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Phytochemicals investigation, hypocholesterolemic and anti-atherosclerotic effects of *Amaranthus viridis* leaf extract in hypercholesterolemia-induced rabbits

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Abstract

Hypercholesterolemia is one of the main causes for coronary heart disease which occurs due to high level of serum cholesterol. Oxidized LDL accumulation leads to atherosclerotic plaque formation which contributes to myocardial infarction and cardiovascular diseases. Consumption of statins lead to adverse health effects such as liver and muscle toxicity, thus attention is now focused on alternative treatment using plant origin. This study is designed to investigate the phytochemical components, hypocholesterolemic and antiatherosclerotic effects of *Amaranthus viridis* (*A. viridis*) using hypercholesterolemic rabbits. Gas Chromatography Mass Spectrometry (GC-MS/MS) analysis revealed 30 compounds while Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) detected the presence of ascorbic acid, rutin, quercetin and catechin. Animal model study was performed on twenty New Zealand white rabbits that were randomly divided into 5 groups and fed with normal diet, 2% high cholesterol diet (HCD), 2% HCD + 10 mg kg^{-1} simvastatin, 2% HCD + 100 mg kg^{-1} *A. viridis* extract and 2% HCD + 200 mg kg^{-1} *A. viridis* extract, respectively. The supplementation with *A. viridis* extract significantly reduced total cholesterol, LDL and triglycerides levels, and increased the levels of HDL and antioxidant enzymes (SOD and GPx). The elevated levels of AST, ALT and creatine kinase indicate liver and muscle injuries. Treatment with *A. viridis* extract also diminished the development of aortic plaque and decreased the intima: media ratio as observed in simvastatin-treated rabbits. The phytocomponents of *A. viridis* have been reported to possess therapeutic effects in treating hypercholesterolemia and atherosclerosis, and the *in vivo* study on *A. viridis* further confirms its potential as an alternative therapeutic agent.

Keywords: *Amaranthus viridis*, phytocomponents, *in vivo* study, alternative treatment, hypercholesterolemia, atherosclerosis

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1. Introduction

Hypercholesterolemia is a condition characterized by increased concentrations of cholesterol particularly low-density lipoprotein (LDL) and triglycerides (TG),[1] which can leads to atherosclerosis, obesity and cancer. Statins are well known synthetic drugs that effective in lowering cholesterol. However, long term usage of statins causes side effects such as liver and muscle damages, and myopathy. Serious side effects such as rhabdomyolysis and acute renal failure may occur if myopathy is not diagnosed and the statins are continued.[2] Thus, usage of natural products developed from medicinal plants was encouraged for treating cardiovascular diseases.[3]

Medicinal plants have been widely claimed on its nutritional values and pharmacological activities such as antioxidant, anti-inflammatory, anti-thrombotic, anti-artherogenic and cardioprotective effects.[4] Phytochemicals are active compounds that naturally exist in plants and known as strong effectors of biological processes. They are capable of lowering the risk of diseases via complementary and overlapping mechanisms. Meanwhile, flavonoids provide significant protections against the progression of chronic illness such as tumor,[5] diabetes[6] and cardiovascular diseases.[7] Flavonoids were demonstrated to decrease LDL oxidation,[8] inhibit lipid peroxidation and suppress the progression of atherosclerotic plaque.[9]

Amaranthus viridis (*A. viridis*) commonly known as green amaranth and "bayam pasir" locally belongs to the family of Amaranthaceae. *A. viridis* is widely found in tropical countries[10] and has been traditionally used to treat respiratory problems, asthma, eczema and psoriasis.[11] In a recent study, *A. viridis* showed high inhibition (about 70 %) on HMG-CoA reductase, a precursor enzyme of cholesterol biosynthesis in liver.[12,13] The prospective of medicinal plants for the prevention and treatment of hypercholesterolemia is still broadly

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unexplored. Alternative therapy for the development of potent and safe hypocholesterolemic agent is highly needed.

The present study was aimed to investigate the phytochemical components, hypocholesterolemic and antiatherosclerotic effects of *Amaranthus viridis* (*A. viridis*) using hypercholesterolemia-induced rabbits. The phytochemicals present in *A. viridis* leaf extract were detected using Gas Chromatography Mass Spectrometry (GC-MS/MS) and Reverse Phase-High Performance Liquid Chromatography (RP-HPLC). The identified phytocomponents have been reported to possess therapeutics effects against hypercholesterolemia and its related cardiovascular diseases. Thus, *in vivo* study on rabbits was performed to further investigate the potential of *A. viridis* as an alternative strategy for the treatment of hypercholesterolemia and atherosclerosis.

2. Materials and methods

2.1. Preparation of *A. viridis* methanol extract

A. viridis leaf was purchased from a local market in Selangor, Malaysia. A voucher specimen was deposited in the Institute of Bioscience, Universiti Putra Malaysia (voucher no. SK 2085/12). *A. viridis* leaf was washed and air dried for overnight at room temperature. The leaf was grounded using a blender (MX 8967, Panasonic) and subjected to methanol 50% (v/v) distillation for 48 hours. The mixture was filtered and isolated using a separatory funnel. The methanolic extract of *A. viridis* leaf was concentrated using rotary evaporator (Heidolph) under reduced pressure at 40 °C and freeze dried at – 40 °C.[12]

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2.2. Phytochemical screening

The phytocomponents of *A. viridis* leaf was determined qualitatively for flavonoids, phenolics, saponins, tannins, alkaloids, diterpenes and steroids. The phytochemical assays were conducted using freeze-dried *A. viridis* leaf extract.

2.2.1. Test for flavonoids

Ethyl acetate (10 mL) was mixed with *A. viridis* extract (0.5 mg) and heated over a steam bath for 3 minutes. The mixture was filtered and the filtrate (4 mL) was shaken with ammonia solution (10%; 1 mL). A yellow coloration indicates the presence of flavonoids.[12]

2.2.2. Test for phenolic content

Folin-ciocalteu reagent (10 fold diluted; 0.75 mL) was mixed with *A. viridis* extract (200 µl; 0.5 mg mL^{-1}). After 5 minutes incubation, sodium carbonate solution (6%; 0.75 mL) was added and the mixture was further incubated for 90 minutes at room temperature. The formation of brown color indicates the presence of phenolic compounds.[14]

2.2.3. Test for saponins

Distilled water (5 mL) was added to *A. viridis* extract (0.5 g) and shaken vigorously. The formation of froth determines the presence of saponins.[15]

2.2.4. Test for tannins

A. viridis extract (0.5 g) was boiled in distilled water (10 mL) and filtered. A few drops of ferric chloride solution (1%) were mixed with the filtrate. The formation of blue black color shows the presence of hydrolysable tannins while brownish green precipitates regarded the presence of condensed tannins.[16]

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2.2.5. Test for alkaloids

A. viridis extract (0.5 g) was partitioned with chloroform and followed by ammoniacal chloroform. Sulphuric acid (10%) was added to the mixture and tested with Mayer's reagent. A white precipitate formation determined the presence of alkaloids.[15]

2.2.6. Test for steroids/triterpenes

Chloroform (1 mL) was mixed with *A. viridis* extract (0.5 mg) followed by addition of a few drops of concentrated sulphuric acid and acetic anhydride. Appearance of blue or green color indicates the presence of steroids while appearance of red or brown color determines the presence of triterpenes. [15]

2.3. Gas chromatography mass spectrometry (GC-MS/MS) analysis

A. viridis leaf extract (1 µl) was analyzed using gas chromatography (TSQ Quantum XLS, Thermo Scientific) which is equipped with a TG-5 MS capillary column (30 m length x 0.25 mm ID x 0.25 µm thickness) and a flame ionization detector (FID). Carrier gas (helium) was used at a constant flow rate of 0.8 mL minute^{-1}. The oven temperature was fixed for 5 minutes at 40 ºC and increased 2 ºC minute^{-1} gradually up to 280 ºC. The injector and detector temperatures were maintained at 200 ºC and 250 ºC, respectively. The mass spectrometer was handled in scan mode from m/z 40 to 450 Da and the mass spectra were taken at 70 eV with a 0.7 seconds scan interval. Individual compounds were identified by comparing the obtained mass spectra with the internal references in mass spectra library, NIST (National Institute of Standard and Technology).[17,18]

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2.4. RP-HPLC analysis of standards

Stock solution of ascorbic acid and eight flavonoids standards (rutin, luteolin, catechin, quercetin, apigenin, naringin, myricetin and hesperidin) (Sigma, Missouri, US) were prepared in methanol at various concentrations (0.2 0.4, 0.6, 0.8 and 1.0 mg mL^{-1}). The standards were filtered through membrane filter (0.45 mm; Millipore) before subjected into HPLC separately. The linear calibration curve was plotted at A_{280} as peak area against standard concentration (mg mL^{-1}).[19]

2.4.1 Gradient RP-HPLC

The ascorbic acid and flavonoids compounds in the sample were analyzed using a RP-HPLC method as mentioned by Wang and Helliwell[20] with slight modifications. The HPLC analyses were operated with a 9486 tunable absorbance UV detector, Water 600 pump controller and equipped with an Eclipes XDR-C18 reverse phase column (25 cm x 4.6 mm ID x 5 µm; Supelco, USA) at room temperature. The samples were eluted with gradient elution of mobile phase solvent A [deionized water-trifluoroacetic acid (TFA); pH 2.5] and solvent B (HPLC-grade methanol). The gradient elution was started with 100% solvent A at 0 minute; followed by 70% solvent A: 30% solvent B for the 10 minutes; 50% solvent A: 50% solvent B for 30 minutes and finally with 100% solvent A for 40 minutes. The flow rate was fixed at 1.0 mL $minute^{-1}$ and the injection volume was 20 µl with a post-time of 15 minutes before the next injection. Detection of flavonoids was read at absorbance 280 nm. Identification of flavonoids in the sample was determined by matching the retention time against the standard.

2.5. Animals and experimental design

The animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Universiti Putra Malaysia (UPM/IACUC/AUP-R011/2013). Twenty male New

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Zealand white rabbits (1.5-1.8 kg) were purchased from a local supplier. The rabbits were housed individually in stainless steel cages and were fed standard rabbit pellets for a week for acclimatization. The rabbits were kept in almost constant room temperature at 23-25 ^{o}C with 12 h light-dark cycle throughout the study.

The animal model study was conducted for 12 weeks. The rabbits were randomly divided into the following groups (n=4 in each group). Group 1: control rabbits fed with standard diet (12 weeks); Group 2: rabbits fed with 2% high cholesterol diet (HCD) (12 weeks); Group 3: rabbits fed with 2% HCD (8 weeks) and treated with simvastatin (10 mg kg^{-1}) (4 weeks); Group 4: rabbits fed with 2% HCD (8 weeks) and treated with *A. viridis* leaf extract (100 mg kg^{-1}) (4 weeks) and Group 5: rabbits fed with 2% HCD (8 weeks) and treated with *A. viridis* leaf (200 mg kg^{-1}) (4 weeks).

The high cholesterol diet was prepared by dissolving the cholesterol (2%; USP grade, anhydrous; Sigma Chemical Co, Missouri, USA) in chloroform (99%) and sprayed on the standard pellets. Butylated hydroxyanisole (0.02%) was dissolved in chloroform to reduce the cholesterol oxidation. The diets were exposed in well-ventilated fume hoods for overnight at room temperature to evaporate the chloroform. The diets were then vacuum-packed and stored in -20 ^{o}C freezer. All the rabbits were fed about 150 g pellets per day, with or without cholesterol supplementation and water was accessible *ad libitum*. Food and water intake were recorded on daily basis, while the rabbits were weighed every 2 weeks. Blood samples collection were performed at 0, 4, 8 and 12th week via ear marginal vein using butterfly needle (23-gauge) and syringes (3 mL) into EDTA and heparinised tubes. At the end of the study, the rabbits were euthanized through intravenous injection with overdose of sodium pentobarbital.

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2.5.1. Measurement of serum lipids

The serum levels of total cholesterol (TC), LDL, high-density lipoprotein HDL and TG levels were evaluated using Roche kit (Penzberg, Germany) and measured spectrophotometrically by Hitachi chemistry analyzer (Tokyo, Japan).

2.5.2. Liver and muscle test

The serum levels of alanine transaminase (ALT), aspartate transaminase (AST) and creatine kinase (CK) were determined using enzymatic kit (Randox Laboratories, Crumlin, UK) by Hitachi chemistry analyzer (Tokyo, Japan).

2.5.3. Serum antioxidant activities

Superoxide dismutase (SOD) activity was evaluated using RANSOD kit (Randox Laboratories, Crumlin, UK) by Vitalab Selectra Analyzer (Merck, Darmstadt, Germany). The collected erythrocytes were washed with 0.9% NaCl solution (3 mL) for four times by centrifugation at 1000 x g for 10 minutes. Cold distilled water was added up to 2 mL to the packed erythrocytes, vortexed for 10 seconds and incubated at 4 ^{o}C for 15 minutes. The lysate was diluted with phosphate buffer (pH 7; 0.01 mol L^{-1}) and mixed well. The absorbance of the mixture was measured at 505 nm. The glutathione peroxidase (GPx) activity was determined by diluting serum (0.05 mL) with RANSEL kit diluting agent (2 mL; Randox Laboratories, Crumlin, UK) and the mixture was read at A_{340} using Vitalab Selectra Analyzer (Merck, Darmstadt, Germany).

2.5.4. Histopathological analysis

The rabbits' aortic arches were carefully removed, cleaned and fixed in neutral buffer formalin (10%). The tissues were embedded in paraffin, cut in 5-µm thick sections and

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stained with hematoxylin and eosin. The thickness of intima, media and intima: media ratio of rabbits (n=4) were analyzed under the light microscope equipped with an image analyzer system (Olympus, Germany).

2.5.5. Statistical analysis

The data was expressed as mean ± standard deviation (SD). All the groups were analyzed using SPSS program version 19.0. Multiple comparisons among the groups were determined using one way analysis of variance (ANOVA) followed by Dunnett's *Post hoc* test. The difference between groups was considered statistically significant when $p< 0.05$.

3. Results

3.1. Phytochemical screening

The phytochemical analysis of *A. viridis* extract revealed the presence of medically beneficial components such as phenolic compounds, flavonoids, condensed tannins and saponins while other components like hydrolysable tannins, alkaloids, steroids and triterpenes were not detected (Table 1).

3.2. GC-MS/MS analysis

GC-MS/MS chromatogram of *A. viridis* leaf extract was shown in Figure 1. *A. viridis* leaf extract revealed the presence of 30 phytocomponents. The molecular formula, molecular weight (MW) and peak area (%) of the phytocomponents were summarized in Table 2.

3.3. RP-HPLC analysis

The chromatographic separations of ascorbic acid and eight flavonoid standards (rutin, luteolin, catechin, quercetin, apigenin, naringin, myricetin and hesperidin) by gradient elution

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at concentration of 0.2 mg mL^{-1} was shown in Figure 2. The HPLC chromatogram of *A. viridis* extract is presented in Figure 3. The compounds detected in *A. viridis* leaf were ascorbic acid, rutin, catechin and quercetin. The concentration of ascorbic acid and each flavonoid were determined from the standard calibration curve and presented as the mean of three replicates (Table 3). Ascorbic acid showed the highest concentration (0.812 mg mL^{-1}), followed by rutin (0.399 mg mL^{-1}), quercetin (0.256 mg mL^{-1}) and catechin (0.173 mg mL^{-1}).

3.4. Effect of *A. viridis* extract on body weight

Significant increase ($p < 0.05$) in body weight was noted in rabbits fed with HCD for 12 weeks compared to the normal diet group (Table 4). Treatment with simvastatin and *A. viridis* (100 and 200 mg kg^{-1}) for 4 weeks showed reduction in the body weight compared to the untreated hypercholesterolemic rabbits.

3.5. Effect of *A. viridis* extract on serum lipids

Rabbits fed with 2% cholesterol diet showed significant increase ($p < 0.05$) in TC, LDL and TG levels compared to the normal diet group after 8 weeks (Table 5). Significant reductions ($p < 0.05$) were noted in the levels of serum TC, LDL and TG at week 12, after 4 weeks of treatment with simvastatin and *A. viridis* extract. Administration of simvastatin (10 mg kg^{-1}; positive control) significantly lowered ($p < 0.05$) TC (55.6%), LDL (52.7%) and TG (39.5%) levels. Administration of *A. viridis* extract (100 and 200 mg kg^{-1}) significantly reduced ($p < 0.05$) levels of TC by 44.4% and 49.9%, respectively, LDL by 43.6% and 50.3%, respectively and TG by 35.4% and 38.3%, respectively. The TC, LDL and TG lowering effects of *A. viridis* extract (200 mg kg^{-1}) were statistically similar with simvastatin. *A. viridis* extract at dose of 200 mg kg^{-1} showed better hypocholesterolemic effect than that of 100 mg kg^{-1}. Meanwhile, at week 12, HDL level in untreated hypercholesterolemic rabbits was

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significantly lower ($p < 0.05$) compared to the normal control and treatment groups. Simvastatin and *A. viridis* (100 and 200 mg kg^{-1}) treated groups showed significant increase ($p < 0.05$) in the levels of HDL, 24.3%, 33.4% and 56.2%, respectively. *A. viridis* extracts raised HDL levels more efficiently than simvastatin.

3.6. Assessment of liver and muscle injuries

Significant increase ($p < 0.05$) in the levels of ALT, AST and CK were observed in the hypercholesterolemic control rabbits (Table 6). The treatment with *A. viridis* extracts (100 and 200 mg kg^{-1}) significantly reduced ($p < 0.05$) ALT (29.2% and 33.4%, respectively), AST (31% and 43%, respectively) and CK (26.5% and 27.2%, respectively) levels. Meanwhile, the treatment with simvastatin (10 mg kg^{-1}) revealed significant elevation ($p < 0.05$) in ALT (68.5%), AST (66.1%) and CK (34.7%) levels.

3.7. Assessment of serum antioxidant levels

As presented in Table 7, the hypercholesterolemic control group caused significant decrease ($p < 0.05$) in SOD and GPx levels throughout the experiment. *A. viridis* (100 and 200 mg kg^{-1}) treated groups showed significant increase ($p < 0.05$) in SOD (2.6% and 5.8%, respectively) and GPx (21.4% and 37.5%, respectively) levels. Meanwhile, treatment with simvastatin demonstrated significant reduction ($p < 0.05$) in SOD and GPx, 4.8% and 7.6%, respectively.

3.8. Evaluation of atherosclerotic lesion

The rabbits' aortic intimal surfaces from 5 groups were stained with hematoxylin and eosinas shown in Figure 4. Normal control group showed healthy aorta with intact endothelial lining and uniform thickness. Meanwhile, hypercholesterolemic control group caused notable alteration and thickening in the intimal surface of aorta with the presence of a large

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atheromatous plaque. No plaques were detected in the aortic wall of simvastatin and *A. viridis*-treated groups. The thickness of intima and media, and intima/media ratio of the different groups were summarized in Table 8. The highest value of intima and media thickness, and intima/media ratio was noted in hypercholesterolemic control group while significant reductions ($p < 0.05$) were observed in the thickness of intima and media as well as intima/media ratio in simvastatin and *A. viridis* (100 and 200 mg kg^{-1}) treated groups. The treatment with simvastatin and *A. viridis* (200 mg kg^{-1}) showed no significant difference in the intima/media ratio.

4. Discussion

Phenolic compounds have been reported to possess antioxidant effect[21], increase the capacity of antioxidant in plasma[22] and suppress LDL oxidation.[23] Flavonoids reported to exhibit anti-inflammatory and cardioprotective effect by improving the endothelial function.[24,25] Meanwhile, tannins were claimed to possess antiplatelet[26] and antihypercholesterolemic activities by inhibiting the cholesterol absorption.[27] Condensed tannins are desirable in therapeutic treatment as they do not interfere in the iron absorption compared to hydrolysable tannins that suppress the absorption of iron which may cause anemia.[28] Several reports on saponins showed that they inhibit absorption of cholesterol in the intestine and lower LDL as well as plasma cholesterol levels without altering the concentration of HDL in experimental animal models.[29] In addition, saponins were also reported to lower the risk of atherosclerosis.[30]

GC-MS/MS offers better sensitivity and selectivity compared to GC-MS through the removal of matrix ion interferences by Selected Reaction Monitoring (SRM). SRM provides highly specific identification even in low levels of compounds with high matrix background.[31] The potential components of *A. viridis* leaf extract that possess beneficial effects in treating

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hypercholesterolemia and atherosclerosis were 9,12,15-octadecatrienoic acid (peak area 12.191%); l-(+)-ascorbic acid 2,6-dihexadecanoate (peak area 10.655%); phytol (peak area 4.722%); phenol, 2,6-bis(1,1-dimethylethyl)- (peak area 2.808%); 2-methoxy-4-vinylphenol (peak area 1.429%) and oleic acid, eicosyl ester (peak area 0.921%) as presented in Table 9.

RP-HPLC is a chromatographic method widely used in separation and quantification of phenolic compounds. The compounds separation were carried out with a reversed-phase column. Besides ascorbic acid, three flavonoids (rutin, catechin and quercetin) were detected in RP-HPLC analysis which can be linked with the treatment of hypercholesterolemia and atherosclerosis based on previous reports. Rutin decreases hepatic triglyceride and cholesterol levels, and attenuates antioxidant enzymes (SOD and GPx) activities in obese rats.[43] Voskresensky and Bobyrev[44] demonstrated that rutin delays the progression of hypercholesterolemia and suppresses the atherosclerotic plaque formation in rabbit's aorta. Meanwhile, catechin was proven to protect against myocardial injury, enhance endothelial function and reduce the production of monocyte chemotactic protein-1 (MCP-1) in coronary vascular endothelial cells, which can reduce the risk of atherosclerosis.[45,46] The anti-atherosclerotic effect of quercetin was revealed by Bhaskar et al.[47], notable suppression was observed in the atherosclerotic plaque of hypercholesterolemic rabbits supplemented with quercetin. Thus, this shows that several compounds of *A. viridis* leaf extract detected by GC-MS/MS and RP-HPLC possess beneficial effects against hypercholesterolemia and atherosclerosis. This suggests the potential of *A. viridis* extract as an alternative therapeutic agent in treating hypercholesterolemia and atherosclerosis.

The present study is the first report that investigated the oral administration of *A. viridis* extract in hypercholesterolemia-induced rabbits. Rabbit is known to be a better model than rat or mouse to study on hypercholesterolemia and its related diseases since rabbit's metabolism and lipoprotein profile are much similar to humans.[48] A synthetic

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hypocholesterolemic drug, simvastatin, was used as a positive control as its mechanism in inhibiting HMG-CoA reductase is well-known.[49]

Feeding with HCD caused elevated levels of TC, LDL and TG, which increase peroxidation of lipid and influence the progress of atherosclerosis, as also demonstrated in several studies.[50] Meanwhile, a significant decrease observed in HDL level of hypercholesterolemia-induced rabbits, was also reported by Baskaran et al.[51] Administration of simvastatin and *A. viridis* extract showed reduction in body weight as well as TC, LDL and TG levels, while significant increase was noted in the level of HDL. LDL cholesterol is a primary target in cholesterol-lowering therapy. Deposition of oxidized LDL plays a role in infiltrating and damaging arterial walls, thus leading to the development of atherosclerotic plaque formation. High cholesterol diet has been reported to lower fatty acid oxidation, resulted in the elevation of TG level which is also considered as risk factor for cardiovascular diseases.[52] In present study, *A. viridis* extract (100 and 200 mg kg^{-1}) exhibited beneficial effects by reducing the serum levels of TC, LDL and TG in the treated rabbits. *A. viridis* extract (200 mg kg^{-1}) attenuates the level of cholesterol including LDL and TG as effective as simvastatin.

HDL plays an important role in transporting excess cholesterol from cells back to the liver through reverse cholesterol transport system. HDL also involves in protecting the biological membranes against oxidative damage.[53] Clinical and epidemiological studies have proven that low HDL level leads to atherogenic development,[54] thus therapeutic approaches to raise the level of HDL is widely encouraged.[55] Remarkable elevation in HDL level as shown in *A. viridis*-treated rabbits is a preferable criteria for an ideal hypercholesterolemic and atherosclerotic agent.

The liver is the main organ that responsible in the regulation of cholesterol homeostasis. The activities of liver marker enzymes (ALT and AST) were assessed in order to

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detect the liver injury while CK was evaluated to diagnose the muscle damage. These enzymes were proven to leak into the blood stream when injury occurs in the cell membranes.[56] Significant elevations were observed in the enzymes' levels of hypercholesterolemic control and simvastatin-treated groups compared to the normal rabbits. This indicates that high level of cholesterol and the administration of simvastatin caused liver and muscle injuries.[57,58] In contrast, treatment with *A. viridis* extract for 4 weeks lowered the elevations of ALT, AST and CK suggests its hepatic and muscle-protective effect.

Antioxidant enzymes (SOD and GPx) play crucial roles in enhancing the dismutation of oxygen free radicals and sustaining the physiological concentrations of oxygen and hydrogen peroxide.[59] A significant reduction in SOD and GPx levels was noted in simvastatin-treated and hypercholesterolemic control rabbits. Trocha et al.[60] also reported reduced GPx activity in simvastatin-treated group, which may be due to the diminished antioxidant capacity in the serum of animal model. High cholesterol feeding increases the oxygen radicals and changes the serum antioxidant status *in vivo* that leads to lipid peroxidation.[61] The present study showed that HCD for 8 weeks causes reduction in SOD and GPx activities. Several reports have also demonstrated that hypercholesterolemia attenuates the antioxidant capacity of SOD[62,63] and GPx[64], which can be associated with the increased risk of cardiovascular diseases.[65,66] The result obtained in this study showed that *A. viridis* leaf extract is capable of improving the activities of SOD and GPx in hypercholesterolemia-induced rabbits, the effect may be due to the presence of polyphenolic compounds. Moreover, plant polyphenols are able to regulate the anti-oxidative status by enhancing the activities of antioxidant enzymes.[60] Thus, this suggests that *A. viridis* extract is capable of ameliorating the antioxidant status and could be beneficial in inhibiting lipid peroxidation and managing oxidative damages.

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Oxidized LDL particles are commonly accumulated in subendothelial surface layer. Accumulation of oxidized LDL in the macrophages stimulates proliferation of monocytes, endothelial cells and smooth muscle cells. When the macrophages' scavenging receptor for oxidized LDL is upregulated, it causes formation of foam cell which are the primary component of fatty streaks. This further leads to formation of atheromatous plaque and intimal layer thickening.[67,68] The histological examination of rabbits' aortas is in agreement with the serum biochemical data. The severity of atherosclerotic plaque correlates with the level of hypercholesterolemia as observed in the aorta of hypercholesterolemic control rabbits. Supplementation with simvastatin and *A. viridis* showed a remarkable reduction in intimal thickening and aortic plaque. In addition, no significant difference in intima/media ratio was observed between *A. viridis* (200 mg kg^{-1}) and simvastatin-treated groups. This indicates that *A. viridis* extract (200 mg kg^{-1}) is as efficient as simvastatin in managing atherosclerosis.

The phytocomponents identified through GS-MS/MS and RP-HPLC support the findings from *in vivo* study. The mechanism by which *A. viridis* extract suppresses the atherosclerotic plaque are not known but could be due to its phytocomponents that possess antioxidant, hypocholesterolemic and antiatherosclerotic effects like diminishing oxidative stress, lowering serum cholesterol, inhibiting LDL peroxidation, reducing inflammation and preventing macrophage accumulation. Therefore, it can be inferred that *A. viridis* leaf extract possesses beneficial therapeutic effects against hypercholesterolemia and atherosclerosis.

5. Conclusion

The potential phytochemicals of *A. viridis* leaf extract that can be associated with the treatment of hypercholesterolemia and atherosclerosis are 9,12,15-octadecatrienoic acid; l-(+)-ascorbic acid 2,6-dihexadecanoate; phytol; phenol, 2,6-bis(1,1-dimethylethyl)-; 2-

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methoxy-4-vinylphenol; oleic acid, eicosyl ester; rutin; quercetin and catechin. In present study, *A. viridis* extract has been proven to lower the levels of TC, LDL and TG, and enhance the HDL and antioxidant enzymes (SOD and GPx) levels. *A. viridis* extract showed non toxic and protective effects on liver and muscle by reducing the levels of AST, ALT and creatine kinase. The atherosclerotic plaque formation in hypercholesterolemic rabbits was successfully inhibited by *A. viridis* extract. Therefore, the *in vivo* study further proved the potential of *A. viridis* leaf extract as an effective alternative strategy for hypercholesterolemia and atherosclerosis. Further study can be carried out on the mechanisms of *A. viridis* extract in suppressing the aortic plaque formation. Investigations on the bioactive compounds of *A. viridis* leaf extract could be useful in the development of prophylactic agent against hypercholesterolemia and atherosclerosis.

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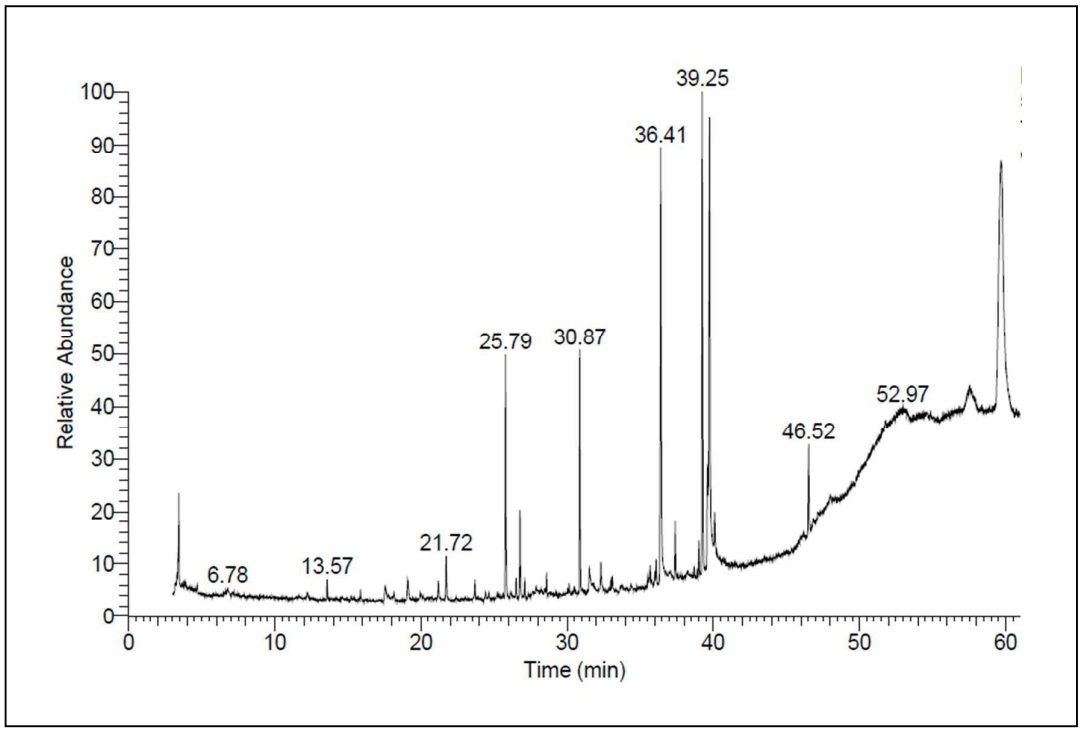

Fig. 1 GCMS/MS chromatogram of *A. viridis* leaf extract.

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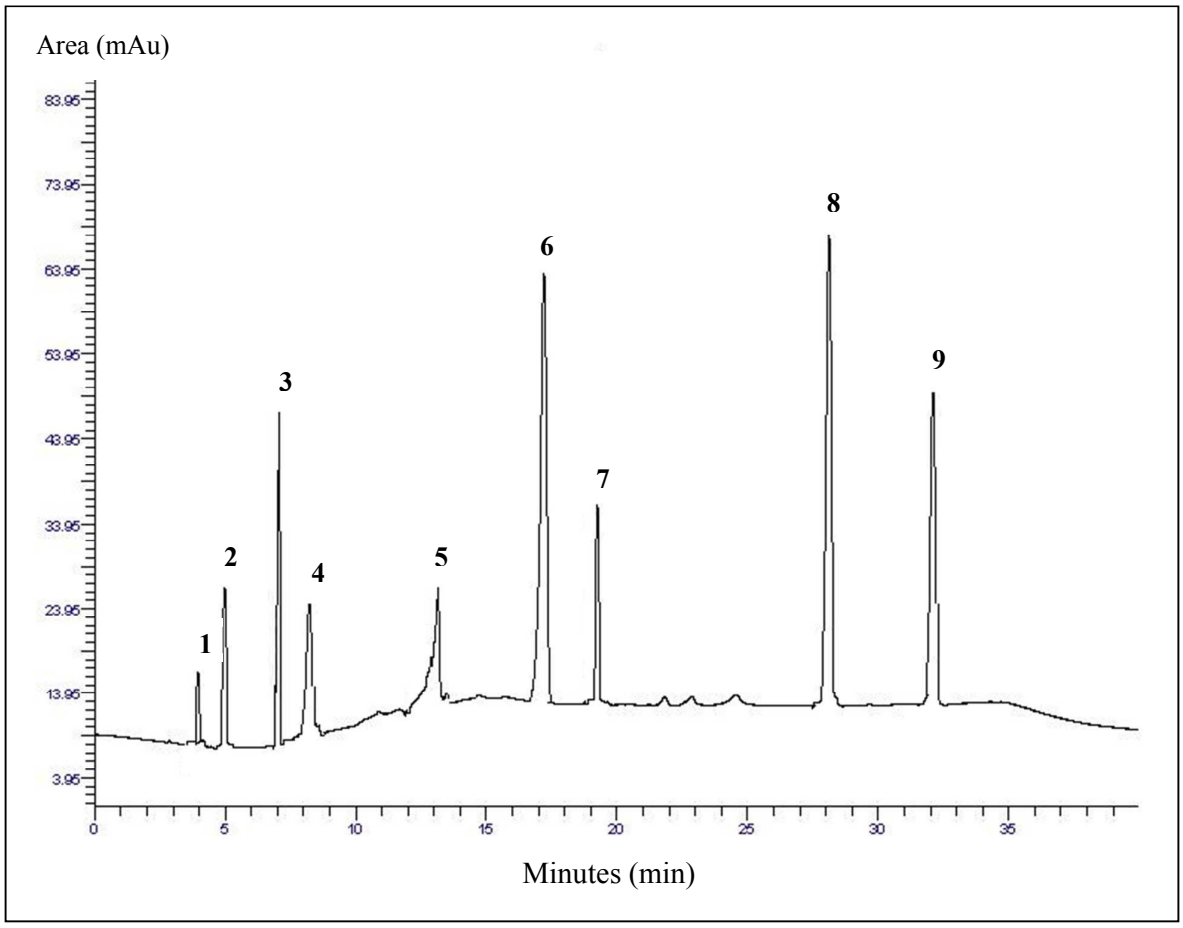

Fig. 2 HPLC chromatogram for ascorbic acid and flavonoids standards at 280 nm: (1) ascorbic acid, (2) rutin, (3) luteolin, (4) catechin, (5) quercetin, (6) apigenin, (7) naringin, (8) myricetin and (9) hesperidin.

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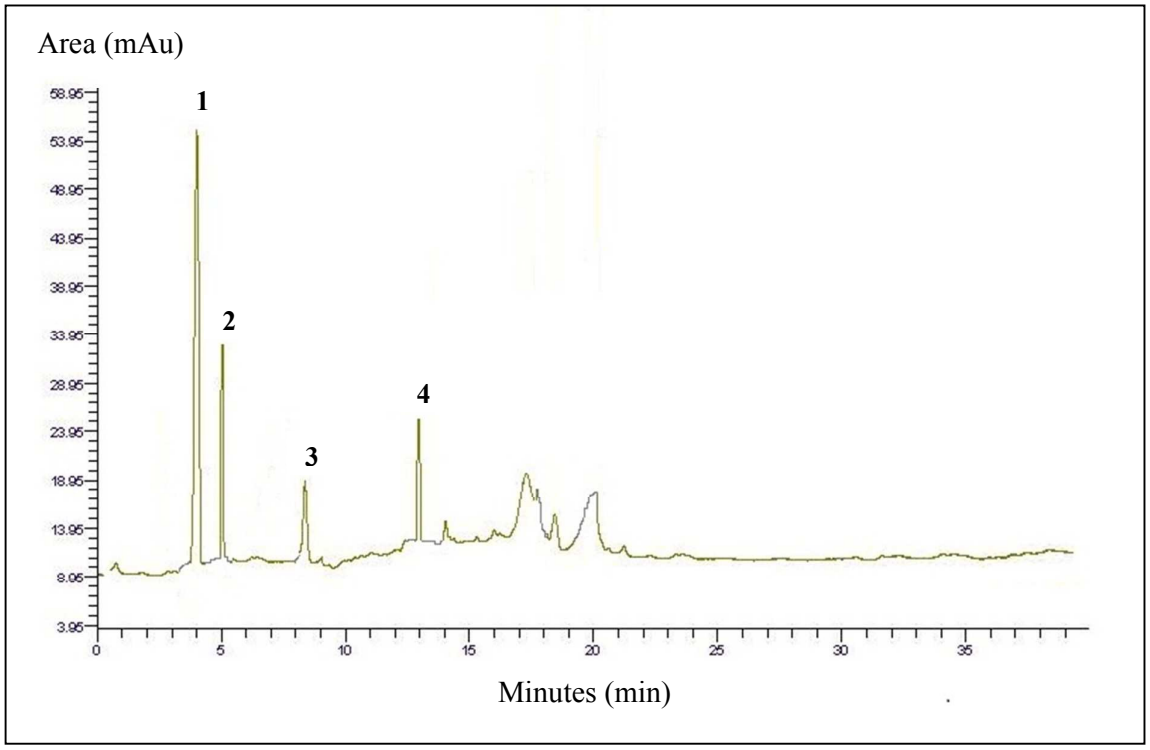

Fig. 3 HPLC chromatogram of *A. viridis* leaf extract at 280 nm: (1) ascorbic acid, (2) rutin, (3) catechin and (4) quercetin.

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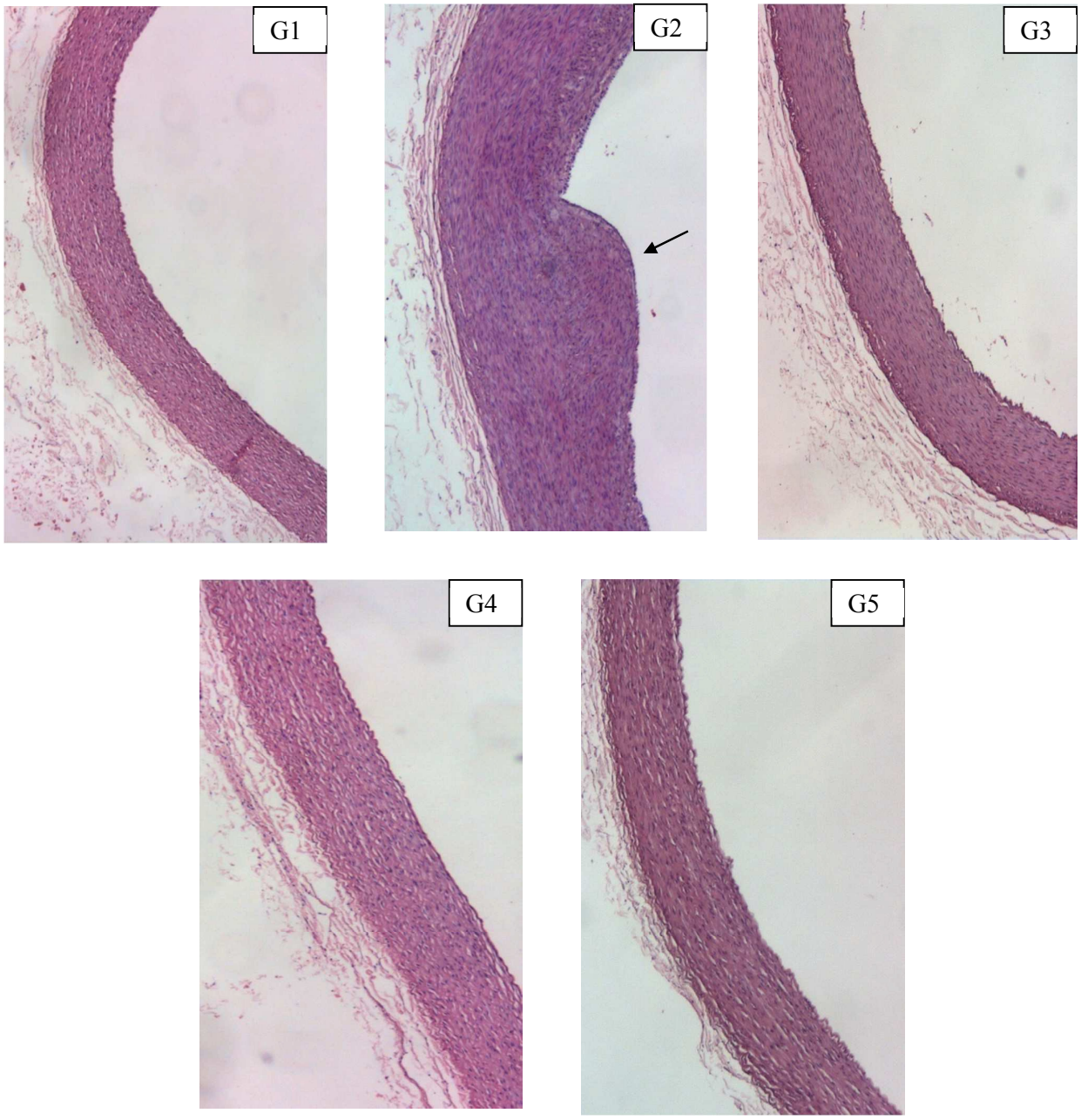

Fig. 4 Representative photographs of rabbits' aorta from 5 groups stained with hematoxylin and eosin. The aortic arch of a control hypercholesterolemic rabbit (G2) showing a large intimal plaque (arrow). G1: normal control, G2: hypercholesterolemic control, G3: simvastatin (10 mg kg^{-1}) treated, G4: *A. viridis* extract (100 mg kg^{-1}) treated, G5: *A. viridis* extract (200 mg kg^{-1}) treated. (Magnification 50x)

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Table 1 Qualitative analysis of phytochemical components of *A. viridis* leaf extract

Phytochemical components	Results
Phenolic	+
Flavonoids	+
Hydrolysable tannins	-
Condensed tannins	+
Saponins	+
Alkoloids	-
Steroids	-
Triterpenes	-

(+: detected, - : not detected)

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Table 2 GC-MS/MS analysis of phytocomponents identified in the *A. viridis* leaf

No.	RT	Compound Name	Molecular Formula	MW	Peak Area (%)
1.	3.42	Allantoic acid	$C_4H_8N_4O_4$	176	0.466
2.	17.55	1H-2,8a-Methanocyclopenta[a]cyclopropa[e]cyclodecen-11-one, 5,6-bis (benzoyloxy)-1a,2,5,5a,6,9,10,10a-octahydro-5a-hydroxy-4-(hydroxymethyl) -1,1,7,9-tetramethyl-,[1aR-(1aà,2à,5á,5aá, 6á,8aà,9à,10aà)]-	$C_{34}H_{36}O_7$	556	0.828
3.	19.10	Benzofuran, 2,3-dihydro-	C_8H_8O	120	0.817
4.	21.20	Tridecanoic acid, 3-hydroxy-, ethyl ester	$C_{15}H_{30}O_3$	258	0.443
5.	21.72	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	150	1.429
6.	25.80	1-Dodecanol	$C_{12}H_{26}O$	186	1.352
7.	26.51	2-Thiophenecarboxylic acid, 5-nonyl-	$C_{14}H_{22}O_2S$	254	0.478
8.	26.78	Phenol, 2,6-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	206	2.808
9.	27.09	Bicyclo[3.2.2]nonane-1,5-dicarboxylic acid, 5-ethyl ester	$C_{13}H_{20}O_4$	240	0.490
10.	27.90	Octadecanoic acid, 4-hydroxy-, methyl ester	$C_{19}H_{38}O_3$	314	0.521
11.	30.14	N,N'-Bis(Carbobenzyloxy)-lysine methyl(ester)	$C_{23}H_{28}N_2O_6$	428	0.517
12.	30.87	3-Chloropropionic acid, heptadecyl ester	$C_{20}H_{39}ClO_2$	346	2.081
13.	31.54	1H-Cyclopenta(b)quinoline, 2,3,5,6,7,8-hexahydro-9-amino-	$C_{12}H_{16}N_2$	188	2.322
14.	32.32	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	0.676
15.	33.10	Cyclobuta[1,2:3,4]dicyclooctene-1,7(2H, 6bH)-dione, dodecahydro-, (6aà,6bà,12aà,12bá)-	$C_{16}H_{24}0_2$	248	0.810
16.	33.70	Heptanoic acid, docosyl ester	$C_{29}H_{58}O_2$	438	0.675
17.	35.68	3-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296	0.800
18.	36.10	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	$C_{18}H_{28}O_3$	292	0.936
19.	36.41	l-(+)-Ascorbic acid 2,6-dihexadecanoate	$C_{38}H_{68}O_8$	652	10.655
20	37.39	Propanoic acid, 3-mercapto-, dodecyl ester	$C_{15}H_{30}O_2S$	274	0.606
21.	39.25	Phytol	$C_{20}H_{40}O$	296	4.722
22.	39.74	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	$C_{18}H_{30}O_2$	278	12.191
23.	40.10	Octadecanoic acid	$C_{18}H_{36}O_2$	284	1.529
24.	45.99	9,12,15-Octadecatrienoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester, (Z,Z,Z)-	$C_{27}H_{52}O_4Si_2$	496	1.074
25.	46.52	1,3-Dioxane, 5-(hexadecyloxy)-2-pentadecyl-, trans-	$C_{35}H_{70}O_3$	538	2.591

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26.	48.00	Oleic acid, eicosyl ester	$C_{38}H_{74}O_2$	562	0.921
27.	51.77	4H-Cyclopropa[5',6']benz[1',2':7,8]azulene no[5,6-b]oxiren-4-one, 8-(acetyloxy)-1,1a,1b,1c,2a,3,3a,6a,6b,7, 8,8a-dodecahydro-3a,6b,8a-trihydroxy-2a -(hydroxymethyl)-1,1,5,7-tetramethyl-, (1aà,1bá,1cá,2aá,3aá,6aà,6bà,7à,8á,8aà)-	$C_{26}H_{34}0_{11}$	522	1.019
28.	53.02	7,8-Epoxylanostan-11-ol, 3-acetoxy-	$C_{32}H_{54}O_4$	502	3.418
29.	57.54	1-Monolinoleoylglycerol trimethylsilyl ether	$C_{27}H_{54}O_4Si_2$	498	4.520
30.	59.69	Propanoic acid, 3,3'-thiobis-, didodecyl ester	$C_{30}H_{58}O_4S$	514	36.988

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Table 3 Compounds detected in *A. viridis* leaf extract

No	Compound	Retention time (RT)	Area	Concentration (mg ml^{-1})
1	Ascorbic acid	4.12 ± 0.031	618460 ± 529.14	0.812 ± 0.0041
2	Rutin	5.07 ± 0.024	318310 ± 1285.23	0.399 ± 0.0023
3	Catechin	8.51 ± 0.037	121357 ± 727.27	0.173 ± 0.0038
4	Quercetin	13.40 ± 0.011	200567 ± 992.03	0.256 ± 0.0026

All data are expressed as Mean ± SD

Table 4 Body weight of rabbits between different groups

Group	Body weight (kg)		
	Initial	Final	Change
G1	1.55 ± 0.13	2.10 ± 0.07	0.55 ± 0.17
G2	1.68 ± 0.19	2.63 ± 0.15	0.95 ± 0.12*
G3	1.73 ± 0.14	2.45 ± 0.19	0.72 ± 0.14
G4	1.61 ± 0.11	2.38 ± 0.09	0.77 ± 0.07
G5	1.65 ± 0.09	2.39 ± 0.11	0.74 ± 0.21

G1: normal control, G2: hypercholesterolemic control, G3: simvastatin (10 mg kg^{-1}) treated, G4: *A. viridis* extract (100 mg kg^{-1}) treated, G5: *A. viridis* extract (200 mg kg^{-1}) treated. All data are presented as the mean ± SD (n=4 for each group). *Significantly different from others ($p < 0.05$).

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Table 5 Serum total cholesterol, LDL, triglycerides and HDL levels of rabbits from various groups

	G1	G2	G3	G4	G5
Total cholesterol (mg dl^{-1})					
Baseline	37.25 ± 3.10^{a}	39.55 ± 3.38^{a}	41.65 ± 2.35^{a}	40.04 ± 2.38^{a}	41.32 ± 4.33^{a}
Week 4	41.12 ± 3.54^{a}	456.08 ± 4.54^{b}	463.45 ± 3.42^{b}	476.23 ± 7.37^{b}	466.68 ± 3.65^{b}
Week 8	45.51 ± 4.46^{a}	$1089.23 \pm 13.22^{b,c}$	1112.70 ± 18.23^{c}	1002.53 ± 13.51^{b}	1038.85 ± 9.35^{b}
Week 12	51.27 ± 2.80^{a}	1399.26 ± 11.29^{d}	494.16 ± 8.67^{b}	557.35 ± 6.34^{c}	520.14 ± 8.32^{b}
LDL level (mg dl^{-1})					
Baseline	27.71 ± 0.64^{a}	29.03 ± 0.87^{a}	28.51 ± 0.53^{a}	29.33 ± 2.35^{a}	26.83 ± 1.62^{a}
Week 4	32.16 ± 0.45^{a}	411.13 ± 3.62^{b}	435.21 ± 2.69^{b}	419.27 ± 3.55^{b}	421.21 ± 2.37^{b}
Week 8	39.42 ± 0.69^{a}	1007.06 ± 11.35^{b}	$1185.62 \pm 11.34^{b,c}$	1024.78 ± 8.70^{b}	$1109.43 \pm 3.06^{b,c}$
Week 12	44.63 ± 1.12^{a}	1321.67 ± 13.78^{d}	560.73 ± 14.99^{b}	578.42 ± 7.41^{c}	551.37 ± 10.05^{b}
Triglyceride level (mg dl^{-1})					
Baseline	121.34 ± 3.15^{a}	109.21 ± 3.32^{a}	119.11 ± 4.27^{a}	117.00 ± 4.36^{a}	105.61 ± 4.25^{a}
Week 4	135.21 ± 5.24^{a}	335.61 ± 5.45^{b}	325.66 ± 3.99^{b}	315.69 ± 2.31^{b}	333.34 ± 3.55^{b}
Week 8	152.66 ± 6.30^{a}	621.46 ± 7.77^{b}	659.28 ± 10.05^{b}	649.04 ± 12.43^{b}	$626.16 \pm 13.01^{b,c}$
Week 12	161.34 ± 4.56^{a}	889.22 ± 8.54^{d}	398.56 ± 9.23^{b}	419.16 ± 7.93^{c}	386.46 ± 6.96^{b}
HDL level (mg dl^{-1})					
Baseline	44.61 ± 0.81^{b}	$42.67 \pm 4.28^{a,b}$	39.26 ± 0.44^{a}	43.21 ± 1.19^{b}	38.43 ± 3.71^{a}
Week 4	45.14 ± 1.15^{c}	$39.11 \pm 3.17^{a,b}$	37.73 ± 0.51^{a}	41.01 ± 1.00^{c}	36.26 ± 4.07^{a}
Week 8	47.77 ± 1.00^{b}	35.67 ± 2.91^{a}	35.82 ± 2.41^{a}	$39.41 \pm 2.22^{a,b}$	35.06 ± 2.95^{a}
Week 12	48.36 ± 0.65^{c}	31.74 ± 4.01^{a}	44.54 ± 2.32^{b}	52.56 ± 2.73^{d}	54.78 ± 3.06^{d}

G1: normal control, G2: hypercholesterolemic control, G3: simvastatin (10 mg kg^{-1}) treated, G4: *A. viridis* extract (100 mg kg^{-1}) treated, G5: *A. viridis* extract (200 mg kg^{-1}) treated. All data are presented as the mean ± SD (n=4 for each group). One way ANOVA was performed followed by Dunnett's *Post hoc* test for multiple comparisons. Within a week, values with different superscript letters are significantly different from each other ($p < 0.05$).

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Table 6 Serum ALT, AST and CK levels of rabbits from various groups

	G1	G2	G3	G4	G5
ALT (U L^{-1})					
Baseline	22.01 ± 2.35^{a}	21.35 ± 0.77^{a}	21.13 ± 1.61^{a}	21.43 ± 1.27^{a}	22.45 ± 1.55^{a}
Week 4	23.09 ± 1.36^{a}	45.32± 2.17^{b}	47.82 ± 1.70^{c}	42.52 ± 1.16^{b}	42.37 ± 1.42^{b}
Week 8	24.72 ± 1.39^{a}	65.99 ± 2.22^{c}	60.08 ± 3.57^{b}	59.95 ± 3.06^{b}	68.35 ± 2.66^{c}
Week 12	26.31 ± 1.52^{a}	95.43 ± 4.15^{c}	101.25 ± 3.63^{d}	42.45 ± 1.64^{b}	45.53 ± 3.58^{b}
AST (U L^{-1})					
Baseline	25.05 ± 0.93^{a}	26.75 ± 2.06^{a}	25.68 ± 2.06^{a}	27.32 ± 2.45^{a,b}	27.04 ± 3.50^{a,b}
Week 4	32.45 ± 2.15^{a}	44.19 ± 3.23^{b}	49.44 ± 2.17^{c}	44.23 ± 3.17^{b}	42.41 ± 2.43^{b}
Week 8	37.19 ± 1.59^{a}	73.98 ± 1.46^{b}	81.34 ± 3.51^{c}	68.31 ± 3.34^{b}	76.23 ± 3.54^{b,c}
Week 12	40.02 ± 1.57^{a}	101.22 ± 3.10^{c}	135.11 ± 4.51^{d}	47.12 ± 2.99^{b}	43.45 ± 4.40^{b}
CK (U L^{-1})					
Baseline	536.28 ± 25.15^{a}	555.92 ± 25.27^{a}	538.25 ± 51.60^{a}	526.45 ± 35.39^{a}	562.13 ± 31.06^{a}
Week 4	561.24 ± 22.67^{a}	875.32 ± 35.33^{b}	796.23 ± 83.99^{b}	809.20 ± 80.26^{b}	824.57 ± 26.30^{b}
Week 8	583.42 ± 27.30^{a}	1345.00 ± 53.42^{b}	1480.23 ± 96.31^{b}	1475.58 ± 74.21^{b}	1359.35 ± 94.35^{b}
Week 12	595.14 ± 16.79^{a}	1843.22 ± 77.37^{c}	1994.24 ± 83.26^{c}	1084.20 ± 110.67^{b}	990.02 ± 83.51^{b}

G1: normal control, G2: hypercholesterolemic control, G3: simvastatin (10 mg kg^{-1}) treated, G4: *A. viridis* extract (100 mg kg^{-1}) treated, G5: *A. viridis* extract (200 mg kg^{-1}) treated. All data are presented as the mean ± SD (n=4 for each group). One way ANOVA was performed followed by Dunnett's *Post hoc* test for multiple comparisons. Within a week, values with different superscript letters are significantly different from each other ($p < 0.05$).

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Table 7 Serum antioxidant enzymes levels of rabbits from various groups

	G1	G2	G3	G4	G5
SOD (U mL^{-1})					
Baseline	5.56 ± 0.14^{a}	5.59 ± 0.32^{a}	$5.73 \pm 0.34^{a,b}$	5.85 ± 0.18^{b}	5.67 ± 0.23^{a}
Week 4	5.69 ± 0.34^{c}	5.45 ± 0.39^{a}	5.59 ± 0.11^{b}	5.69 ± 0.20^{b}	5.54 ± 0.11^{a}
Week 8	5.73 ± 0.27^{c}	5.21 ± 0.10^{a}	5.36 ± 0.25^{b}	5.37 ± 0.13^{b}	5.39 ± 0.09^{a}
Week 12	5.79 ± 0.35^{c}	4.89 ± 0.19^{a}	5.10 ± 0.25^{a}	5.51 ± 0.32^{b}	5.70 ± 0.25^{c}
GPx (U L^{-1})					
Baseline	1351.03 ± 101.31^{b}	1409.25 ± 125.69^{b}	1223.10 ± 107.98^{a}	1192.95 ± 192.95^{a}	1336.67 ± 148.03^{b}
Week 4	1397.95 ± 123.31^{c}	1051.58 ± 133.73^{a}	1049.15 ± 121.07^{a}	972.17 ± 151.28^{a}	1142.57 ± 104.34^{b}
Week 8	1431.05 ± 144.13^{c}	952.84 ± 162.43^{b}	975.24 ± 112.13^{b}	870.15 ± 134.20^{a}	851.30 ± 115.55^{a}
Week 12	1532.45 ± 179.36^{c}	899.23 ± 146.94^{a}	901.24 ± 168.11^{a}	1056.13 ± 131.38^{b}	1170.36 ± 122.35^{b}

G1: normal control, G2: hypercholesterolemic control, G3: simvastatin (10 mg kg^{-1}) treated, G4: *A. viridis* extract (100 mg kg^{-1}) treated, G5: *A. viridis* extract (200 mg kg^{-1}) treated. All data are presented as the mean ± SD (n=4 for each group). One way ANOVA was performed followed by Dunnett's *Post hoc* test for multiple comparisons. Within a week, values with different superscript letters are significantly different from each other ($p < 0.05$).

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Table 8 Thickness of intima, media and intima/media ratio of rabbits at week 12

Groups	Intima thickness (μm)	Media thickness (μm)	Intima/media
G1	1173.101 ± 126.29^{a}	3111.675 ± 183.13^{a}	0.377 ± 0.021^{a}
G2	5051.567 ± 143.56^{d}	6123.112 ± 124.43^{d}	0.825 ± 0.056^{d}
G3	1591.159 ± 115.47^{b}	3765.245 ± 154.25^{b}	0.404 ± 0.011^{b}
G4	1840.976 ± 134.50^{c}	4211.556 ± 146.32^{c}	0.556 ± 0.038^{c}
G5	1701.625 ± 129.05^{b}	3889.732 ± 102.34^{b}	0.459 ± 0.015^{b}

G1: normal control, G2: hypercholesterolemic control, G3: simvastatin (10 mg kg^{-1}) treated, G4: *A. viridis* extract (100 mg kg^{-1}) treated, G5: *A. viridis* extract (200 mg kg^{-1}) treated. All data are presented as the mean ± SD (n=4 for each group). One way ANOVA was performed followed by Dunnett's *Post hoc* test for multiple comparisons. Within a column, values with different superscript letters are significantly different from each other ($p < 0.05$).

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Table 9 Potential effects of *A. viridis* leaf compounds in treating hypercholesterolemia and atherosclerosis

No	Retention Time	Compound Name	Compound Nature	Biological Activity
1.	21.72	2-Methoxy-4-vinylphenol	Phenolic compound	- Antioxidant[21] - Anti-inflammatory, suppresses the activation of NF-κB and MAPK[22]
2.	26.78	Phenol, 2,6-bis(1,1-dimethylethyl)-	Aromatic and phenolic compound	-Anti-inflammatory[23] -Anti-atherosclerotic, lowers plasma cholesterol level and inhibits LDL cholesterol peroxidation[24]
3.	36.41	l-(+)-Ascorbic acid 2,6-dihexadecanoate	Reductone	-Antioxidant and reduces the triglycerides level[25] - Protects LDL against peroxidation and inhibits the progression of atherosclerosis[26]
4.	39.25	Phytol	Diterpene	- Lowers and maintains the level of LDL cholesterol and triglycerides, possess anti-cholesterol effects and reduces the risk of atherosclerosis[27]
5.	39.74	9,12,15-Octadecatrienoic acid	Polyunsaturated omega-3 fatty acid	- Cholesterol reducing agent and lowers the risk of cardiovascular diseases[28] - Anti-inflammatory effects, lowers triglycerides level, retards the atherosclerotic plaque progression[29]
6.	48.00	Oleic acid, eicosyl ester	Monounsaturated omega-9 fatty acid	- Inhibits the activity of HMG COA reductase[30,31]

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