King Saud University

Saudi Pharmaceutical Journal

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ORIGINAL ARTICLE

Antioxidant and anti-inflammatory effects of *Marrubium alysson* extracts in high cholesterol-fed rabbits

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Abstract The antioxidant and anti-inflammatory effects of hexane (HEXA), chloroform (CHLORO), ethyl acetate (EA) and total alcoholic (T. ALCOH) extracts of *Marrubium alysson* in hypercholesterolemic-fed rabbits were evaluated. Hypercholesterolemia was induced in male rabbits by high cholesterol diet (HCD) (350 mg/kg) for 8 weeks. Hypercholesterolemic rabbits were allocated into groups, treated with simvastatin (SIM 5 mg/kg), different extracts of *M. alysson* at two doses of 250, 500 mg/kg. A normal control group and an HCD control one were used for comparison. Lipid profile, as well as oxidized low density lipoprotein-cholesterol (ox-LDL-C), myeloperoxidase activity (MPO) and superoxide anion production ($O_2^{\cdot-}$), C-reactive protein (CRP) and monocyte chemoattractant protein-1 (MCP-1) were also evaluated. In addition, histological examination of ascending aorta was performed. We found dyslipidemia associated with significant increases in ox-LDL-C 123.5 ± 9.8 nmol MDA/mg non-HDL, MPO activity 0.08 ± 0.05 U/100 mg tissue and $O_2^{\cdot-}$ production 3.5 ± 0.3 nmol cytochrome C reduced/min/g tissue $\times 10^{-4}$ in

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hypercholerterolemic rabbits. In addition, there was a significant increase in CRP 6.6 ± 0.49 μmol/L and MCP-1 190.9 ± 6.4 pg/ml and its mRNA expression in HCD. Intima appeared thick with thick plaques surrounding the intima and luminal narrowing. SIM, EA and HEXA extracts of *M. alysson* had lipid lowering effect, decrease in ox-LDL-C, MPO, $O_2 \bullet^-$, CRP and MCP-1 mRNA expression with improvement of the pathological picture. *M. alysson* enhanced the stability of plaque, had lipid lowering, anti-inflammatory and antioxidant activities.

1. Introduction

Marrubium alysson (*M. alysson*) is an asterid dicot genus of Old World aromatic herbs; includes horehound, a genus of about 40 species of flowering plants in the family Lamiaceae. It has been used in traditional medicine for various purposes. It was chosen in our study for its wide distribution in Egypt; it is reported to have a hypoglycemic effect and influence on bile secretion (Blumenthal et al., 2000). It is used also in the treatment of cold, cough and asthma (Louhaichi et al., 2011), as a diuretic (Caceres et al., 1999), appetizer, astringent, gastroprotective (Paula et al., 2011) and antiviral (Edziri et al., 2011).

High cholesterol diet and oxidative stress increase serum total cholesterol (TC) and LDL-C levels (Jeon et al., 2001). Oxidative modification of LDL-C plays a major role in the pathogenesis of atherosclerosis. The first stage of atherogenesis is characterized by an influx and accumulation of LDL-C in the intima, followed by recruitment of blood-derived monocytes and lymphocytes to the developing lesion (Steinberg, 2005). Subsequently, LDL-C is oxidized by free radicals; ox-LDL-C induces a multitude of cellular responses which lead to vascular dysfunction (Hulten et al., 2005).

MCP-1, a member of the C–C chemokine β subfamily, causes the recruitment of monocytes, and as such may contribute to the initiation and maintenance of inflammatory reactions in the vascular tissues (Feng et al., 2005). Moreover, MCP-1 has broader roles in adipocyte physiology than inflammatory cell recruitment (Sartipy and Loskutoff, 2003). MCP-1 has a direct angiogenic effect on endothelial cells (Low et al., 2001), it contributes indirectly to inflammation by acting on the liver to produce acute phase proteins (Hug and Lodish, 2005). Among these acute phase proteins is CRP. In recent years, circulating levels of CRP have been clearly identified as a powerful independent risk factor for cardiovascular diseases (Ridker et al., 2000).

Since the involvement of free radicals in the pathophysiology of atherosclerosis was proposed, antioxidant supplementation arose as a potential strategy for the management of this disease (Hakimoğlu et al., 2007). The antioxidant activity is higher in medicinal plants, and because of their perceived effectiveness, with minimal side effects in clinical experience and relatively low costs, herbal drugs are prescribed widely (Miliauskas et al., 2004).

Our study aimed to investigate the potential benefits of total alcoholic (T. ALCOH), ethyl acetate (EA), chloroform (CHLORO), hexane (HEXA) extracts of *M. alysson* for their antioxidant and anti-inflammatory effects in hyper-cholesterolemic rabbits in order to verify the activities for which the plant is used in traditional medicine. Also, the toxicity of different extracts was evaluated.

2. Materials and methods

2.1. Plant collection and preparation of extract

Marrubium alysson was collected from Burg El-Arab at Alexandria during April to June 2010. The collected plant was identified by Dr. Saneia Kamal, Assistant Professor, Faculty of Science, Alexandria University. A voucher sample (*M.A.* – 1) was kept at the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Suez Canal University, Egypt. The plant was air-dried, finely powdered (4 kg of the dry plant), then extracted. The cold maceration technique was used for extraction of the plant. The powdered plant was soaked in methanol at room temperature. After seven days, the extract was filtered under vacuum through Whatman filter paper No. 1. The residue was again dipped in methanol for an additional seven days and filtered thereafter. The filtrate was combined and methanol was evaporated under vacuum, using a rotary evaporator (Buchi Rotavapor R-200) at 55 °C to yield viscous greenish-colored extract. The quantity of the extract obtained from *M. alysson* was 300 g (15%).

2.2. Fractionation

Distilled water was added to the methanol solution in a ratio of 2:1, followed by successive fractionation with HEXA (3×200 ml), CHLORO (3×200 ml) and EA (3×200 ml). Each extract was concentrated separately using vacuum rotary evaporator and stored at 4 °C till use. Two doses of 250 and 500 mg/kg were selected and used in this study. The two doses were prepared by dissolving appropriate amount of these viscous extracts in 1 ml Tween 20. This was followed by adding 9 ml of 0.9% NaCl to each mixture. The vehicle was obtained by dissolving 1 ml of Tween 20 in 9 ml of 0.9% NaCl (Irshaid and Mansi, 2009).

2.3. Animals

Eighty-eight male New Zealand White rabbits (2–2.5 kg) were obtained from the Egyptian Organization for Biological Products and Vaccines. All the animals were housed in individual cages, left for 7 days prior to the study to acclimatize and received standard pellets (15% protein, 2.5% lipid, 15% cellulose, 14% clay, 13% water) (Sezer et al., 2011) during this time. The animals were maintained on normal light–dark schedule and temperature 25 ± 3 °C throughout the experiment and given free access to water. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the Faculty of Pharmacy, Suez Canal University (Ismailia, Egypt).

2.4. Experimental design

Rabbits were fed either a standard chow diet (control group, $n = 8$) or a high-cholesterol diet (HCD, $n = 80$) consisting of standard chow supplemented with cholesterol (350 mg/kg/day) (Oxford Lab, Mumbai, India), dissolved in cottonseed oil (Egyptian market) for 8 weeks. This dose was proved to induce hypercholesterolemia (Riezebos et al., 1994). Cotton seed oil is a natural oil that contains a high proportion of polyunsaturated fatty acids and proved to have no effects on the tissues (Mayes, 2000). Hypercholesterolemic rabbits were, then, randomly divided into nine treatment groups. During treatment, all animals continued to receive the HCD. Animals were treated once daily by oral gavage with the compounds suspended in Tween 20 (vehicle) according to the following schedule:

Group 1 (control group): fed a standard pellet 100 g/kg/d.
Group 2 (HCD): fed a HCD and received no additional treatment.
Group 3 (SIM): HCD received simvastatin (Sigma Chemical Company, Egypt) at doses of 5 mg/kg (Zhang et al., 2009).

Group 4 (EA 250): HCD received 250 mg/kg EA extract.
Group 5 (EA 500): HCD received 500 mg/kg EA extract.
Group 6 (HEXA 250): HCD received 250 mg/kg HEXA extract.
Group 7 (HEXA 500): HCD received 500 mg/kg HEXA extract.
Group 8 (CHLORO 250): HCD received 250 mg/kg CHLORO extract.
Group 9 (CHLORO 500): HCD that received 500 mg/kg CHLORO extract.
Group 10 (T. ALCOHOL250): HCD received 250 mg/kg T. ALCOHOL extract.
Group 11 (T. ALCOHOL500): HCD received 500 mg/kg T. ALCOHOL extract.

2.5. Toxicity study

The toxicity of four used *M. alysson* extracts were tested using four doses (100, 250, 500 and 1000 mg/kg) (three rabbits for each dose). Three control rabbits were kept under the same conditions without any treatments. The animals were observed continuously during the first hour, and then every hour for 6 h, then after 12 and 24 h, and finally after every 24 h, up to 3 weeks, for any physical signs of toxicity such as writhing, gasping, salivation, diarrhea, cyanosis, pupil size, any nervous manifestations, or mortality (Elberry et al., 2011).

2.6. Blood sampling and biochemical analysis

At the end of the study, rabbits were fasted overnight, anesthetized with thiopental sodium (50 mg/kg) (Vogler, 2006). Blood samples were collected by cardiac puncture. Blood was centrifuged at $2000 \times g$ for 15 min after 30 min of collection and stored at $-80\,°C$ until assayed.

2.7. Lipid profile

Serum triglycerides (TGs), TC, LDL-C and high density lipoprotein cholesterol (HDL-C) were measured colorimetrically using assay kits from (Stanbio, Texas, USA) according to the manufacturer instructions using UV–visible spectrophotometer (UV-1601PC, Shimadzu, Japan). Additionally atherosclerosis index was calculated by the formula: atherosclerosis index = (serum TC−HDL-C) /HDL-C (Hua et al., 2009).

2.8. Plasma oxidized LDL-C (ox-LDL-C)

It was measured according to the principle of (Dujovne et al., 1994). Briefly, very low density lipoprotein cholesterol (VLDL-C) and low LDL-C were precipitated from 500 μl of plasma by 100 μl dextran sulfate/magnesium chloride. The pellet was dissolved in 4% saline solution. A volume of the re-dissolved precipitate containing 100 μg non HDL-C was mixed with 4% sodium chloride to give 500 μL of total solution. Copper solution (0.5 mM $CuCl_2$) was added and incubated at 37 °C for 5 h in a shaking water bath. The solution was assayed for thiobarbituric acid reactive substances as an index for oxidation.

2.9. Serum CRP and MCP-1

Serum CRP was measured according to the principle of (Ben Assayag et al., 2009) by ELISA technique using kits purchased from DIA MED (Belgium). Serum MCP-1 was determined using Rat MCP-1 ELISA kit (Biosource International, California, USA) as previously described (Sacerdoti et al., 2005).

2.10. Determination of liver enzymes

Commercial kit Purchased from BioMed Diagnostics (Oregon, USA), was used for determination of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) using UV–visible spectrophotometer (UV-1601PC, Shimadzu, Japan).

2.11. Measurement of blood urea nitrogen (BUN), creatinine and total protein

Creatinine and BUN were determined enzymatically using commercially available kits (Spinreact, Gerona, Spain).Total Protein was determined using Diamond Diagnostic Kit (Egypt), according to the manufacturer's protocol. Serum levels of BUN, creatinine and total protein were determined by colorimetric methods using UV–visible spectrophotometer (UV-1601PC, Shimadzu, Japan).

2.12. Processing of aortic tissue

After the end of the experiment, animals in each group were sacrificed, their abdomens and chests were opened and the thoracic aorta of each animal was rapidly removed, washed out of blood using ice-cold (4 °C) phosphate-buffered saline (pH = 7.4) and cleaned of adherent connective tissue. Three specimens of the ascending aorta, starting just above the left main coronary artery, was obtained; one specimen was quickly directed for the analysis of $O_2{\cdot}^-$, second specimen was placed in an autoclavable test tube, frozen and stored at $-70\,°C$ for biochemical analysis. The other specimen possessed for histopathological examination.

2.13. Assay of aortic myloperoxidase (MPO) activity

Assaying of MPO activity was described by (Philip et al., 2004). Briefly, aortic tissue was homogenized in (1/20 wt., g/volume ml) homogenization buffer pH (4.7) [0.1 mol/L NaCl, 0.02 mol/L NaHPO₄ and 0.015 mol/L sodium ethylenediamine tetraacetic acid (EDTA)], centrifuge at 260×g for 10 min and the pellet underwent (dissolved) hypotonic lysis (0.2% NaCl) solution followed 1 min later by adding an equal volume of solution containing (1.6% NaCl and 5% glucose). After further centrifugation, the pellet was then suspended in resuspension buffer pH 5.4 (0.05 mol/L NaHPO₄ containing 0.5% hexadecyltrimethylammonium bromide) and re-homogenized. The suspension was frozen and thawed three cycles in liquid nitrogen, then centrifuged for 15 min at 3000×g. The pellet was discarded. MPO activity was assayed by measuring the change in optical density at 450 nm using tetramethylbenzidine, (1.5 mmol/L) and H_2O_2 (0.5 mmol/L) (Sigma Chemical Co) by UV–visible spectrophotometer (UV-1601PC, Shimadzu, Japan).

2.14. Assay of superoxide anion ($O_2 \cdot^-$) production in aortic tissue

Tissue $O_2 \cdot^-$ was determined according to the modified method of (Hassoun and Stohs, 1996). Briefly, aortic tissue was homogenized in phosphate buffer saline and centrifuged at $16000 \times g$ for 15 min in cooling centrifuge. The supernatant was added to 0.5 mM cytochrome C (Sigma–Aldrich). This mixture was re-centrifuged at $3000 \times g$ for 10 min; the supernatant fractions were collected for subsequent evaluation using a UV–visible spectrophotometer (UV-1601PC, Shimadzu, Japan).

2.15. RT-PCR for detection of MCP-1 mRNA

Total RNA was isolated from aortic tissues using an RNA extraction kit (Qiagen, Germany). Concentration of the extracted RNA was measured by NanoDrop ND-1000 (NanoDrop Tech., Wilmington, USA). Specific primers for rat MCP-1 cDNA fragment: primer 5′-GTC TCT GCA ACG CTT CTG TGC C′-3 and 5′-AGT CGT GTG TTC TTG GGT TGTGG-3′. β-actin primers sense: 5′-ATGGAT GATGATATCGCCGCG-3′ antisense5′-TGAAGGTAGTTT CGTGGATGC-3′ were used (Sekalska et al., 2007). PCR was performed using one step RT-PCR kit (Qiagen). Cycling parameters for amplifying RT products were as follows: 95 °C, 1 min; 60 °C, 1 min; 72 °C, 1 min, for 30 cycles, and then extended at 72 °C for another 5 min. After amplification, PCR products were electrophoresed on 1.2% agarose gel, stained with ethidium bromide, and photographed by using the gel documentation system (Bio Doc Analyze, Biometra).

2.16. Quantification of the PCR product

The PCR products were then quantified by using a quantification kit (from Promega Corporation, Madison, WI, USA). This method depends on the purification of the PCR using Promega Wizard PCR preps DNA purification kit (Promega Corporation, Madison, WI, USA). The mixture for quantization consisted of DNA quantification buffer, sodium pyrophosphate, 7 NDPK enzyme solution, T4 DNA polymerase and DNA. All these contents were incubated at 37 °C for 10 min. Then, 100 µL of Enliten L/L reagent was added. Immediately, the reaction was read using a luminometer. The same steps were done on DNAs of known concentrations provided by the kit, and a standard curve was performed by plotting the readings of the luminometer against the concentrations. Then, the readings of the amplified PCR products of the five different genes after using the luminometer were read from the standard curve. The results were expressed as µg/mg wet tissue (El-Seweidy et al., 2011).

2.17. Histopathological study

Then aortic specimens were fixed overnight in 10% formaldehyde, and after routine processing, were embedded in paraffin and cross-sectioned into slices of 4–5 µm thickness at 5 mm intervals. Sections were subjected to histological techniques for the general architecture of the aorta. The cross sections of the aortic tissue were stained with hematoxylin and eosin (H&E) using an Olympus BX51 light microscope (Tokyo, Japan). Aortic specimens were stained also with oil red O staining-to evaluate the atheroma plaque area. Quantitative histopathological parameters were determined using IMA-GEJ 1.45F (National Institutes of Health, Bethesda, MD, USA) for evaluating the percentage of the intima affected by atherosclerosis (the ratio of the perimeter or the length of the atherosclerotic lesions/the perimeter of intima), intima to media ratio and the ratio of the positive surface area occupied by oil red O-stained lesions to the total aortic surface area. Intimal thickening of each aorta was estimated as an index of the extent of atherosclerosis by measuring the cross-sectional area of intima and media. Four to six sections were used. Fifteen captures per section were taken from each aortic segment. All histological examinations were performed in a blinded fashion.

2.18. Statistical analysis

All data were expressed as mean ± SEM and analyzed using the Statistical Package of Social Sciences (SPSS) program version 17, (Chicago, IL, USA). For all parameters, comparisons among groups were carried out using one-way analysis of variance (ANOVA) followed by Dunnat's multiple comparison test (Katz, 2006). All P values reported are two-tailed and $P < 0.05$ was considered significant.

3. Results

3.1. Toxicity study

The toxicity study revealed the non-toxic nature of EA, CHLOR, HEXA and T. ALCOHOL extracts of *M. alysson* at doses up to 1000 mg/kg. Rabbits did not show any drug-induced physical signs of toxicity during the whole experimental period, and no deaths were reported. CHLOR and T. ALCOH extracts of *M. alysson* showed non-significant beneficial effects on all measured parameters.

3.2. Effect of M. alysson extracts on serum lipid profile and atherosclerosis index

Serum lipid profile was significantly increased by feeding HCD. Table 1 shows a significant increase in TC, TG and LDL-C and a non-significant decrease in HDL-C in HCD group as compared to control group. SIM significantly ameliorated dyslipidemia, while serum HDL-C slightly (but not significantly) increased in rabbits treated with SIM. On the other hand, M. alysson could reduce the serum level of TC, TG and LDL-C as compared to HCD group. EA extract showed better results than HEXA extract. Additionally, the difference in the calculated atherosclerosis index between the HCD group and the treated groups was significant ($P < 0.05$).

3.3. Effect of M. alysson extracts on plasma ox-LDL-C, aortic MPO activity and $O_2 .^-$ production

Regarding, ox-LDL-C, it significantly increased by 2.5-fold at $P < 0.05$ in HCD group compared to controls. In consequence, two and three fold significant elevation in both aortic MPO activity and $O_2 .^-$ production respectively was detected in comparison with control group ($P < 0.05$, Table 2).

Treatment with SIM significantly reduced serum ox-LDL-C and both MPO activity and $O_2 .^-$ production in aortic tissue compared to HCD group. However, treatment with M. alysson EA and HEXA extracts significantly reduced the all measured oxidative stress parameters. EA extract (500 mg/kg) showed significantly better results than HEXA extract regarding MPO activity and $O_2 .^-$ production. ($P < 0.05$, Table 2).

3.4. Effect of M. alysson extracts on serum CRP and MCP-1

In response to oxidative stress, Table 3 shows that, CRP was significantly increased (6.6 ± 0.49 in HCD group vs. 1.3 ± 0.15, $P < 0.05$), in addition to MCP-1 (190.9 ± 6.4 vs. 69.2 ± 4.1, $P < 0.05$) as compared with the control group. In the meanwhile, both SIM and M. alysson extracts could significantly decrease these inflammatory markers compared to HCD group ($P < 0.05$, Table 3).

3.5. Effect of M. alysson extracts on MCP-1 mRNA expression

MCP-1 mRNA was significantly enhanced in HCD rabbits in comparison with the normal control rabbits (750.2 ± 30.2 vs. 150.2 ± 8.9 $P < 0.05$). Oral administration of SIM signifi-

Table 1 Effect of ethyl acetate and hexane extracts of M. Alysson on serum lipid profile and atherosclerotic index in hypercholesterolemic rabbits.

Groups	TG (mg/dl)	TC (mg/dl)	LDL-C (mg/dl)	HDL-C (mg/dl)	Atherosclerotic index
Control	88.2 ± 5.6	100 ± 4.5	96.2 ± 4.7	40.9 ± 1.7	1.48 ± 0.13
HCD	$138.4 \pm 7.1^*$	$412.5 \pm 23.4^*$	$143.4 \pm 5.2^*$	37.1 ± 1.0	$9.9 \pm 0.9^*$
HCD + SIM 5 mg/kg	$110.3 \pm 1.7^{*,\#}$	$273.0 \pm 27.0^{*,\#}$	$114.3 \pm 3.2^{*,\#}$	38.0 ± 1.5	$6.35 \pm 0.7^{*,\#}$
HCD + EA 250 mg/kg	$125,5 \pm 3.4^{*,S}$	$305.9 \pm 34.1^{*,\#}$	$129.2 \pm 4.9^{*,\#,S}$	38.1 ± 1.4	$8.29 \pm 0.34^*$
HCD + EA 500 mg/kg	$119.1 \pm 3.1^{*,\#}$	$298.0 \pm 3.8^{*,\#}$	$123.8 \pm 5.6^{*,\#}$	38.4 ± 1.6	$6.9 \pm 0.4^{*,\#}$
HCD + HEXA 250 mg/kg	$127.9 \pm 5.2^{*,S}$	$361.0 \pm 14.4^{*,S}$	$134.3 \pm 2.6^{*,S}$	36.6 ± 1.4	$8.7 \pm 0.45^{*,S}$
HCD + HEXA 500 mg/kg	$121.1 \pm 2.9^{*,\#}$	$338.0 \pm 14.2^{*,\#,S}$	$124.7 4.1^{*,\#}$	37.3 ± 1.3	$7.2 \pm 1.0^{*,\#}$

TG: triglycerides, TC: total cholesterol, LDL-C: low density lipoprotein cholesterol, HDL-C: high density lipoprotein cholesterol, HCD, high cholesterol diet, SIM: simvastatin, EA: ethyl acetate extract, HEXA: hexane extract. Rabbits were treated for eight weeks. Results were expressed as mean $\pm$ SEM and analyzed using one-way ANOVA followed by Duncan's post hoc test.
\# $P < 0.05$ compared to HCD group.
$ $P < 0.05$ compared to simvastatin group, $n = 8$.
* $P < 0.05$ compared to control group.

Table 2 Effect of ethyl acetate and hexane extracts of M. alysson on serum ox-LDL-C, aortic MPO and $O_2 .^-$ in hypercholesterolemic rabbits.

Groups	ox LDL-C (nmol MDA/ mg non HDL)	MPO (U/100 mg tissue)	$O_2 .^-$ (nmol cytochrome C reduced/min/g tissue $\times 10^{-4}$)
Control	49.2 ± 3.9	0.04 ± 0.03	1.09 ± 0.14
HCD	$123.5 \pm 9.8^*$	$0.08 \pm 0.05^*$	$3.5 \pm 0.3^*$
HCD + SIM 5 mg/kg	$53.9 \pm 1.9^\#$	$0.05 \pm 0.04^\#$	$2.4 \pm 0.2^\#$
HCD + EA 250 mg/kg	$62.5 \pm 2.6^\#$	$0.06 \pm 0.04^{*,\#,@}$	$3.05 \pm 0.2^{*,@}$
HCD + EA 500 mg/kg	$59.2 \pm 1.9^\#$	$0.045 \pm 0.03^\#$	$2.1 \pm 0.1^\#$
HCD + HEXA 250 mg/kg	$71.0 \pm 3.6^{*,\#,S}$	$0.07 \pm 0.04^{*,S,@}$	$3.2 \pm 0.2^{*,S,@}$
HCD + HEXA 500 mg/kg	$69.6 \pm 2.8^{*,\#,S}$	$0.06 \pm 0.05^{*,\#,@}$	$2.8 \pm 0.1^{*,\#,@}$

Ox-LDL-C: oxidized low density lipoprotein cholesterol, MPO: myloperoxidase $O_2 .^-$: superoxide anion, HCD: high cholesterol diet, SIM: simvastatin, EA: ethyl acetate extract, HEXA: hexane extract. Rabbits were treated for eight weeks. Results were expressed as mean $\pm$ SEM and analyzed using one-way ANOVA followed by Duncan's post hoc test.
$ $P < 0.05$ compared to simvastatin group.
@ $P < 0.05$ compared to EA 500 mg group, $n = 8$.
* $P < 0.05$ compared to control group.
\# $P < 0.05$ compared to HCD group.

Table 3 Effect of ethyl acetate and hexane extracts of *M. alysson* on serum CRP, MCP-1 in hypercholesterolemic rabbits.

Groups	Serum CRP (μmol/L)	MCP-1 (pg/ml)
Control	1.3 ± 0.15	69.2 ± 4.1
HCD	6.6 ± 0.49*	190.9 ± 6.4*
HCD + SIM 5 mg/kg	2.8 ± 0.2*,#	77.8 ± 2.9*,#
HCD + EA 250 mg/kg	3.5 ± 0.25*,#	92.7 ± 6.2*,#,$
HCD + EA 500 mg/kg	3.4 ± 0.2*,#	85.3 ± 6.5*,#
HCD + HEXA 250 mg/kg	3.7 ± 0.31*,#,$	151.5 ± 5.6*,#,$
HCD + HEXA 500 mg/kg	3.5 ± 0.2*,#	150.0 ± 3.9*,#,$

CRP: C-reactive protein, MCP-1: monocyte chemoattractant protein-1, HCD, high cholesterol diet, SIM: simvastatin, EA: ethyl acetate extract, HEXA: hexane extract. Rabbits were treated for eight weeks. Results were expressed as mean ± SEM and analyzed using one-way ANOVA followed by Duncan's post hoc test.
$ $P < 0.05$ compared to simvastatin group, $n = 8$.
# $P < 0.05$ compared to HCD group.
* $P < 0.05$ compared to control group.

the normal control group. SIM and *M. alysson* extracts showed non-significant changes in these levels (Table 4).

3.7. Effect of M. alysson extracts on BUN, serum creatinine and total protein

At the end of the study period, hypercholesterolemic rabbits showed a non-significant change in BUN, serum creatinine and total protein as compared to normal controls. Oral administration of SIM and *M. alysson* extracts did not show any significant change in these serum levels (Table 5).

3.8. Effect of M. alysson extracts on histopathological picture and perimeter ratio and intima/media ratio

The thoracic aorta of rabbits from control group appeared normal with intact intimal surface. The intima appears thin and intact with no atherosclerotic plaque on the aorta luminal surface (Fig. 2A).

In HCD group, H&E stain in all animals of this group revealed thickened intima with thick atherosclerotic plaques observed surrounding the intima and nearly 50% luminal narrowing in aortic tissue as well as hypertrophic degeneration of the arterial intima. In all treatment groups, there was a decrease in the intimal thickening and plaque formation (Fig. 2A).

There was a significant dose dependent decrease in the perimeter of the lumen covered by atherosclerotic lesions in the animals treated with EA extract of *M. alysson* compared to HCD non-treated group, HEXA extracts caused a less inhibition of atherosclerosis. However, a further reduction ($P < 0.05$, Fig. 2B (of atherosclerotic lesions was observed in the SIM treated group. SIM resulted in a significant decrease
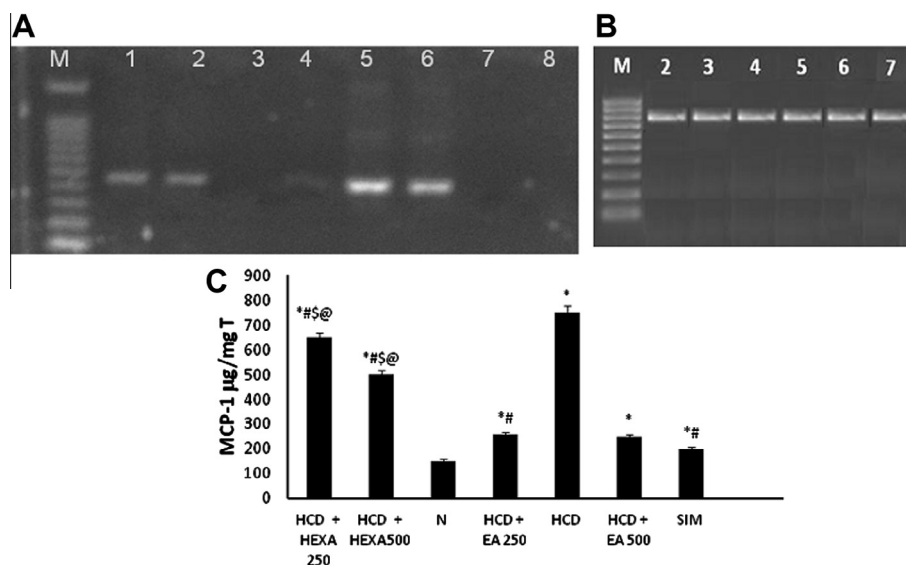
cantly reduced this increment as compared to the HCD group (200.4 ± 8.4 vs. 750.2 ± 30.2, $P < 0.05$, Fig. 1C). EA and HEXA (250, 500) extracts of *M. alysson* showed significant reduction in MCP-1mRNA expression in comparison to HCD rabbits ($P < 0.05$), notably, EA extract resulted in a significant improvement in MCP-1 mRNA expression compared to HEXA extract.

3.6. Effect of M. alysson extracts on liver enzyme activity

Feeding of the HCD for 8 weeks resulted in a non-significant increase in serum levels of ALT, AST and ALP compared with

Figure. 1 MCP-1 mRNA expression in aortic tissue homogenate of studied groups. (A) RT-PCR. 100 bp marker, lane 1; HEXA 250, lane, 2, HEXA 500 (lane 3, normal, lane 4, EA 250, Lane 5, 6 HCD group, lane 7, EA 500, lane 8 SIM.MCP-1: monocyte chemoattractant protein-1, N: normal control, HCD: high cholesterol diet, SIM: simvastatin, EA 250, 500: Ethyl acetate extract (250, 500 m/kg) respectively, HEXA 250, 500: hexane extract (250, 500 m/kg) respectively. (B) β-Actin mRNA expression for each sample was used as internal control. (C) Mcp-1 m RNA in μg/mg tissue. Rabbits were treated for eight weeks. Results were expressed as mean ± SEM and analyzed using one-way ANOVA followed by Duncan's post hoc test. *$P < 0.05$ compared to control group, #$P < 0.05$ compared to HCD group, $$P < 0.05$ compared to SIM group, @$P < 0.05$ compared to EA 500 mg group, $n = 8$.

Table 4 Effect of ethyl acetate and hexane extracts of *M. alysson* on liver enzymes in hypercholesterolemic rabbits.

Groups	Serum ALT (U/L)	Serum AST (U/L)	Serum ALP (U/L)
Control	27.5 ± 1.4	52.1. ± 2.6	60.1 ± 2.0
HCD	35.7 ± 2.5	60.4 ± 3.1	64.7 ± 2.7
HCD + SIM 5 mg/kg	37.2 ± 1.8	59.0 ± 2.0	63.1 ± 2.4
HCD + EA 250 mg/kg	30.5 ± 2.8	55.8 ± 4.1	61.4 ± 2.4
HCD + EA 500 mg/kg	31.0 ± 2.4	54.5 ± 3.8	60.9 ± 2.3
HCD + HEXA 250 mg/kg	26.4 ± 0.9	57.3 ± 3.7	58.2 ± 2.4
HCD + HEXA 500 mg/kg	29.5 ± 1.9	58.9 ± 2.5	60.5 ± 2.1

ALT: alanine aminotransferase, AST: aspartate aminotransferase, ALP: alkaline phosphatase, HCD, high cholesterol diet, SIM: simvastatin, EA: ethyl acetate extract, HEXA: hexane extract. Rabbits were treated for eight weeks. Results were expressed as mean ± SEM and analyzed using one-way ANOVA followed by Duncan's post hoc test. $n = 8$.

Table 5 Effect of ethyl acetate and hexane extracts of *M. alysson* on blood urea nitrogen, serum creatinine and total protein in hypercholesterolemic rabbits.

Groups	BUN (mg/dl)	Creatinine (mg/dl)	Total protein (g/dl)
Control	29.6 ± 1.4	1.29 ± 0.1	8.0 ± 0.4
HCD	30.9 ± 2.2	1.3 ± 0.17	9.9 ± 0.4
HCD + SIM	27.6 ± 1.2	1.2 ± 0.08	8.1 ± 0.4
HCD + EA 250 mg/kg	29.6 ± 0.9	1.3 ± 0.1	7.8 ± 0.3
HCD + EA 500 mg/kg	32.4 ± 1.7	1.2 ± 0.08	8.2 ± 0.4
HCD + HEXA 250 mg/kg	29.0 ± 1.3	1.2 ± 0.07	7.9 ± 0.5
HCD + HEXA 500 mg/kg	31.2 ± 1.3	1.4 ± 0.08	8.0 ± 0.5

BUN: blood urea nitrogen, HCD: high cholesterol diet, SIM: simvastatin, EA: ethyl acetate extract, HEXA: hexane extract. Rabbits were treated for eight weeks. Results were expressed as mean ± SEM and analyzed using one-way ANOVA followed by Duncan's post hoc test. $n = 8$.

in perimeter ratio compared to *M. alysson* HEXA extract in the lower dose.

HCD resulted in a significant decrease in intima/media ratio and obliteration in arterial lumen. The intima/media ratio was significantly decreased after SIM treatment to 0.7 ± 0.05 compared with rabbits in the HCD group 1.4 ± 0.1 (Fig. 2C). *M. alysson* extracts in both doses induced a reduction in this ratio also compared to HCD group ($P < 0.05$, Fig. 2C).

The differences in the degree of atherosclerotic plaque by cross-sectional microscopic examination of the thoracic aorta are shown in Fig. 3A. The section showed Oil Red O-stained particles, mainly foam cells, in the aortic wall intima, with findings consistent with those observed in Fig. 3B. SIM and *M. alysson* EA and HEXA extracts improved the atherosclerotic formation.

Average aortic fatty streak accumulation, measured as the percentage of Oil Red O staining relative to the total area surveyed (Fig. 3B), was significantly increased in hypercholesterolemic non-treated rabbits, while it decreased significantly on SIM administration (44.2% ± 3.9 vs. 89.2% ± 7.9). *M. alysson* extracts in both doses induced a significant reduction in this ratio also compared to HCD group ($P < 0.05$, Fig. 3B).

4. Discussion

The purpose of this study was to evaluate the antioxidant and anti-inflammatory effects of *M. alysson* in hypercholesterolemic-fed rabbits.

In the animal model used, the hypercholesterolemic diet severely increased plasma TC and LDL-C levels reaching about

4 times and 1.5 times the values in control animals respectively, consistent with the results of another study reported by (Tavridou et al., 2007). HCD induced also a moderate increase in TGs and a non significant decrease in HDL-C, consistent with the results reported by (Hernandez-Presa et al., 2003; Nachtigal et al., 2005).

The significant increase in serum ox- LDL-C as well as aortic MPO and $O_2.^-$ levels in the hypercholesterolemic rabbits represents the effect of hypercholesterolemia on increasing susceptibility to oxidation (Witztum and Steinberg, 2001). The oxidatively modified lipids and their degradation products are believed to have pro-inflammatory, immunogenic, and cytotoxic properties which contribute to both the initiation and progression of atherosclerotic lesions (Kato et al., 2009) as they contribute to the recruitment of monocytes and T cells, directly or indirectly, via induction of chemokines and endothelial cell adhesion molecules (Witztum and Steinberg, 2001).

In accordance, the increased oxidative stress was accompanied by increased serum MCP-1 and its mRNA expression in aortic tissue. It was proved that platelet-activating factor lipids derived from ox-LDL-C stimulate 5-Lipooxygenase expression in leukocytes and is accompanied by biosynthesis of MCP-1 (Silva et al., 2002).

In the current study, serum CRP was increased suggesting that the liver had switched to a predominantly inflammatory state responding to high plasma cholesterol as previously described (Kleemann et al., 2007). It is well known that CRP is an excellent marker of inflammation in atherosclerosis (Libby and Ridker, 2004). There is also evidence indicating that high levels of CRP may be potentially atherogenic (Jialal et al., 2006).
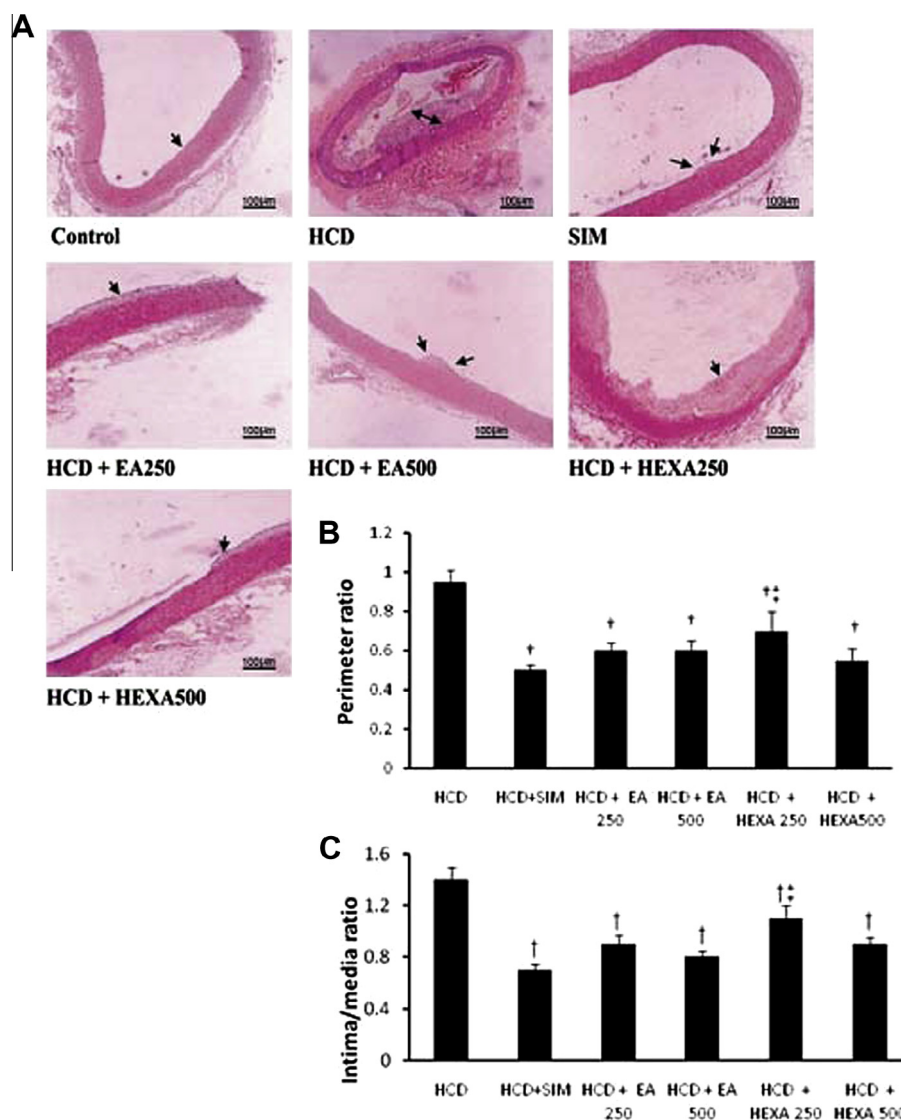
Figure. 2 Histopathological examination of ascending aortic tissue. (A) The microscopic images of the ascending aortic sections of the control group appeared normal, with organized intimal surfaces. HCD resulted in huge atherosclerotic plaques surrounding the lumen. SIM group showed thin and limited atherosclerotic plaque formation. EA and HEXA extracts of *M. Alysson* showed decrease in intimal thickening and atherosclerotic plaque formation to a lesser degree than SIM. Scale bar = 100 μm. (B) The perimeter ratio of the studied groups. (C) Intima/media ratio of the studied groups. HCD: high cholesterol diet, SIM: simvastatin, EA 250, 500: ethyl acetate extract (250, 500 m/kg) respectively, HEXA 250, 500: hexane extract (250, 500 m/kg) respectively. Rabbits were treated for eight weeks. Results were expressed as mean ± SEM and analyzed using one-way ANOVA followed by Duncan's post hoc test. $^*P < 0.05$ compared to control group, $^\#P < 0.05$ compared to HCD group, $^\$P < 0.05$ compared to SIM group, $^@P < 0.05$ compared to EA 500 mg group, $n = 8$.

These biochemical findings in HCD group were supported by histopathological picture of increased atherosclerotic plaque formation and nearly 50% luminal narrowing in aortic tissue and increased number of inflammatory cells and hypertrophic degeneration of the arterial intima (Steinberg, 2005).

As expected, SIM achieved a 33% and 21% decrease in TC and LDL-C levels respectively, consistent with previous studies (Ray et al., 2006). In accordance to (Tavridou et al., 2007), non-significant changes were observed following 4 weeks of treatment by SIM regarding levels of HDL-C, whereas a sig-

nificant but modest decrease was observed in TG levels. LDL levels in the hypercholesterolemic diet group were higher than in the control group in spite of treatment with SIM, probably due to the excessive amount of cholesterol added to the diet. However, this gave us the opportunity to examine the beneficial effects of SIM additional to lowering of cholesterol levels.

The current study showed that treatment with SIM for 4 weeks, revealed a significant decrease in serum ox-LDL-C, aortic MPO level and $O_2{}^{\bullet-}$ production compared with HCD animals. Interestingly, there is clinical evidence that inhibition
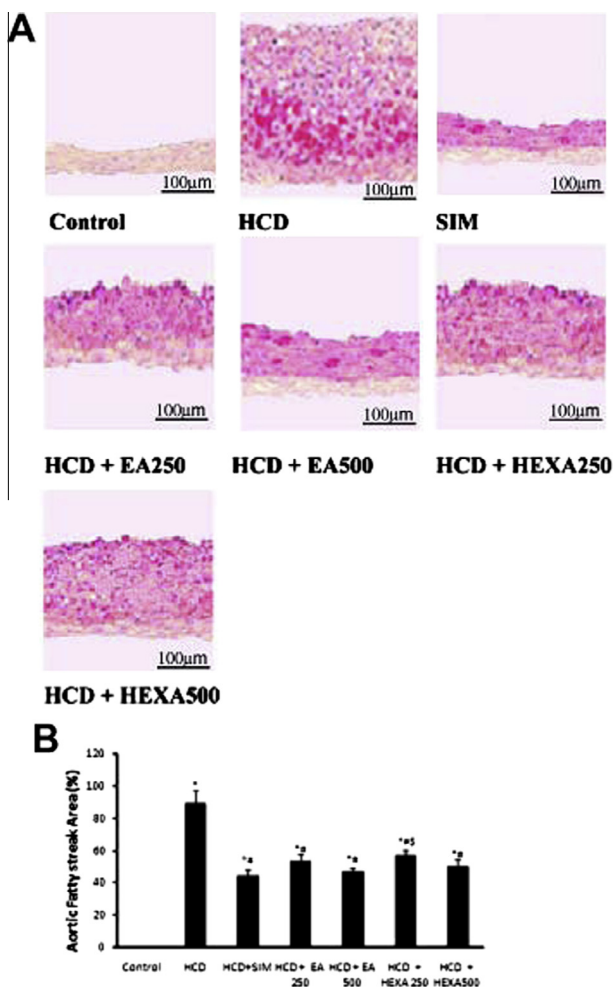
Figure. 3 Histopathological examination of ascending aortic tissue. (A) Microscopic images of the ascending aortic sections of the control group appeared normal. HCD showed the oil red O-stained particles of fat deposition. SIM group showed thin and limited atherosclerotic plaque formation. EA and HEXA extracts of *M. Alysson* showed decrease in intimal thickening and atherosclerotic plaque formation to a lesser degree than SIM. Scale bar = 100 μm. (B) Quantification of aortic fatty streak area percentage of the studied groups. HCD: high cholesterol diet, SIM: simvastatin, EA 250, 500: ethyl acetate extract (250, 500 m/kg) respectively, HEXA 250, 500: hexane extract (250, 500 m/kg) respectively. Rabbits were treated for eight weeks. Results were expressed as mean ± SEM and analyzed using one-way ANOVA followed by Duncan's post hoc test. $^*P < 0.05$ compared to control group, $^{\#}P < 0.05$ compared to HCD group, $^{\$}P < 0.05$ compared to SIM group, $n = 8$.

of LDL-oxidation can inhibit atherosclerosis independently of lowering plasma cholesterol levels (Sezer et al., 2011). It has been suggested that the inhibitory effect of statins on LDL oxidation may result from enhanced removal from plasma of "aged" LDL, which is more prone to oxidation than newly synthesized LDL (Franzoni et al., 2003). In addition, we found significant reductions of atherosclerotic lesions in animals treated with SIM compared with HCD animals. To elucidate the mechanism(s) by which SIM could reduce plaque inflammation, we investigated its effect on MCP-1 which is not only a

marker of the development of atherosclerosis, but also of destabilization of atherosclerotic plaques. Our results showed that SIM decreased serum MCP-1-and MCP-1 mRNA expression and so may inhibit monocyte migration. Previous studies have demonstrated that statins reduce the expression of several adhesion molecules most likely via increased endothelial nitric oxide synthase expression and activity (Sasaki et al., 2003; Nachtigal et al., 2005). SIM also down regulates serum CRP. Support for our results has come from the report of (Albert et al., 2001) who found that patients exhibiting lower serum levels of CRP after statin therapy have better clinical outcomes than those with higher CRP levels.

By reducing hyperlipidemia and chronically elevated levels of circulating and local inflammatory proteins statins may minimize the risk of atherosclerotic plaque rupture or arterial occlusion (Patel et al., 2007; Yu et al., 2012).

The genus *Marrubium* is represented by 97 species which are widely spread over the temperate and warm regions. Many *Marrubium* species are reported in the literature to be used in folk medicine (Karioti et al., 2005, 2003).

Similar to SIM group, *M. alysson* groups – mainly EA extract- exhibited a low serum level of TC, LDL-C and ox-LDL-C. Additionally, CRP serum level, found increased in untreated rabbits, was also reduced by *M. alysson* extracts. CRP, a classical plasma protein marker, is related to cardiovascular risk, and its reduction after cholesterol lowering is associated with improved clinical outcomes (Libby, 2002; Ridker et al., 2005).

It is well known that lesion size is not predictive of plaque stability in humans, and that inflammation is related to plaque instability (Libby, 2002).

The lipid lowering, antioxidant and anti-inflammatory properties of *M. alysson* contribute to its anti-atherosclerotic effect by several different manners. For example, decreased LDL-oxidation may suppress the expression of adhesion molecules by endothelial cells and the expression of tissue factor, colony-stimulating factor, and MCP-1 in macrophages, molecules that modulate the atherosclerotic process which may be due to the cumulative or synergistic effects of multiple compounds present in the *M. alysson* extracts such as phenylpropanoid glycosides (Çalis et al., 1992), diterpenes (Savona et al., 1979) and flavonoids (Karioti et al., 2003).

The results of the current study demonstrated that EA extract exhibited the major activity followed by HEXA extract, on the other hand CHLORO and T. ALCOHOL extracts showed less-significant beneficial effects on all measured parameters (data not shown). A previous work demonstrated a very preliminary study of the antioxidant activity of different extracts of *M. alysson* in vitro using DPPH reagent and anti-inflammatory effect using rat paw edema test, where the study revealed the activity of all the extracts in different strengths (Ghazy et al., 2011)

Although hepatotoxicity remains one of the concerns of statin use and frequent follow-up of liver markers is recommended. The results of the current study revealed a non-significant increase in serum ALT, AST and ALP in SIM treated group. To exclude such an adverse effect with *M. alysson* treatment, we examined the effect of the four extracts of *M. alysson* on serum liver enzymes. EA and HEXA extracts showed a more favorable effect both on liver transaminasese and ALP enzymes, indicating a lack of hepatotoxicity. Moreover, there was no evidence of toxicity of these extracts on the kidney as

manifested by non-significant change in BUN, serum creatinine and total protein. Overall, the safety profile of *M. alysson* extracts on this animal model was good and this is encouraging given the safety concerns raised for many of the therapeutic agents aiming at the cardiovascular system.

The results of our study coincide with previous study of the chemical constituents of *M. alysson*, where it was proved that the plant possesses a number of flavonoids accumulated mostly in the ethyl acetate extract including diosmetin, diosmetin-7-rhamnoside, chryseriol, luteolin, luteolin-7-rutinoside, acacetin, acacetin-7-glucoside and quercetin, moreover, all these flavonoids showed significant free radical scavenging activities when tested in the same study using DPPH reagent (Mohamed, 2010).

Our study contains some limitations. First, although lipid-lowering, antioxidation, anti-inflammation and decrease in plaque size effects of *M. alysson* have been clarified; the detailed molecular mechanisms underlying these effects require further investigation. Second, the potential additive effects of simvastatin and *M. alysson* on hypercholesterolemia plaque stabilization need to be tested.

5. Conclusion

Both SIM and *M. alysson* enhanced the stability of plaque in rabbits, had lipid lowering, anti-inflammation and anti-oxidation activities that may explain the mechanisms underlying the beneficial effects of SIM and *M. alysson*.

6. Funding

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