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Standardisation of Vitexin and Isovitexin in *Ficus deltoidea* var. *Kunstleri* (FDK) Extract and Anti-Hyperlipidaemic Properties of FDK Hydrogel in High Cholesterol Diet-Induced Early and Established Atherosclerosis Rabbits

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Abstract

Hyperlipidaemia is a major risk factor for cardiovascular disease (CVD) due to its role in forming atherosclerotic plaques. Finding natural products with anti-hyperlipidaemic properties is important for preventing atherosclerosis. *Ficus deltoidea* var *kunstleri* extract (FDK) is traditionally used to regulate blood glucose and blood pressure, however its effect on lowering lipid is scarce. Previous study showed neutral effects of FDK extract on lipids. Hydrogel delivery is commonly used to enhance absorption of active compounds therefore enhancing their therapeutic effect. This study aimed to develop a FDK hydrogel from standardised FDK extract and evaluate its effectiveness in lowering lipids and atherogenic index (AI) in high cholesterol (HC)-induced-early and established atherosclerosis rabbits. Standardised FDK extract against vitexin and isovitexin was produced according to a standard guideline using Liquid chromatography-tandem mass spectrometry (LCMS/MS) method prior to the preparation of FDK hydrogel (FDKH). Forty-rabbits were fed with 1 % high cholesterol diet for 4 and 8 weeks to induce early and established atherosclerosis, respectively. Each group was subdivided into 4 intervention arms: 1) FDKH 125 mg/kg; 2) FDKH 250 mg/kg; 3) Simvastatin 5 mg/kg; and 5) placebo for 8 weeks. Lipid profile and atherogenic index (AI) were measured at the baseline (B0), post-HC diet and post-treatment in both early and established atherosclerosis rabbits. Quantitative analysis of FDK using LC-MS/QQQ reveals the presence of 3.5 ± 0.04 mg/g of vitexin and 3.1 ± 0.8 mg/g isovitexin, respectively. In the early atherosclerotic group, at week 12, all treatment groups showed significant reductions in total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and triglycerides (TG) compared to week 4. The reductions varied by treatment type. FDKH 250 mg/kg resulted in the highest reductions in TC and LDL-C, followed by FDKH 125 mg/kg and simvastatin, which is still higher than placebo. Interestingly, simvastatin led to the greatest reduction in TG, followed by FDKH 125 mg/kg and FDKH 250 mg/kg. In the established atherosclerotic group, FDKH 250 mg/kg again demonstrated the highest reductions in TC, LDL-C and TG levels, confirming its efficacy in more advanced stages of atherosclerosis. Both FDKH dosages significantly improved AI compared to the placebo, with FDKH 250 mg/kg showing the lowest AI value. These findings suggest that FDKH, particularly at the 250 mg/kg dosage, is highly effective in reducing lipid levels and improving AI, indicating its potential as a potent alternative to traditional treatments like simvastatin. Further clinical trials are warranted to confirm these results and explore the long-term effects and safety of FDKH in humans. In conclusion, FDKH have significant potential to mitigate serum lipid and atherogenesis in both early and established atherosclerosis rabbits. This advancement represents a promising adjunct treatment option, offering a natural and effective solution for hyperlipidaemia.

Keywords: Anti-hyperlipidaemic, Hydrogel, *Ficus deltoidea* var *kunstleri*, Standardised extract, Atherosclerosis

Introduction

Atherosclerosis (AS) is one of the major risk factors of cardiovascular disease (CVD) and stroke. It is estimated that in 2030, 58 % of CVD death were associated with cardiac events and ischemic stroke [1]. In 2019, Malaysia accounts for increased atherosclerosis related implications such as hypertension, hypercholesterolemia and obesity by 30, 38 and 50.1 %, respectively [2]. It is known that reducing serum cholesterol levels contribute to the AS progression. Dyslipidaemia is an abnormality of lipid metabolism characterized by an elevation of circulating total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), triglycerides (TG) and reduction of high-density lipoprotein cholesterol (HDL-C) [3,4].

Modulating the dysregulation of lipid metabolism and alleviates the TC, LDL-C and TG levels are taking the prime consideration in preventing the development of AS and CVD [5]. Currently the available therapy for dyslipidaemia includes PCSK9 and HMG-CoA reductase inhibitors, bile acid sequestrants, fibric acid derivatives and nicotinic acids. These drugs are used as monotherapy or in combination to attenuates the elevated lipid profile. However, these conventional synthetic drugs usually come with contradictions for long-term used such as rhabdomyolysis, myopathy, and increased risk of gallstone. Therefore, herbal medicine popularity had emerged in various countries as an alternative treatment for dyslipidaemia with no or minimum side effects [4,6]. Therefore, this study aims to standardize FDK extract and evaluate its hypolipidemic effects in an atherosclerosis model.

One of the most popular herbs with a long history of ethnopharmacological use among Malaysian and Southeast Asian is *Ficus deltoidea* (FD) or locally known as Mas Cotek. The legendary reputation of FD is scientifically proven by its pharmacological properties as antidiabetic drugs through regulation of insulin resistance via hepatic PTP1B [7]. Ham *et al.* [8] reported the suppression of several hypercholesterolemic related proteins such as Apo A1, Apo E, C3 and C1 following intervention with FD leaves extract in rats. Previously, our group reported a superior anti-atherogenic actions of FD var *kunstleri* (FDK) as compared to other FD varieties such as FD var. *trengganuensis* (FDT), FD var. *deltoidea* (FDD) and FD var. *intermedia* (FDI) through the NF-κβ and e-NOS pathways in stimulated human coronary artery endothelial cells [9].

Extract standardisation is one of the pivotal steps in food and nutraceutical industry to ensure the quality and safety of herbal remedies. Although FDK has been widely cultivated and commercialised as part of functional food in Malaysia, however, documentation of extract standardization of FDK is rather scarce [10]. Ideally, extract standardisation relies on the bioactive principles of the species [11]. Vitexin and isovitexin has been regarded as the chemical markers across all FD varieties. Pharmacological activities of vitexin and isovitexin against diabetes mellitus and atherosclerosis by regulating inflammatory cytokines has been widely documented [12-14]. Therefore, extract standardization against bioactive components is vital to ensure therapeutic efficacy and safety of the plant extract [11].

In addition to the aforementioned concern, the poor water solubility and low bioavailability of FDK often hindered its therapeutic efficacy *in vivo*. Previously, we have reported the neutral effects of FDK leaves extract towards lipid profile in New Zealand White Rabbits fed with a high cholesterol diet [15]. Looking into the potential of FDK as hypolipidaemic agent, the issue on the neutral effects of FDK towards lipid profile can be circumvented by developing a drug delivery system that can maximize the stability of active components as well as enhancing the gastrointestinal absorption of active principles [16]. In recent years, carboxymethyl chitosan-based hydrogel has been widely used in pharmaceutical and biomedical fields to increase penetration of phototherapeutic components, minimizing toxicity, and increase stability. In this context, carboxymethyl chitosan is considered as the perfect fit to tackle the delivery of FDK in gastrointestinal tract due to its pH sensitivity and its potential of sustain release [17]. Therefore, it is postulated that incorporation of FDK into CMCS may enhance its hypolipidaemic action *in vivo* and thus producing a bioenhanced formulation of FDK. Hence, the objective of this study is to standardise FDK against vitexin and isovitexin via a validated LC MS/MS method followed by loading the standardised extract into carboxymethyl chitosan to produce FDK hydrogel (FDKH). Next, the effects of FDKH (125 and 250

mg/kg) on lipid profile and atherogenic index (AI) in high-cholesterol-diet-induced early and atherosclerosis was being investigated. To the best of our knowledge, this is the first investigation on hypolipidaemic and AI effects of FDKH.

Materials and methods

Plant material and chemicals

Dried leaves of *Ficus deltoidea* var *kunstleri* (FDK) were acquired from Herbagus Trading, Penang, Malaysia, and deposited at Herbarium, University Kebangsaan Malaysia (voucher specimen number; ID011/2021). The botanist indicated that the FDK are from the family of *Moracea*.

The leaves were milled (RT-CR50S, Kimah Industrial Supplies Sdn Bhd) to a powdered form and passed through a 5 mm sieve. Dimethyl sulfoxide 95 % (DMSO), formic acid 99.5 %, acetonitrile 99 % and ethanol 95 % (HPLC grade) were purchased from Merck (Darmstadt, Germany) and chosen based on their polarity and solubility characteristics. Certified reference material (CRM) of vitexin (95 %) and isovitexin (99 %) were purchased from HWI Pharma, Ruelzheim, Germany. FDK hydrogel (FDKH) was prepared in the laboratory of Institute of Medical Molecular Biotechnology and Nexus Wise Sdn. Bhd., Malaysia.

Extraction of FDK

The samples were extracted using ultrasonic assisted extraction method [15,18] with slight modifications. Ultrasonic assisted extraction (Kudos SK8210HP, Shanghai, China) was performed to extract 9.3 kg of FDK dried leaves using an ultrasonic cleaning bath apparatus (UC-10, Jeiotech Lab Companion, Seoul, Korea) operated at a constant input power of 430 W at a frequency of 40 kHz. Sample were placed in a volumetric flask at three-fourth of the capacity of the tank and soaked with 50 % ethanol with liquid to solid ratio of 1:10 g/mL. The volumetric flask was introduced to ultrasonic cleaning bath for 30 min while the temperature was set using in-built thermostats water bath at 40 °C. Upon extraction, the samples were filtered through filter funnel using Whatman No. 01 filter paper and the filtrates were collected in a volumetric flask. Excess solvents were evaporated using rotary evaporator (Buchi R-210 MX07R-20, PolyScience, USA) and subsequently lyophilized in a freeze dryer (RV-8, Edwards, Czech) at pressure of 0.1 mbar and temperature of −20 °C to remove traces of water to yield dark brown solid crude extract (752 g). The percentage yield of crude extract (8.1 %) was calculated according to equation as follows:

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

LC MS/MS analysis of FDK extract

LCMS analysis in this study were conducted at Integrative Pharmacogenomics Institute (iPROMISE), UiTM Puncak Alam, Malaysia. The LC/MS-QQQ system comprised of an Agilent 1,200 liquid chromatography system, equipped with a binary pump, a vacuum degasser unit, an auto sampler and 6,460 triple quadrupole mass spectrometers with an ESI negative and positive ionization mode with Agilent Jet Stream source. Column used was Agilent Eclipse XDB-C18 (2.1×50 mm^2×1.8 µm). Since vitexin and vitexin are isomers, isocratic elution of 40 % mobile phase B (methanol) and 0.1 % formic acid in dH_2O (mobile phase A) was found the best to separate the components. The sample injection volume was set at 2 µL and the flow rate of mobile phase was set at 1 mL/min. The analytes were quantified by multiple-reaction-monitoring (MRM) mode. The precursor-to-product ion pairs, the fragmentor and collision energy (CE) for vitexin and isovitexin were observed at m/z 431 to product ion m/z 340.9, 311 and 282. Mass spectrometer was operated in Electrospray ionization (ESI) with Agilent jet stream technology with optimum gas temperature at 350 °C, gas flow at 11 L/min, nebulizer at 45 psi and capillary at 3.5 kV, respectively.

LC-MS/MS standardisation of FDK

Standardisation of FDK using LC MS/QQQ method was validated according to The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Q2(R1) guideline.

Primary stock solution was prepared at concentration of 1,000 ng/mL and filtered via a 0.22 µM pore size syringe filter. The working standard solutions, quality control and samples were prepared fresh. All the samples prepared were injected in triplicate. Extract

standardization and method validation were performed according to The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Q2(R1) guideline. Six concentrations (5, 10, 20, 40, 80 and 160 ng/mL) of vitexin and isovitexin were analysed in triplicate to construct a calibration curve. The quantitation of each analyte was performed based on their individual calibration curve.

Method validation

The method was validated based on its linearity, precision, accuracy, limit of detection (LOD) and limit of quantitation (LOQ). Linearity was assessed by evaluating the correlation coefficient (R^2) of the calibration curve. The LOD and LOQ were determined using standard deviation of the response and the slope of calibration curve and expressed as (3.33×SD)/s and LOQ (10×SD)/S), respectively. Precision was evaluated by using repeatability (intra-day assay) and intermediate precision (inter-day assay) with 9 determinations (3 concentrations/3 replicates each). The intra-day and inter-day assay of this study were expressed as the relative standard deviation (% RSD) at 3 different concentrations, 15 ng/mL low quality control (LQC), 65 ng/mL for medium quality control (MQC) and 130 ng/mL for high quality control (HQC). Determination of accuracy were accomplished determined by measuring the percentage of recoveries by using the following equation:

$$\text{Recovery (\%)} = \frac{\text{Found amount} - \text{Original amount}}{\text{Spiked amount}} \times 100.$$

Preparation of FDK hydrogel

CMCS-FDK were prepared freshly by dissolving 373 mg of CMCS in 6.8 mL distilled water (pH 5.5) under continuous stirring on a magnetic stirrer for 1 h to get a homogenized hydrogel. Standardized extract of FDK (310 mg) was added to the hydrogel slowly while mixing. All the ingredients were weighed and added to a mixing bowl. The mixture was then homogenized using a laboratory homogenizer (IKA T25 digital UltraTurrax) and was stored in a sealed bag at 4 °C until further analysis.

Sample size calculation

Calculation below is based on the crude method based on the law of diminishing return. E is the degree of freedom of analysis of variance (ANOVA). The value of E should lie between 10 and 20 to be considered adequate. If E is less than 10, it is necessary to add more animals to increase the chance of getting more significant result. However, if E is more than 20, it will be considered more than necessary and does not increase the chance of getting significant results. E can be measured by the following formula:

$$\text{E} = \text{Total number of animals} - \text{Total number of groups}$$

Previous study conducted by our team found that 700 and 800 mg·kg^{-1} FDK extract showed neutral effects [15]. Additional groups (product containing 125 and 250 mg standardised FDK extract/kg are added to compare with the effectiveness of this extract with the assistance of carboxymethyl (CM) chitosan nanoparticles as carrier. This study wants to see the effect of 700 and 800 mg·kg^{-1} FDK extract/bioenhanced product with 125 and 250 mg·kg^{-1} of standardized FDK extract/3 mg·kg^{-1} Simvastatin/FDK-free hydrogel/Placebo consumption and there are 7 groups in both treatment arms with 3 rabbits each. In this case, E will be; Early AS: E = (3×7) − 7 = 14; Established AS: E = (3×7) − 7 = 14. E of both treatment arms are within the acceptable limit, hence can be considered adequate. Corrected sample size [15] = Sample size/(1 − [% attrition/100])

= 3/(1 − [10/100])

= 3.33

Therefore, the corrected sample size is n = 4 per group. The estimated number of rabbits required for both treatment arms will be 56 (2 treatment arms×4 rabbits×7 groups). Since this study is just a continuation from previous study, the addition of 3 groups gives a total of 24 rabbits (2 treatment arms×4 rabbits×3 groups) to be used.

Experimental animals and treatments

All animal experimental protocols were approved by Institutional Laboratory Animal Care Unit (Universiti Teknologi MARA, Malaysia). Forty New Zealand white rabbits aged 3 months and weighing 2.5 - 3.0 kg were purchased from A Saphire Sdn Bhd,

Malaysia. The animals were acclimatized for 1 week under controlled light (12 h light: dark) at 20 - 22 °C in metallic wire individual cages with food and water ad libitum. In terms of animal care, the cages were cleaned at least once in 2 days, Health check-up are performed routinely every week, water was added every day. In addition, the rabbits were groomed twice a week and enriched with small boxes and small balls.

The protocol in this study was according to method by Abdul Rahman *et al.* [19] where the rabbits were randomised and either 1) induced with 1 % HCD for 4 weeks for early atherosclerosis or 2) induced with 1 % HCD for 8 weeks for established atherosclerosis. Upon induction with 1 % HCD, each early and established atherosclerotic groups were further randomly assigned into 4 intervention arms (n = 4) and supplemented with normal diet (ND) for subsequent 8 weeks as the following:

FDKH 125 group (n = 4): ND + FDKH 125 mg/kg body weight

FDKH 250 group (n = 4): ND + FDKH 250 mg/kg body weight

Simvastatin group (n = 4): ND + Simvastatin 5 mg/kg body weight

Placebo group (n = 4): ND + Placebo

Blood collection and biochemical analysis

Lipid profile (TC, LDL-C, TG and HDL-C) were measured at baseline, before and post-FDK hyrogel intervention. All rabbits were undergone overnight fasting (12 h). Fasting blood samples were collected from marginal ear vein into yellow blood tubes and were separated by centrifugation at 4,000 rpm for 10 min and stored at −20 °C until further analysis. Serum TC, TG and HDL-C were measured by enzymatic reference methods on an automated analyser (Cobas 400 PLUS, Roche, USA). LDL-C was analysed by direct method using the same analyser.

Atherogenic index value

Atherogenic index (AI) was measured to predict the risk of atherosclerosis and calculated by the following formula according to Kazemi *et al.* [20]:

$$\mathrm{AI} = \mathrm{LDL} - \mathrm{C}/\mathrm{HDL} - \mathrm{C}.$$

LDL/HDL ratio was chosen because this study would like to explore the impact of FDKH on lipid profiles, particularly LDL, to potentially reduce atherosclerosis and coronary artery disease. The LDL/HDL ratio is highlighted as a superior indicator of arterial health because it reflects lipoprotein balance, predicts cardiovascular risk, indicates lipoprotein particle size, provides a comprehensive risk assessment, and is easily modifiable through lifestyle changes. Therefore, the LDL/HDL ratio serves as a valuable and practical measure for assessing and managing cardiovascular risk [20].

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed by using SPSS software (25.0 Version). Significant value was set at $p < 0.05$. Firstly, normality test (Kolmogorov-smirnov) test was performed to define the distribution of data. Since the data was normally distributed, parametric test was being selected for data analysis. Student paired *t*-test was used to evaluate the effect of treatment in each intervention arm. One-way ANOVA was performed to assess overall differences between different groups of treatment followed by post-hoc analysis (Bonferroni).

Results

Extract standardization

A well-defined composition of active principles is one of the most important prerequisites to produce herbal medicines. Phytochemical screening of FDK revealed the presence of 18 polyphenols with vitexin and isovitexin as the major components (**Table 1**) which is consistent with previous metabolomics study [21]. Therefore, it is safe to say that vitexin and isovitexin are the important arrays of biological attributes in FD (**Figure 1**). After several mobile phase trials, an isocratic system of 40 % methanol (mobile phase B) and 0.1 % formic acid in dH_2O (mobile phase A) provides a good separation of vitexin and isovitexin, with symmetrical Gaussian-shaped peaks in a short analysis time (4 min). The LC-MS/QQQ was conducted in positive and negative ionisation modes, however the analytes were better ionized in the negative mode. Vitexin and isovitexin were simultaneously eluted at t_R 1.5 and 1.9 min, respectively.

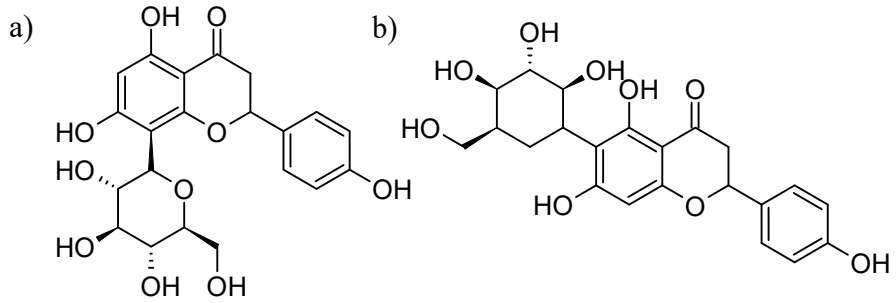

Figure 1 Chemical structure of a) vitexin and b) isovitexin.

The linearity of the developed method was expressed with squared correlation coefficient (R^2) of 0.997 and 0.998 for vitexin and isovitexin, respectively. The regression equations are y = 66.95x + 136.86 for vitexin, and y = 42.03x + 169.69 for isovitexin (**Table 2**). Concentration of vitexin and isovitexin interpreted using standard calculations of regression equations and the result suggest that the extract contains 3.5 ± 0.04 and 3.1 ± 0.8 mg/g (w/w) of vitexin and isovitexin, respectively. From the slope equation, LOD and LOQ of FDK were determined to be 10.1 and 30.8 ng for vitexin while 11.1 and 33.5 ng for isovitexin. The standardized FDK was found to be stable at room temperature for 24 h.

Table 1 Chemical constituents of FDK extract detected by LC-MS QQQ in negative ionization mode.

Number	Name	$[M - H]^-$ (m/z)	t_R	Molecular formula
1	Gallocatechin	305.069	7.948817	$C_{15}H_{14}O_7$
2	Epicatechin	289.0739	8.0453	$C_{15}H_{14}O_6$
3	Epi afzelechin epi catechin	561.1429	8.238283	$C_{30}H_{25}O_{11}$
4	Catechin	289.0739	9.733833	$C_{15}H_{14}O_6$
5	Orientin 2 rhamnoside	593.1542	9.97505	$C_{27}H_{30}O_{15}$
6	Vicenin-2	593.1544	10.58613	$C_{27}H_{30}O_{15}$
7	Coumaroyl acid derivative	337.0603	10.85952	$C_{16}H_{18}O_8$
8	Coumaroyl acid derivative	337.0593	11.14898	$C_{16}H_{18}O_8$
9	Orientin	447.0964	11.3902	$C_{21}H_{20}O_{11}$
10	Schaftoside	563.1436	11.47062	$C_{26}H_{28}O_{14}$
11	Isorientin	447.0965	11.53493	$C_{34}H_{34}O_{18}$
12	Luteolin h7-rhamnosyl(1->6)galactoside	593.1545	11.66358	$C_{27}H_{30}O_{15}$
13	Vitexin	431.1017	11.95305	$C_{21}H_{20}O_{10}$
14	Isovitexin 2’’-O-rhamnoside	577.1593	12.04953	$C_{27}H_{30}O_{14}$
15	Isoschaftoside	563.1436	12.11387	$C_{26}H_{28}O_{14}$
16	Isovitexin	431.1017	12.35508	$C_{21}H_{20}O_{10}$
17	Rhoifolin	577.1599	12.4194	$C_{27}H_{30}O_{14}$
18	Orientin-O-rhamnoside	577.1594	13.33603	$C_{27}H_{30}O_{14}$

Table 2 Calibration curve equation, linearity, LOD and LOQ of vitexin and isovitexin in FDK.

Analyte	Regression equation	Correlation coefficient (R^2)	LOQ (ng)	LOD (ng)	Range of quantitation
Vitexin	66.957042x + 136.862407	0.997	30.8	10.1	10.1 - 30.7
Isovitexin	42.030623x + 169.698925	0.998	33.5	11.054	11.0 - 33.5

Two of the most important aspect in chromatography analysis are precision and accuracy. The RSD values for intra-day assay ranging between 0.78 - 2.37 % (**Table 3**) for both components suggesting that the data obtained are acceptable for all concentrations assayed for intraday assays [22]. Inter-day assay of 3 concentrations of analyte revealed RSD values ranging between 0.38 and 6.61 % indicating that the said method was reliable and repeatable [23]. In this study, the proposed method demonstrates good accuracy as the percent of recoveries ranged between 112.5 - 119.21 and 119.77 - 125.25 % for vitexin and isovitexin, respectively (**Table 4**).

Table 3 RSD values (%) for intra-day and inter-day precision for vitexin and isovitexin.

	Intra-day assay		Inter-day assay	
	Vitexin	Isovitexin	Vitexin	Isovitexin
LQC (15 ng/mL)	2.37	2.10	3.97	6.61
MQC (65 ng/mL)	1.96	0.79	1.84	0.38
HQC (130 ng/mL)	0.78	1.20	2.57	2.47

Table 4 Recovery of vitexin and isovitexin in FDK.

Analyte	Exp	Sample concentration	Recovery (%) ± SD
Vitexin	1	LQC (15 ng/mL)	112.5 ± 0.99
	2	MQC (65 ng/mL)	119.5 ± 8.38
Isovitexin	1	LQC (15 ng/mL)	119.7 ± 1.13
	2	MQC (65 ng/mL)	126.25 5.53

Fasting serum lipid on early atherosclerotic group

Figure 2 illustrated the percentage (%) delta change (Δ) of TC, LDL-C, TG and HDL-C levels post-treatment at week 12. All treatment group showed significant reduction of TC, LDL and TG at week 12 compared to Week 4. However, the degree of reduction for each group was different depending on the type of treatment and presented as percentage (%) delta change (Δ) as shown in **Figure 2**.

There were significantly higher % Δ TC, LDL-C and TG reduction following 8 weeks treatment with FDKH 125 mg/kg (% Δ TC: −87.2 ± 4.8 %, Δ LDL-C: −90.4 ± 5.4 %, Δ TG: −50.5 ± 3.9 %), FDKH 250 mg/kg (Δ TC: −91.1 ± 1.1 %, Δ LDL-C: −96.1 ± 1.3 %, Δ TG: −23.3 ± 5.6) and simvastatin (Δ TC: −82.3 ± 4.1 %, Δ LDL-C: −74.9 ± 6.5 %, Δ TG: −55.7 ± 4.1) compared to placebo. FDKH 250 had significantly higher % Δ TC and LDL-C reduction compared to FDKH 125 ($p < 0.05$). In contrast, FDKH 125 had significantly higher % Δ TG reduction compared to FDKH 250 ($p < 0.05$). In terms of comparison with simvastatin, interestingly, FDKH 250 had higher % Δ TC and LDL reduction compared to simvastatin ($p < 0.05$). Both FDKH 250 and 125 had significantly lower % Δ TG reduction compared to Simvastatin ($p < 0.05$).

In overall, treatment with FDKH 250 mg/kg indicated as the highest % Δ TC and LDL-C reduction among all other treatment group. It was followed by treatment with FDKH 125 and simvastatin and most importantly there were still higher than the placebo group. Interestingly, simvastatin had the highest % Δ TG followed by FDKH 125 and then 250.

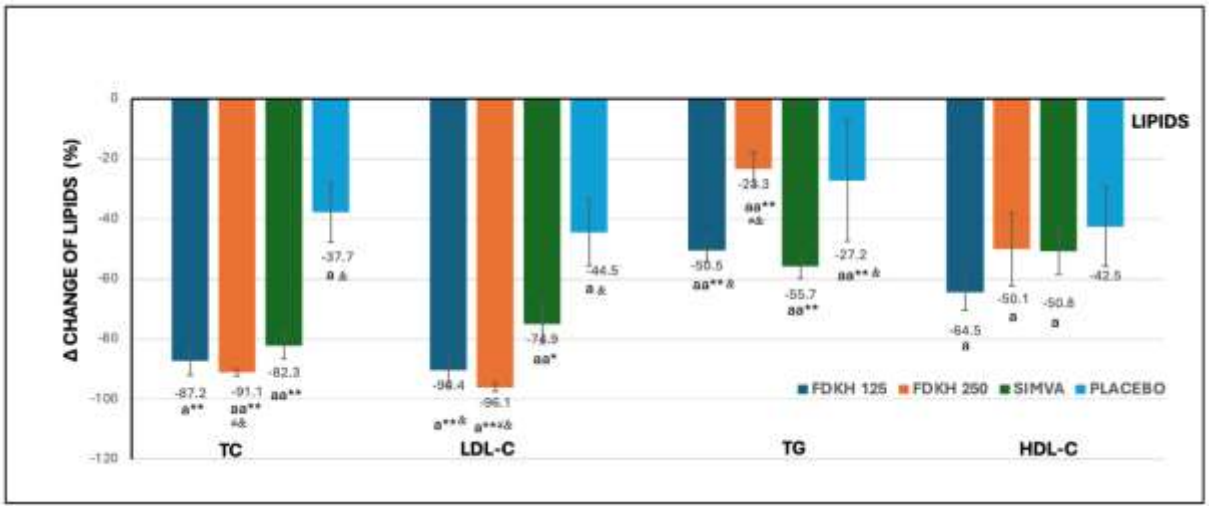

Figure 2 Percentage delta change (% Δ change) of TC, LDL, TG and HDL at week 12 over week 4 for early atherosclerosis group. Data presented as mean ± SEM (n = 4). $^{a}p < 0.05$ and $^{aa}p < 0.01$ indicates significant different between week 12 vs. week 4 in within treatment group; $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to placebo group, $^{\#}p < 0.05$ compared to FDKH125, $^{\&}p < 0.05$ compared to simvastatin.

Fasting serum lipid on established atherosclerotic group

Figure 3 illustrated the percentage (%) delta change (Δ) of TC, LDL-C, TG and HDL levels post-treatment at week 16. Except for placebo group, all other treatment group showed reduction of TC, LDL, TG (only FDKH 250 and HDL (FDKH 250 and simvastatin) at week 16 compared to week 8. However, the degree of reduction for each group was different depending on the type of treatment and presented as percentage (%) delta change (Δ) as shown in **Figure 3**.

There were significantly higher % Δ TC, LDL-C and TG reduction following 8 weeks treatment with FDKH 125 mg/kg (% Δ TC: −86.4 ± 8.5 %, Δ LDL-C: −61.0 ± 19.1 %, Δ TG: −45.2 ± 1.9), FDKH 250 mg/kg (Δ TC: −87.5 ± 2.6 %, Δ LDL-C: −78.9 ± 10.3 %, Δ TG: −78.5 ± 7.7) and simvastatin (Δ TC: −69.7 ± 22.8 %, Δ LDL-C: −56.6 ± 21 %, Δ TG: −62.8 ± 2.2) compared to placebo.

FDKH 250 had significantly higher % Δ TC, LDL and TG reduction compared to FDKH 125 ($p < 0.05$). In terms of comparison with simvastatin, interestingly, FDKH 250 had higher % Δ TC, LDL and TG reduction compared to simvastatin ($p < 0.05$). FDKH 125 exhibited significantly lower % Δ TG reduction compared to Simvastatin ($p < 0.05$).

In overall, treatment with FDKH 250 mg/kg indicated as the highest % Δ TC, LDL-C and TG reduction among all other treatment group. It was followed by treatment with FDKH 125 (except for TG) and simvastatin (except for TG) and most importantly there were still higher than the placebo group.

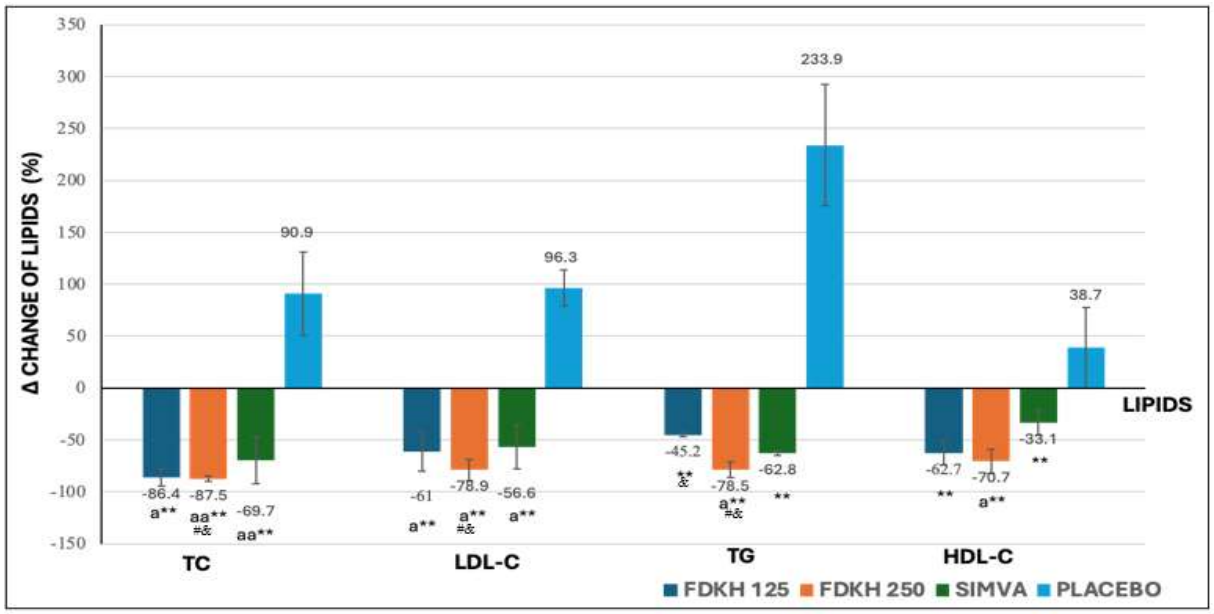

Figure 3 Percentage delta change (% Δ change) of TC, LDL, TG and HDL at week 16 over week 8 for established atherosclerosis group. Data presented as mean ± SEM (n = 4). [a] $p < 0.05$ and [aa] $p < 0.01$ indicates significant different between week 16 vs. week 8 in within treatment group; [*] $p < 0.05$ and [**] $p < 0.01$ compared to placebo group, [#] $p < 0.05$ compared to FDKH125, [&] $p < 0.05$ compared to simvastatin.

Atherogenic index in early atherosclerosis group

Table 5 illustrates atherogenic index (AI) of each group at baseline (B0), week 4 (W4) and week 12 (W12) in early atherosclerosis rabbits. Administration of HCD for 4 weeks had significantly increased AI value in all treatment group to induce early atherosclerosis in rabbits. At week 12 (W12), FDKH 125, FDKH 250 and simvastatin had significantly lower AI value than week 4 (W4). All treatment group had significantly lower AI compared to placebo at W12. FDKH 250 had significantly lower AI compared to FDKH 125 ($p < 0.05$). In terms of comparison with simvastatin, interestingly, FDKH 250 had comparable AI with simvastatin. However, FDKH 125 had significantly higher AI compared to Simvastatin ($p < 0.05$). In overall, treatment with FDKH 250 mg/kg indicated as the lowest AI value among all other treatment group. It was followed by treatment with simvastatin and FDKH 125 mg/kg and most importantly there were still lower than the placebo group.

Table 5 Atherogenic index of each treatment group at Baseline (B0), week 4 and week 12 in early atherosclerosis rabbits.

Groups	Early Atherosclerosis		
	B0	W4	W12
FDKH 125	1.18 ± 0.16 [aa]	10.38 ± 1.15	4.67 ± 2.29 [aa**&]
FDKH 250	0.87 ± 0.25 [aa]	10.65 ± 3.37	2.34 ± 0.71 [a**#]
Simvastatin	1.54 ± 0.68 [a]	5.05 ± 2.15	2.93 ± 0.87[a**]
Placebo	3.37 ± 1.09 [a]	5.72 ± 0.7	6.63 ± 3.17[&]

Data expressed in mean ± SEM (n = 4). [a]$p < 0.05$ and [aa]$p < 0.01$ compared to W4 in within treatment group. [*]$p < 0.05$ and [**]$p < 0.01$ compared to placebo group, [&] $p < 0.05$ compared to Simvastatin, [#] $p < 0.05$ compared to FDKH 125.

Atherogenic index value in established atherosclerosis group

Table 6 illustrates atherogenic index (AI) value of each group at baseline (B0), week 8 (W8) and week 16 (W16) in established atherosclerosis rabbits. Administration of HCD for 8 weeks had significantly increased AI value in all treatment group compared to B0 ($p < 0.01$). Treatment with FDKH at 125 mg/kg (FDKH 125) significantly cause reduction in AI value after 8 weeks treatment period (W16) compared to W8. At W16, it is noted that, FDK 125 ($p < 0.01$) and simvastatin ($p < 0.001$) had significantly lower AI value than placebo. FDKH 125 had significantly lower AI compared to FDKH 250 ($p < 0.05$). In terms of comparison with simvastatin, interestingly, FDKH 125 had comparable AI with simvastatin. However, FDKH 250 had significantly higher AI compared to Simvastatin ($p < 0.05$). In overall, treatment with FDKH 125 mg/kg indicated as the lowest AI value among all other treatment group. It was followed by treatment with simvastatin and FDKH 250 mg/kg and most importantly there were still lower than the placebo group.

Table 6 Atherogenic index of each treatment group at Baseline (B0), week 8 (W8) and week 16 (W16) in established atherosclerosis rabbits.

Groups	Established Atherosclerosis		
	B0	W8	W16
FDKH 125	1.11 ± 0.24^{aa}	15.35 ± 1.1	$3.4 \pm 1.2^{a**}$
FDKH 250	0.93 ± 0.16^{aa}	14.78 ± 2.7	$5.5 \pm 0.4^{\#\&}$
Simvastatin	1.35 ± 0.32^{aa}	22.9 ± 5.7	$4.8 \pm 1.7^{a**}$
Placebo	1.08 ± 0.15^{a}	2.58 ± 1.8	6.2 ± 0.8

Data expressed in mean ± SEM (n = 4). $^{a}p < 0.05$ and $^{aa}p < 0.01$ compared to W8 in within treatment group. $^{**}p < 0.01$ compared to placebo group, $^{\#}p < 0.05$ compared to FDKH 125, $^{\&}p < 0.05$ compared to Simvastatin.

Discussion

Development of robust and efficient quantitative and qualitative method to standardise crude botanicals against bioactive markers to ensure their therapeutic consistency, extract quality and safety of phytochemicals constituents is one of the essential steps in food and health supplement industry [24]. Extract standardisation can be done by various phytochemical assays such as total phenolic and total flavonoids content. However, to guarantee the therapeutic consistency of plant extract, quantitation analysis of bioactive components using modern spectroscopic techniques are known to offers better accuracy, feasibility and repeatability [25]. Optimizing the pH and composition of the mobile phase have always been the hallmark to achieve reasonable selectivity especially when separating phenolic components in reverse phase chromatography [26]. In this study, a slightly acidic mobile phase systems (0.1 %) performed very well as compared to higher pH of mobile phases. This finding is supported by a fact where reverse-phase chromatography is often accompanied by lower pH of mobile phase to ensure ionisation equilibrium of solute and mobile phase [27]. Similarly, this present study demonstrates that vitexin and isovitexin are better ionised with better selectivity in negative mode which is in agreement with previous study [21]. The application of MRM in this method lowers LOD values and allowed vitexin and isovitexin to be distinguished and quantified as previously describe in another study by [28].

The standardisation of FDK against vitexin and isovitexin using proposed LC-MS/QQQ method demonstrated excellent linearity ($R^2 > 0.99$) and good reliability of the proposed method at 5 calibration points [11]. The low values of LOD and LOQ for vitexin and isovitexin attests that the proposed method is reliable to determine and separate very low concentration of vitexin and isovitexin. Considering the low concentration range of analyte (ng/mL), RSD values for both intra (< 2.37 %) and inter-day (< 6.61 %) assays are within acceptable range indicating a good reproducibility and laboratory certainty [29]. The

slightly higher RSD value (6.61 %) of LQC (15 ng/mL) for isovitexin could be affected by the variations in experimental conditions such as reagents, column efficiency and operator skill [28,29]. All recoveries are within acceptable range of 70 - 120 % except for HCQ of isovitexin (126.25 ± 5.53 %). The possible explanation was the sample preparation and dilution method could cause interference to the sample [30].

Development of atherosclerosis involves different states of lesion leading to manifestation of plaque. Early stage of atherosclerosis involves fatty streak formations starting with accumulation of LDL-C in the vascular intima, activation of endothelial and monocytes cells and eventually leads to formation of foam cells [31]. As the process progresses to established atherosclerosis, smooth muscle cells migrate to the luminal side of vessel wall which causes severe damage to vascular tissue, resulting in atherosclerosis plaque, and reduce blood flow [32]. To enhance the validity of our hyperlipidemia model, we incorporated the approach described by Lu *et al.* [33], which combines a high-fat diet with cryofluid-induced endothelial injury to establish a rabbit model of atherosclerotic vulnerable plaque. This method effectively simulates the necessary conditions for atherosclerosis development, providing a more comprehensive understanding of the disease mechanism. In addition, prolonged LDL in the blood can increase oxidative stress, leading to endothelial injury and inflammation. Oxidized LDL (oxLDL) enters the tunica intima due to increased endothelial permeability, contributing to atherosclerosis. In this study, we induced hypercholesterolemia (HC) through a high-cholesterol diet for 4 weeks to induce early atherosclerosis and for 8 weeks to establish advanced atherosclerosis, as previously reported [15].

The results of this study provide compelling evidence for the efficacy of FDKH in reducing serum lipid levels and improving atherogenic index (AI) in both early and established atherosclerotic models. The significant reductions in total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and triglycerides (TG) observed in the FDKH treatment groups highlight its potential as a potent lipid-lowering agent.

In the early atherosclerotic group, FDKH 250 mg/kg demonstrated the highest percentage reductions in TC and LDL-C levels, outperforming both FDKH 125 mg/kg and simvastatin. This suggests that a higher dosage of FDKH is more effective in managing cholesterol levels. Interestingly, FDKH 125 mg/kg showed the greatest reduction in TG levels, indicating that different dosages of FDKH may have varying effects on different lipid parameters. Simvastatin, while effective, was generally outperformed by FDKH 250 mg/kg in terms of TC and LDL-C reduction but showed the highest reduction in TG levels. This highlights the potential of FDKH as a superior alternative to traditional statin therapy, particularly for patients who require significant reductions in TC and LDL-C.

In the established atherosclerotic group, FDKH 250 mg/kg again demonstrated the highest reductions in TC, LDL-C and TG levels, confirming its efficacy in more advanced stages of atherosclerosis. The significant reductions in AI observed with FDKH 250 mg/kg further support its potential in improving overall cardiovascular health. FDKH 125 mg/kg also showed notable efficacy, particularly in reducing TG levels, although it was less effective than FDKH 250 mg/kg in reducing TC and LDL-C.

A study on the correlation between dietary fat quality indices and lipid profile with AI in obese and non-obese volunteers found that higher AI was associated with poor dietary fat quality [34]. This aligns with our findings that significant reductions in AI can be achieved through effective lipid-lowering treatments like FDKH. Another study comparing calculated lipid indices, including AI, with conventional lipid profile parameters in predicting atherosclerosis in obesity, highlighted the importance of precise measures beyond routine lipid parameters [35]. Our study supports this by demonstrating that FDKH significantly improves AI, a crucial marker for cardiovascular risk. The differential effects of FDKH dosages on lipid parameters suggest that personalized treatment regimens could be developed to optimize therapeutic outcomes. For instance, patients with elevated TG levels might benefit more from FDKH 125 mg/kg, while those with high TC and LDL-C levels might achieve better results with FDKH 250 mg/kg.

Other finding from this study is that progression of early atherosclerosis is partially reversible with lifestyle intervention which justifies the amelioration of lipid profile upon conversion from high-cholesterol diet (HCD) to normal diet (ND) [36]. Contrary, the lipid

profile of placebo group in established atherosclerosis group continue to increase after diet conversion can be explain by the severe plaque accumulation in the endothelium caused by the prolong administration of 1 % HCD for 8 weeks in established group as report in our previous study [15]. Excess TC and LDL-C are the known to be the central aetiology to the evolution of endothelium plaque lesion [37]. In this study, FDKH at 250 mg/kg shows the highest TC (96.1 and −87.5 %) and LDL-C % (−91.1 and −78.9 %) % Δ change reduction as compared to other treatment groups in both early and established atherosclerosis rabbits, respectively. Our previous study reported that FDK had a neutral effect on the lipid profile in both early and established high-cholesterol-diet (HCD)-induced rabbits [15]. This led to the development of an improved formulation of FDK to enhance its efficacy in lipid reduction. Findings from this present study applauded the encapsulation of FDK in a hydrogel formulation enhanced its stability, possibly due to improved gastrointestinal absorption of vitexin and isovitexin. A previous study in humans reported that FD leave extract significantly decreased total and LDL cholesterol concentrations in healthy normolipidemic adults with pre-diabetes [38]. In contrast, our study investigates the effects of FDK in hypercholesterolaemia condition.

HDL-C plays important role in reverse cholesterol transport (RCT) by inhibiting the formation of foam cells and prevent vascular endothelial oxidation [39]. In summary, our findings suggest that treatment with FDKH does not significantly alter high-density lipoprotein (HDL) levels in rabbits fed a high-cholesterol diet. This neutral effect indicates that while FDKH exhibit TC, LDL and TG levels reduction, its impact on HDL levels in the context of a high-cholesterol diet appears to be minimal. A possible mechanism by which FDK attenuates cholesterol biosynthesis is through the reduction of cholesterol absorption, achieved by enhancing bile acid excretion in hypercholesterolemic rats [8].

The lipid lowering effects of FDK are suggested to be contributed by the presence of vitexin and isovitexin through various mechanisms. It has been reported that vitexin and isovitexin, both flavone C-glycosides that are present in FDK have shown promising lipid-lowering effects through activation of AMP-activated Protein Kinase (AMPK) by decreasing lipogenesis, increasing fatty acid oxidation thus reducing lipid levels. Vitexin has been shown to increase the expression of PPAR-γ, and reducing lipid accumulation in the liver. Vitexin are also reported to reduce the HMG coA reductase activity, a regulatory enzyme for cholesterol synthesis in the rats fed on HFAD [40]. Isovitexin has been reported to inhibit lipase activity for tryglycerides hydrolysis [41]. Many studies have reported Vitexin and isovitexin is a potent antioxidant and anti-inflammatory agents, however more study are needed to investigate its lipid lowering property and mechanism, especially *in vivo* [42].

In this study, we have reported the lipid lowering property by FDKH hydrogel. There are several natural products that exhibit lipid lowering effects including soy bean and flexseed [43]. Plant sterols and stanols have been shown to reduce LDL levels. In this study, FDK was incorporated in the hydrogel system to improve its bioavailability and enhance its efficacy for lipid lowering. Hydrogel formulations, particularly those incorporating lipid nanoparticles, have been explored for their potential in drug delivery systems. Recent studies have highlighted the effectiveness of lipid nanoparticle-hydrogel composites in enhancing the delivery and efficacy of lipophilic drugs [44]. Previously, curcumin incorporated into hydrogel was developed and showed improved drug released in alkaline conditions, highlighting its potential for target delivery in GIT [45]. Curcumin loaded with chitosan encapsulated curcumin has been reported in total cholesterol level reduction in streptozotocin-induced type-1 diabetes in mice, but neutral effects with TG and VLDL [46]. Interestingly, our study showed reduction in TC, LDL and TG with FDKH hydrogel formulation. The advantage of hydrogels is the prolonged release profile of the carried substance (natural product) [47].

New Zealand White (NZW) rabbits are chosen as the model of HCD induced atherosclerosis in this study. The reasons being is that NZW rabbits are often considered a superior model for studying atherosclerosis compared to other animal such as rats and mice. Their lipid metabolism closely resembles that of humans, leading to the development of hyperlipidaemia and atherosclerotic lesions when fed a high-cholesterol diet. These lesions are more comparable to human ones in size and composition, making the rabbits a more relevant model for research. Their plasma lipoprotein

profiles, particularly the distribution of LDL and HDL, are also similar to humans, which is crucial for studying atherosclerosis. Furthermore, these rabbits provide consistent results in the induction and evaluation of atherosclerosis, making them a reliable model for research [48]. In contrast, other animal such as rats do not develop atherosclerosis as readily and their lipid metabolism differs significantly from humans, making them less ideal for this specific type of research [49].

In our study, we evaluated atherogenic index (AI) value as an additional parameter to reflect the presence of cholesterol transport from peripheral cells to the liver for irreversible disposal [20]. Kazemi *et al.* [20] reported the positive correlation of AI with TC, LDL and TG and suggested that AI may become as a strong marker for predicting the risk of atherosclerosis and CVD. In our present study, Both FDKH treatment groups showed significant reduction of AI. This data justifies the potential efficacy of FDKH in improving diet-induced metabolic disturbances such as hyperlipidaemia, diabetes mellitus and metabolic syndrome.

Conclusions

The study successfully standardized Ficus deltoidea var kunstleri (FDK) extract using a validated LC-MS/QQQ method to quantify vitexin and isovitexin. This is the first study to demonstrate the serum lipid reduction effects of FDK incorporated into a hydrogel system (FDKH) in both early and established atherosclerosis rabbits. Treatment with FDKH 250 mg/kg resulted in the most significant reductions in total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and atherogenic index (AI) in both groups. FDKH 125 mg/kg also showed notable reductions, particularly in triglycerides (TG) for the early atherosclerotic group. Simvastatin, while effective, was generally outperformed by FDKH 250 mg/kg in terms of TC and LDL-C reduction but showed the highest reduction in TG levels. These findings suggest that FDKH, especially at the 250 mg/kg dosage, is highly effective in reducing lipid levels and improving AI in both early and established atherosclerosis.

Given these promising results, FDKH could be further developed as a clinical treatment for atherosclerosis. Its significant impact on lipid levels and AI suggests it could be a valuable addition to current therapeutic options. However, further clinical trials are essential to confirm its efficacy and safety in humans. These trials should focus on long-term outcomes, potential side effects, and optimal dosing strategies. Future studies should investigate specific mechanistic pathways, including cholesterol metabolism, lipoprotein metabolism, triglyceride metabolism, inflammatory pathways, oxidative stress pathways and liver function related to lipid regulation. Understanding these pathways could provide insights into FDKH's broader therapeutic potential and optimize its clinical use.

Human trials should be structured to evaluate the effectiveness of FDKH in diverse populations, considering factors such as age, gender and comorbid conditions. These trials should also compare FDKH directly with existing lipid-lowering drugs to establish its relative efficacy and safety. Beyond its potential as a pharmaceutical treatment, FDKH could be developed for the nutraceutical market, offering a natural alternative to traditional lipid-lowering drugs. Its benefits over conventional treatments, such as potentially fewer side effects and a natural origin, could make it an attractive option for health-conscious consumers. This could open new avenues for market development and provide additional options for managing lipid levels and atherosclerosis.

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