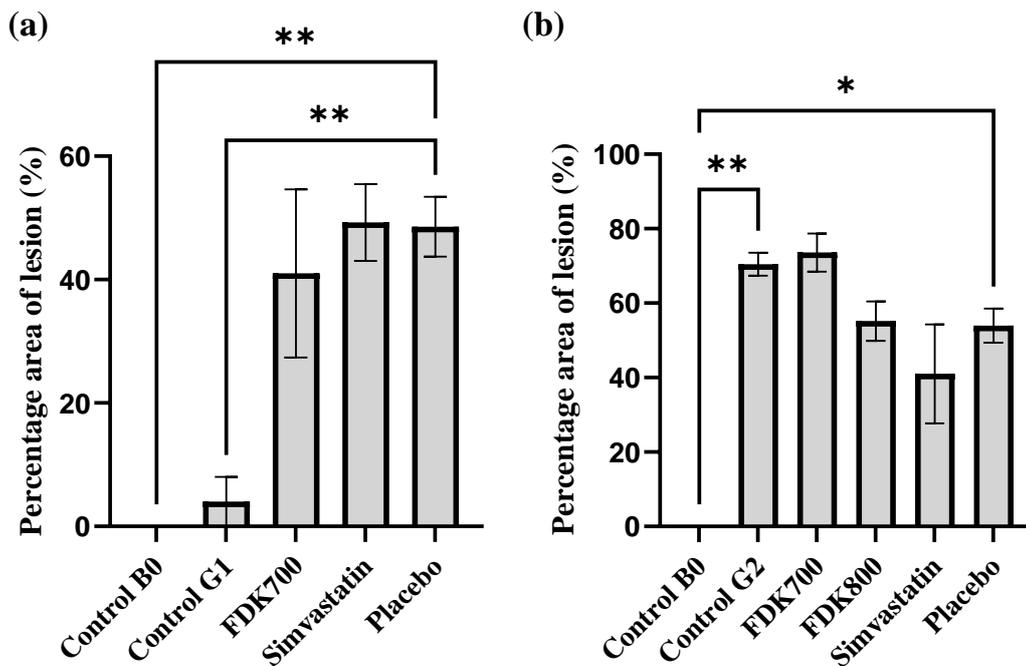
Fig. 8. Quantitative atherosclerotic lesion analysis between all treatment groups in **(a)** G1 and **(b)** G2. Data are expressed as Mean ± SEM. *$p<0.05$ and **$p<0.01$.

DISCUSSIONS

This study used 1% HCD to induce hypercholesterolaemic state in a rabbit model experiment. To the best of our knowledge, the present study is the first attempt to explore whether FDK is safe to be consumed by high-cholesterol diet-induced atherosclerotic rabbits. Cholesterol-fed rabbits were used in investigating the effects of treatment on atherosclerosis as they represent an established animal model for this disease (Fan *et al.*, 2015; Finking & Hanke, 1997; Niimi *et al.*, 2020). The rabbit model is notable due to the rapid development of atherosclerotic lesions upon consumption of a cholesterol-rich diet and having similar lipid metabolism as humans (Fan *et al.*, 2015).

Several acute toxicity studies conducted in rats found no significant change in behaviour or mortality with FDK ingestion. The median lethal dose (LD_{50}) of FDK extract was found to be above 2000 mg/kg body weight (Choo *et al.*, 2012; Noor *et al.*, 2016) and is even safe at 5000 mg/kg in rats (Farsi *et al.*, 2014). Similarly, longer duration (sub-chronic) studies, where 200 mg/kg FD methanol extract was administered for 28 days in diabetic rats, have shown no significant adverse effects on body weight, food and water consumption, behaviour, urination and defecation pattern, mortality, organ weight, histopathology, and clinical chemistry values compared to controls was observed (Ilyanie *et al.*, 2011; Farsi *et al.*, 2013). Additionally, administration of FD (1000 mg) in pre-diabetic, otherwise healthy adults for 8 weeks showed no adverse effects (Kalman *et al.*, 2013).

The above findings were also replicated in our study. The doses used in this study were well below the doses reported in the toxicity studies (Ilyanie *et al.*, 2011; Farsi *et al.*, 2013). The dose range of 700–800 mg/kg/day FDK extract did not exhibit any significant adverse effects as reflected by the insignificant body weight gain between FDK-treated hypercholesterolaemic rabbits that was comparable to that of the placebo, and the absence of any mortality in the treated animals in both treatment arms. It is also essential to highlight that FDK administration did not affect nor worsen the blood pressure at these doses. Abdullah et al. reported that FD fruit extract produced inhibitory effects on Angiotensin-I converting enzyme (ACE), suggesting antihypertensive properties (Abdullah *et al.*, 2008). Another study that used aqueous ethanolic FDK leaf extract found that it reduced blood pressure in spontaneous hypertensive rats, which might involve the renin-angiotensin-aldosterone system (RAAS) (Ab Azis *et al.*, 2019). Similarly, FD var. *angustifolia* given daily at doses of either 800 or 1000 mg/kg/day reduced the blood pressure in spontaneously hypertensive rats (Kamal *et al.*, 2019). Despite these reports, we found that FDK did not cause significant changes in blood pressure measurement. This could be due to the absence of inherent blood pressure control abnormalities in the animals used in this study; the previous studies were performed on spontaneously hypertensive rats, which have intrinsically abnormal blood pressure control.

TC, HDL and LDL levels of FDK700 and simvastatin in G1 were significantly reduced after the cessation of HCD, but the reductions were comparable to the placebo group. The reductions seen in all treatment groups were probably attributed to switching the rabbits' diet from HCD to ND after 4 weeks. Similarly, in G2, both doses of FDK treatments were unable to reduce TC and LDL levels after 8 weeks. The abnormality of increased HDL after dietary cholesterol intake was possibly due to an increase in HDL-c lipid hyperoxide content, a key feature of dysfunctional HDL (Morgantini *et al.*, 2018). The reduced TG levels after dietary cholesterol intake and even after the treatments were possibly due to the presence of niacin in the rabbits' chow diet. Dietary niacin has been shown to regulate lipid metabolism in the kidney and adipose tissue, as well as lipid transportation in plasma (Cho *et al.*, 2010; Fabbrini *et al.*, 2010). Overall, FDK treatments in both treatment arms have neutral effects on lipid profile compared to placebo. The findings are in contrast to a study by Kalman *et al.* (2013) in which FD leaf extracts significantly decreased total and LDL-c concentrations in a group of healthy adults with pre-diabetes.

It has been suggested that high plasma cholesterol concentrations, especially LDL-c levels result in atherosclerotic formation (Niimi *et al.*, 2020). The lesions produced are morphologically similar to those seen in humans (Dornas *et al.*, 2010). Our findings showed that there is increased lesional formation in G2 compared to G1. The alteration of plaque growth in both treatment arms was due to the duration of the diet; G2 received HCD for a longer period. The extent of the lesions developed in the aortic wall in rabbits is proportional to the quantity of cholesterol consumed (Yanni, 2004). There was no reduction in the atherosclerotic lesion in all treatment groups, including the positive control, compared to the placebo in G1 and G2. This is in contrast to a study by Alfon *et al.,* which showed that statin reduced aortic fatty streak surface coverage which correlated positively with the reduction of total cholesterol, triglycerides and LDL levels in the dyslipidaemic rabbit model (Alfon *et al.*, 1999). The finding of an insignificant change of atherosclerotic lesion showed by simvastatin is not surprising as it could be due to the shorter period of treatment of 8 weeks, compared to 10 weeks in the former study. A longitudinal study by Corti *et al.* showed that there were no changes in atherosclerotic lesions in the lumen area, vessel wall thickness, or vessel wall area in asymptomatic hypercholesterolaemic patients after 6 months receiving simvastatin, although it caused a significant reduction in total and LDL cholesterol levels at 6 weeks that was sustained thereafter. However, at 12 months, substantial decreases in vessel wall thickness and vessel wall area were detected in both the aortic and carotid arteries (Corti *et al.*, 2001). Since the results following FDK treatments were no different to that of placebo, these findings suggest that the oral administration of FDK extract did not ameliorate either early or established atherosclerosis, as evidenced by lipid profiles and the regression of plaques. We postulate that the *in vivo* effect of FDK on atherosclerotic lesions in the treatment of early and established atherosclerosis is not appreciated because of the short time frame of the study.

The degree of exposure to cholesterol led to high inflammatory levels. To assess the inflammatory levels of the rabbits, ELISA experiment was performed to measure the sCRP levels. In contrast to simvastatin,

the present study demonstrated that the intervention with FDK700 in G1 failed to reduce sCRP level. Although FDK700 significantly reduced the sCRP level in G2, the result was not comparable to the placebo. The finding is in contrast with Yu et al. that found significantly higher CRP levels in cholesterol-fed rabbits than in control rabbits (Yu *et al.*, 2012).

Histopathological examination showed only mild steatosis and mild inflammation of the liver areas. As the abnormal morphology was observed exclusively in the established atherosclerosis model irrespective of the intervention (FDK or placebo), the changes were possibly due to the longer HCD feeding period. In the early atherosclerosis group, HCD was given for 4 weeks whereas in the established atherosclerosis it was given for 8 weeks. The liver is a vital organ in lipid metabolism (Liu *et al.*, 2012). A prolonged HCD diet may lead to an excessive burden on the liver to metabolise cholesterol, thus leading to chronic inflammation and liver steatosis. Fortunately, the changes are mild and do not affect the function of the liver and the integrity of liver cells.

FDK treatment also did not induce renal toxicity as evidenced by the normal level of serum and urine creatinine and normal kidney morphology on histopathology. The changes seen in the heart were the most striking, especially in the established atherosclerosis groups. These changes are those seen in cardiovascular disease, and they are seen in animals treated with a placebo and FDK. This shows that the abnormality was due to high-cholesterol diet feeding and not FDK administration. The spleen showed an accumulation of foam cells (lipid laden cells) within the splenic sinusoids (red pulp). The changes were seen in rabbits given 8 weeks of HCD and not in the other groups. There is no difference between FDK or placebo supplementation in this aspect. The pancreas is not affected and appeared normal in all groups.

Although FDK is found to have anti-adipogenic (Woon *et al.*, 2014), anti-atherogenic (Mohd Ariff *et al.*, 2020) and anti-inflammatory (Omar *et al.*, 2011), the results in this experimental atherosclerosis utilising rabbits did not support the previous positive findings. These findings suggest that the oral administration of FDK extract did not suppress acute hypercholesterolemia; 8 weeks of FDK supplementation in the treatment of early and established atherosclerosis had neutral effects on lipid profiles, inflammation, and the regression of plaques. It was postulated that the neutral effects of FDK may be attributable to the formulation utilised in this study, which reduces the extract's bioavailability and hence hampers the anti-atherosclerotic action of FDK *in vivo*. Further studies on the bioavailability and development of better FDK formulation are warranted. Our preliminary study utilising a bioenhanced formulation of FDK extract to enhance the efficacy of aqueous ethanolic extract has shown promising results. Despite its neutral effect, FDK was found safe to be consumed up to 800 mg/kg/day for 8 weeks in HCD-induced atherosclerotic rabbits. FDK did not affect nor worsen the blood pressure at these doses. FDK consumption in early and established atherosclerosis showed no significant difference in biochemical and histological parameters compared to placebo.

CONCLUSIONS
Administration of FDK up to 800 mg/kg/day for 8 weeks is safe in HCD-induced atherosclerotic rabbits. FDK extract has neutral *in vivo* effects on lipid profiles, inflammation, and atherosclerotic plaque formation in early and established atherosclerosis. This study adds to the current body of knowledge on the safety and efficacy of FDK use, specifically in a rabbit atherosclerotic model. Further studies on the bioavailability of FDK and the development of bioenhanced FDK formulation are necessary.

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ETHICAL STATEMENT
Approved by the Committee on Animal Research and Ethics (UiTM CARE: 155/2016).

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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