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Gynura procumbens ethanol extract and its fractions inhibit Th1, Th2 and Th17 but induce Treg cells differentiation during atherosclerosis development

Manimegalai Manogaran[1], Vuanghao Lim[2], Doblin Sandai[3], Rafeezul Mohamed[1*]

[1]Regenerative Medicine Cluster, Advanced Medical and Dental Institute, Universiti Sains Malaysia, Bertam, 13200 Kepala Batas, Pulau Pinang, Malaysia
[2]Integrative Medicine Cluster, Advanced Medical and Dental Institute, Universiti Sains Malaysia, Bertam, 13200 Kepala Batas, Pulau Pinang, Malaysia
[3]Infectomic Cluster, Advanced Medical and Dental Institute, Universiti Sains Malaysia, Bertam, 13200 Kepala Batas, Pulau Pinang, Malaysia

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ABSTRACT

Introduction: *Gynura procumbens* (Lorr.) Merr. (GP) displays cardio-protective effect, which may hinder atherogenesis induced by oxidized low-density lipoprotein (oxLDL) and leukocytes. The current study was undertaken to elucidate the chemical constituents of GP ethanol extract and its aqueous, chloroform, ethyl acetate, and hexane fractions, and their effects on CD4+ T cell differentiation during atherogenesis.

Methods: Initially, the bioactive constituents in GP ethanol extract and its fractions were analysed using gas chromatography-mass spectrometry (GC-MS). Generated mouse bone marrow dendritic cells (BMDC) were loaded with oxLDL and GP ethanol extract and its fractions for 24 hours and co-cultured with mouse CD4+ T cells for 72 hours. For the determination of T-bet, GATA-3, RORγt, Foxp3, DLL-3, and Jagged-1 mRNA gene expression, the floating cells (CD4+ T cells) and adherence cells (BMDC) were isolated from their total RNAs and reverse transcribed into cDNA.

Results: GC-MS analysis showed that GP ethanol extract and its fractions contained various volatile compounds. GP ethanol extract and its fractions also increased the DLL-3 gene but suppressed Jagged-1 gene expression in oxLDL-treated BMDC. Furthermore, GP ethanol extract and its fractions suppressed T-bet, GATA-3, and RORγt gene expression but increased the expression of the Foxp3 gene in differentiated CD4 + T cells.

Conclusion: GP ethanol extract and its fractions are composed of various bioactive chemical components that can induce anti-atherogenic effects by inhibiting pro-atherogenic cells such as Th1, Th2, and Th17 cells while increasing anti-atherogenic cells, Treg cells.

Implication for health policy/practice/research/medical education:
Anti-atherogenic effects of *Gynura procumbens* ethanol extract and its fractions observed in the current study showed their great potential as herbal products for utilisation in halting atherosclerosis development.

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Introduction

Gynura procumbens (Lour.) Merr. (GP) was originated from Astereceae family and primarily found in South East Asian countries such as Thailand, Indonesia, Malaysia, and China (1). GP is well known as Pokok sambung nyawa among Malaysian people (2). GP is utilised among old folk as a traditional medicine to treat kidney disease, cancer, constipation, migraines, constipation, and eruptive fever (3). Further studies showed that GP also had various pharmacological and therapeutic effects such as anti-hyperglycaemic, anti-hypertensive, anti-oxidative, anti-herpes simplex virus, and cardioprotective properties (4). These pharmacological benefits were induced by chemical constituents found in GP such as kaempferol, flavonoids, terpenoids, tannins, saponins, rutin, and sterol glycosides (4,5). Recently, LC-MS/MS analysis of GP ethanol extract and its fractions exhibited various biochemical compounds originated from flavonoids, fatty acids, sesquiterpenoids,

***Corresponding author**: Rafeezul Mohamed,
Email: rafeezul@usm.my

and the products of chlorophyll breakdown (6).

Atherosclerosis is a chronic inflammatory disease in the medium and large-sized arteries, which causes plaque formation, rupture of plaque, and ischemic injury leading to various clinical complications such as myocardial infraction, stroke, ischemic heart disease, and aneurysm (7). The innate and adaptive immune responses play pivotal roles in the initiation and progression of atherosclerosis development (8). The oxidative stress elicits LDL oxidation and stimulates endothelial cells to express P-selection, intercellular adhesion molecule 1 and vascular cell adhesion molecule 1, and potentiates chemoattractant factor and monocyte chemoattractant protein-1 that accelerate the migration of monocyte into the intima region of arteries (8). Within intima, monocyte transform to macrophage under influence of M-CSF will engulf oxidized low-density lipoprotein (LDL) via scavenger receptor and become foam cells to develop an early atherosclerotic plaque (9). On the other hand, dendritic cells (DCs), other innate immune cells, act as integral cells for innate and adaptive immune responses within the intima of arteries either by accelerating or suppressing atherosclerosis development (10). The activation of DCs by oxLDL stimulates the differentiation of naïve CD4+ T cells into T helper 1 (Th1), T helper 2 (Th2), Th17, and regulatory T cells (Treg) depending on the certain cytokine milieu of the microenvironment. Th1 cells mainly secrete interferon (IFN)-γ, Th2 cells secrete interleukin (IL)-4, IL-5, and IL-13, Th17 cells produce IL-17 and IL-22, and Treg cells secrete transforming growth factor (TGF)-β and IL-10. T-box transcription factor (T-bet), GATA-3, retinoic acid receptor-related orphan receptor gamma t (RORγt), and forkhead box P3 (Foxp3) are transcription factors that control the differentiation of Th1, Th2, Th17, and Treg cells, respectively (11). Th1, Th2, Th17 cells were demonstrated to promote atherogenesis whereby Treg cells inhibited atherosclerotic plaque development.

There is still no study to elucidate the effects of GP extract and its fractions on atherogenesis. GP ethanol extract at 500 mg/kg has displayed a better effect compared to a statin in lowering lipid deposition in the thoracic aorta (12). GP ethanol extract also had the capacity to lessen the activity of reactive oxygen species and hinder the destruction of membrane cell via modification of the antioxidant enzyme action in postmenopausal rats induced with atherogenic stimuli (12). Manogaran et al recently discovered that GP ethanol extract and its fractions inhibited the development of macrophage-derived foam cells by suppressing TNF-α and IL-1β expression, which was found to be high in atherosclerotic lesions. They also discovered that GP ethanol extract and its fractions suppressed the expression of the lectin-like oxLDL receptor-1 (LOX-1) gene, which interacts with atherogenic stimuli, oxLDL. Finally, they demonstrated that GP ethanol extract and its fractions stimulated the ATP-binding cassette transporter ABCA-1 gene that promoted the removal of lipids from macrophages (13). The present study was performed to determine the effect of GP ethanol extract and its fractions on CD4+ T cells differentiation during atherosclerosis development.

Material and Methods

Reagents and kits

Roswell Park Memorial Institute (RPMI) 1640 with L-glutamine, fetal bovine serum (FBS), penicillin and streptomycin, and phosphate-buffered saline (PBS) were purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). Recombinant mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) was obtained from R&D systems (Minneapolis, Minnesota, USA). Mouse CD4+ T cell isolation kit, LS column, and Midi MACS separator were bought from Miltenyi Biotech (Bergisch Gladbach, Germany). Tetro cDNA synthesis kit and SensiFAST SYBR Hi-Rox kit were purchased from Meridian Bioscience (Memphis, Tennessee, USA). OxLDL was bought from Alfa Aesar, Haverhill, Massachusetts, USA. Hamster Anti-Mouse CD11c PE and Rat-anti-mouse MHC II FITC were obtained from BD Bioscience, San Jose, California, USA.

Animals

Six weeks-old C57BL/6 male mice were purchased from Animal Research and Service Centre (ARASC), Universiti Sains Malaysia. Splenocytes, femur and tibia were collected at termination for downstream experiments. All procedures were carried out after obtaining an ethical approval from Institutional Animal Care and Use Committee (IACUC) of Universiti Sains Malaysia [USM/Animal Ethics Approval/2015/ (705)].

Preparation of GP ethanol extract and its fractions

Fresh leaves of GP were purchased from Herbagus Sdn. Bhd. at Kepala Batas, Penang, Malaysia (GPS coordinate: 5.5210° N, 100.4370° E) and a sample of the plant was identified by a botanist from the School of Biological Sciences, Universiti Sains Malaysia and deposited at the USM Herbarium with voucher number of 11753. The preparation of GP ethanol extract and its fractions were described by Manogaran et al (6,13).

Gas chromatography–mass spectrometry (GC-MS)

The GC-MS analysis was performed using Agilent Technologies gas chromatography 6890 coupled with an Agilent 5973 mass spectrometry with HP-5MS column (30 m long × 0.25 mm ID × 0.25 μm film thickness). The oven temperature was initiated at 70°C and maintained at this temperature for 2 minutes. The oven temperature was then raised to 280°C at a rate of 20°C/min and sustained for 30 minutes. One μL of GP ethanol extract and fraction samples were injected into the column at a temperature of 250°C, with helium as the carrier gas at a flow rate of 1.2

mL/min. The ionisation voltage was set to 70 eV, and the samples were injected in a 5:1 split mode. Identification and interpretation on mass spectra of GC-MS of GP extract and fractions were carried out by comparing the database from National Institute Standard and Technology (NIST).

Isolation of CD4+ T cells

The spleens of C57BL/6 mice were collected after they were euthanized by cervical dislocation and held in full RPMI medium. The collected spleens were gently teased apart to generate a single cell suspension in a complete RPMI medium containing 10% FBS and 1% antibiotics (250 U/mL penicillin, 250 µg/mL streptomycin) in a petri dish using a syringe plunger. The cells were transferred into a 70 µm cell strainer fitted on a 50 mL tube and centrifuged for 10 minutes at 300 × g. The cell pellet was resuspended in 1x M-lyse buffer after centrifugation and thoroughly mixed by vortexing before incubation at room temperature for 10 minutes. The cells were washed by centrifugation in complete RPMI medium for 10 minutes at 300 × g. The supernatant was removed, and the cell pellet was resuspended in one mL complete RPMI medium for haemocytometer counting. Subsequently, CD4+ T cells were isolated using mouse CD4+ T cell isolation kit according to manufacturer's instructions. Briefly, 2×10^8 cells were resuspended in 400 µL of PBS followed by labelling with 100 µL of the biotin-antibody cocktail and were incubated for 5 minutes at 4°C. The cells were then combined with 300 µL of PBS and 200 µL of anti-biotin microbeads and incubated at 4°C for 10 minutes. Subsequently, the CD4+ T cells were isolated by loading labelled cells onto LS separation column placed in the magnetic field of MACS separator. Unlabelled cells were collected as enriched CD4+ T cells, while labelled cells (non-CD4+ T cells) remained in the column due to magnetic beads attached to their surface. The suspension was carefully removed after separation and centrifuged for 10 minutes at 300 × g. The cells were counted after being resuspended in 1 mL of complete RPMI medium.

Generation of bone marrow-derived dendritic cells (BMDCs)

The femur and tibia from the hind legs of C57BL/6 mice were collected after they were euthanized. The excess tissues were removed from the femur and tibia, which were then kept in full RPMI medium. The femur and tibia were disinfected by rinsing them twice with 70% ethanol, then flushing them through 10 mL of complete RPMI medium 2-3 times with a syringe and a 25-gauge needle into a centrifuge tube. The cells were then washed twice with complete RPMI medium before being centrifuged at 300 ×g for 10 minutes. The contaminant erythrocytes were eliminated by resuspending the pellet in 1x M-lyse buffer for 5 minutes. The cells were vortexed and incubated at room temperature for 10 minutes. After full red cell lysis, the cells were centrifuged at 300 ×g for 10 minutes in a complete RPMI medium. Prior to cell counting with a haemocytometer, the cell pellet was resuspended in one mL complete RPMI medium. The bone marrow derived cells (BMDC) were cultured at a concentration of 2×10^7 in 75 cm^2 cell culture flasks for seven days in a complete RPMI medium supplemented with 25 ng/mL rmGM-CSF. The cultures were replenished with fresh complete medium added with 25 ng/mL rmGM-CSF for every 3 days. The semi-suspended cells and loosely attached cells were collected on day seven by gently pipetting the medium against the plate and collecting them in a 50 mL tube before centrifugation at 300 × g for 10 minutes to wash them. The cells were counted after being resuspended in one ml of complete RPMI medium. The generated BMDC was confirmed by surface staining of CD11c and MHCII using flow cytometry. The cell density was adjusted to 1×10^6 cells/mL in a complete RPMI medium and plated about 2 mL/well in 6-well plates. BMDC was treated with oxLDL, GP ethanol extract and its fractions, and a combination of both for 24 hours in an incubator at 37°C and 5% CO_2.

Co-culture of CD4+ T cells with BMDC

The purified CD4+ T cells (2×10^6 cells/well) were co-cultured with treated BMDC (2×10^6 cells/well) at a ratio of 1:1 (BMDC: CD4+ T cells) and were incubated for three days. On day 3, CD4+ T cells (non-adherence) and BMDC (adherence) were harvested, and total RNA was extracted followed by cDNA synthesis for mRNA gene expression quantification using qRT-PCR.

Total RNA extraction

Total RNA of differentiated CD4+ T cells (non-adherence) and activated BMDC (adherence) were extracted using TRIzol® reagent based on the manufacturer's instructions. The cells were lysed and homogenized by adding 1 mL of TRIzol® reagent directly to the cells and by passing the cell lysate several times through a pipette. Following homogenization, 0.2 mL chloroform was added, vigorously mixed, and incubated for 5 minutes at room temperature. To allow the homogenate to form a mixture of lower red phenol-chloroform, interphase, and colourless upper aqueous phase, it was centrifuged at 12 000 ×g for 20 minutes at 4°C. The RNA-containing aqueous phase was carefully transferred to a new tube, and 0.5 mL of isopropanol was applied to the RNA, which was incubated for 10 minutes at room temperature before being centrifuged at 12 000 × g for 20 minutes at 4°C. The pellet was vortexed and centrifuged for 10 minutes at 7500 × g at 4°C after being washed with 75 % ethanol. The RNA pellets were air-dried before being dissolved in 40 µL of RNase-free water. By loading one µL of sample onto Nano Drop, the concentration and purity of RNA were determined.

Complementary DNA (cDNA) synthesis

The extracted RNA was reverse transcribed into cDNA using the Tetro cDNA synthesis kit following the manufacturer's protocol. Briefly, 2000 ng of total RNA was mixed with 1 µL oligo (dT)18, 1 µL of 10 mM dNTP, 4 µL of 5x RT buffer, 1 µL of RiboSafe RNA inhibitor, 1 µL of Tetro reverse transcriptase (200/µL), and DEPC-treated water with a total volume of 20 µL in a tube. The mixture was briefly vortexed and incubated at 45°C for 30 minutes followed by incubation at 85°C for 5 minutes before being chilled on ice to terminate the reaction of the reverse transcriptase.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

A SensiFAST SYBR Hi-Rox kit was used to conduct a quantitative real-time polymerase chain reaction (qRT-PCR) to detect relative mRNA expression level. The fluorescence intensity was measured by StepOne Plus QPCR system. Reaction master mix was prepared in a final volume of 20 µL (10 µL 2x SensiFAST SYBR® Hi-ROX Mix, 0.8 µL primer pair mixture [10 µM of each primer], 1 µL cDNA, and 7.4 µL H_2O). The cycling conditions was performed under the following conditions: 95°C for 2 minutes, followed by 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds. The primer sequences of target genes were as follows:
T-bet: 5'-CCTGGACCCAACTGTCAACT-3' (F)
5'-AACTGTGTTCCCGAGGTGTC-3' (R),
GATA-3: 5'-CCGAAACCGGAAGATGTCTA-3'
(F) 5'-AGGGCTCTGCCTCTCTAACC-3' (R),
RORγt: 5'TGCAAGACTCATCGACAAGG-3'
(F) 5'-AGGGGATTCAACATCAGTGC-3' (R),
Foxp3: 5'-CAACCTAGCCCCAAGATGAA-3' (F)
5'-CCAGATGTTGTGGGTGAGTG-3' (R), Jagged-1:
5'-AGAAGTCAGAGTTCAGAGGCGTCC-3'
(F) 5'-AGTAGAAGGCTGTCACCAAGCAAC-3'
(R), DLL-3: 5'-TCGTACGTGTGCCCTTCC-3'
(F) 5'-TGCTCTCCAGGTTTCAATG-3' (R),
GAPDH: 5'-AGAACATCATCCCTGCATCC-3' (F)
5'-CACATTGGGGGTAGGAACAC-3' (R).

Relative mRNA expression was calculated using the ΔΔCt method and the housekeeping gene GAPDH was used for normalization.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism Version 8.0 software. Values of the data were presented as mean ± standard deviation (SD) from three independent experiments and analysed using one-way analysis of variance (ANOVA) followed by the Tukey post hoc test.

Results

GC-MS analysis of GP ethanol extract and fractions

The identification of the volatile bioactive compounds of GP ethanol extract and its fractions were performed by comparing the mass of the unknown compounds with the mass of known compounds in the NIST library. The volatile bioactive compounds with more than 90% matching quality were presented. The total ion chromatogram (TIC) of GP ethanol extract is shown in Figure 1A. The chemical constituents present in GP ethanol extract were phenol, 2,4-bis(1,1-dimethylethyl), alpha-calacorene, 1-tetradecene, naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-, hexadecanoic acid, methyl ester, hexadecanoic acid, ethyl ester, linoleic acid ethyl ester and gamma-sitosterol (Table 1). The TIC for GP hexane fraction is presented in Figure 1B. The volatile

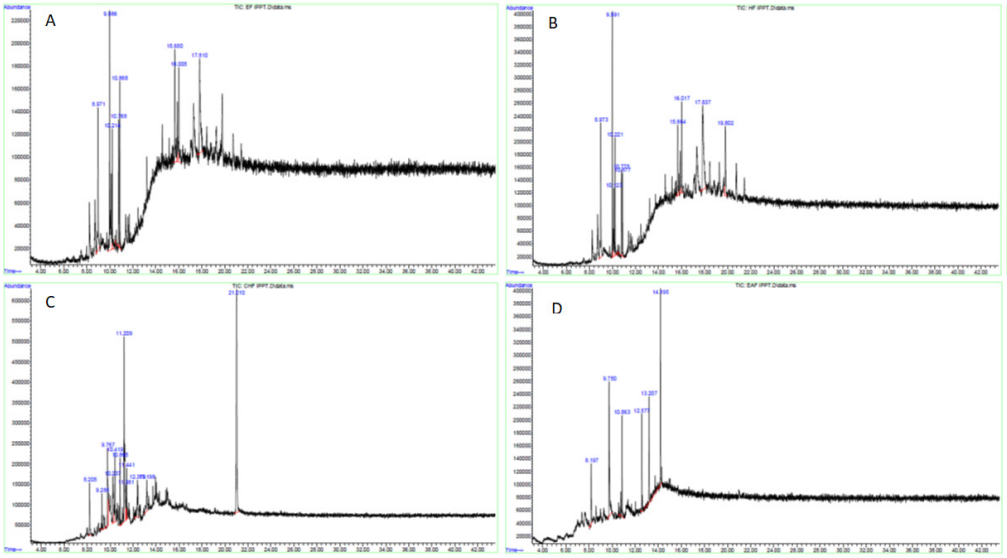

Figure 1. The total ion chromatogram (TIC) generated from gas chromatography–mass spectrometry (GC-MS) analysis. The TIC generated for *Gynura procumbens* (GP) ethanol extract (A), hexane fraction (B), chloroform fraction (C) and ethyl acetate fraction (D).

bioactive compounds detected in the GP hexane fraction were hexadecanoic acid, ethyl ester, phytol, stigmasterol and stigmasterol, 22,23-dihydro- (Table 2). The TIC for GP chloroform fraction is displayed in Figure 1C. The chemical compounds found in the GP chloroform fraction were phenol, 2,4-bis(1,1-dimethylethyl), pentadecane, 2-methyl-, cyclotetradecane and 1-octadecene (Table 3). The TIC for GP ethyl acetate fraction is shown in Figure 1D. The bioactive compounds detected in the GP ethyl acetate fraction listed in Table 4 were Hexanedioic acid, bis(2-ethylhexyl) ester, decanedioic acid, bis(2-ethylhexyl) ester and hexadecane. However, no compounds were detected in the GP aqueous fraction because the peaks were not identifiable.

GP ethanol extract and its fractions reduced Jagged-1 gene but increased DLL-3 gene expression in oxLDL-treated BMDC

The interaction of notch ligands on the surface of APCs with notch receptors on T-cells plays a crucial role in T-cell differentiation. Jagged 1 and delta-like 3 (DLL-3) are well recognised membrane bound notch ligands. Initially, the flow cytometry analysis revealed that generated BMDC expressed 75% of CD11c and 35% of major histocompatibility complex (MHC) class II (Figure 2). Subsequently after treatment of BMDC and co-culturing with CD4+ T cells, Jagged-1 gene was highly expressed in oxLDL-treated BMDC for 72 hours (Figure 3A). GP ethanol extract and its fractions significantly inhibited Jagged-1 gene expression ($P<0.001$) in oxLDL-treated BMDC after 72 hours (Figure 3A). In contrast, low expression of DLL-3 gene was detected in oxLDL-treated BMDC after 72 hours (Figure 3B). However, GP ethanol extract and its fractions significantly stimulated DLL-3 gene expression in oxLDL-treated BMDC after 72 hours ($P<0.001$) (Figure 3B).

CD4+ T cells co-cultured with combination of oxLDL and GP ethanol extract and its fractions-treated BMDC reduced T-bet, GATA-3 and RORγt genes but increased Foxp3 gene expression

The stimulation of signal transducer activator of transcription 4 (STAT-4) and STAT-1 signalling pathway induced the expression of Th1 transcription factor, T-bet. T-bet gene was highly expressed when CD4+ T cells were co-cultured with oxLDL-treated BMDC for 72 hours (Figure 4A). However, T-bet gene expression was significantly inhibited when CD4+ T cells were co-cultured with the combination of oxLDL and GP ethanol extract and its fractions-treated BMDC for 72 hours ($P<0.001$) (Figure 4A). GATA-3 gene is a stimulant for Th2 related cytokine, IL-4. GATA-3 gene was highly stimulated when CD4+ T cells were co-cultured with oxLDL-treated BMDC for 72 hours (Figure 4B). However, GATA-3 gene expression was significantly reduced when CD4+ T cells were co-cultured with the combination of

Table 1. The detected volatile bioactive compounds of *Gynura procumbens* (GP) ethanol extract from gas chromatography–mass spectrometry (GC-MS) analysis

Chemical constituents	Chemical formula	Retention time (min)	Composition (%)
Phenol, 2,4-bis(1,1-dimethylethyl)	$C_{17}H_{30}OSi$	8.155	93
Alpha-calacorene	$C_{15}H_{20}$	8.412	96
1-Tetradecene	$C_{14}H_{28}$	8.572	93
Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-	$C_{15}H_{24}$	8.919	96
Hexadecanoic acid, methyl ester	$C_{29}H_{50}O$	10.398	96
Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	10.724	93
Linoleic acid ethyl ester	$C_{20}H_{36}O_2$	11.529	98
Gamma–Sitosterol	$C_{29}H_{50}O$	21.089	94

Table 2. The volatile bioactive compounds of GP hexane fraction identified using GC-MS

Chemical constituents	Chemical formula	Retention time (min)	Composition (%)
Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	10.731	98
Phytol	$C_{20}H_{40}O$	11.328	90
Stigmasterol	$C_{29}H_{48}O$	20.054	97
Stigmasterol, 22,23-dihydro-	$C_{29}H_{50}O$	21.075	97

Table 3. The volatile bioactive compounds of GP chloroform fraction identified using GC-MS

Chemical constituents	Chemical formula	Retention time (min)	Composition (%)
Phenol, 2,4-bis(1,1-dimethylethyl)	$C_{17}H_{30}OSi$	8.162	93
Pentadecane, 2-methyl-	$C_{16}H_{34}$	9.259	90
Cyclotetradecane	$C_{14}H_{28}$	10.175	95
1-Octadecene	$C_{18}H_{36}$	11.168	97

Table 4. The volatile bioactive compounds of GP ethyl acetate fraction detected using GC-MS

Chemical constituents	Chemical formula	Retention time (min)	Composition (%)
Hexanedioic acid, bis(2-ethylhexyl) ester	$C_{22}H_{42}O_4$	10.766	91
Decanedioic acid, bis(2-ethylhexyl) ester	$C_{26}H_{50}O_4$	14.709	94
Hexadecane	$C_{16}H_{34}$	15.653	94

oxLDL and GP ethanol extract and its fractions-treated BMDC for 72 hours ($P<0.001$) (Figure 4B). In addition, Foxp3 is a well-known transcription factor for Treg cells. When CD4+ T cells were co-cultured with oxLDL-treated BMDC for 72 hours, the expression of the Foxp3 gene was low (Figure 4C). However, Foxp3 gene expression was significantly increased when CD4+ T cells were co-cultured with the combination of oxLDL and GP ethanol extract and its fractions treated BMDC for 72 hours ($P<0.001$) (Figure 4C). RORγt is also a key regulator that promotes the differentiation of Th17 cells. When CD4+ T cells were co-cultured with oxLDL-treated BMDC for 72 hours, the RORγt gene was highly detectable (Figure 4D). However, the significant reduction of RORγt gene expression was observed when $CD4^+$ T cells were co-cultured with the combination of oxLDL and GP ethanol extract and its fractions-treated BMDC for 72 hours ($P<0.001$) (Figure 4D).

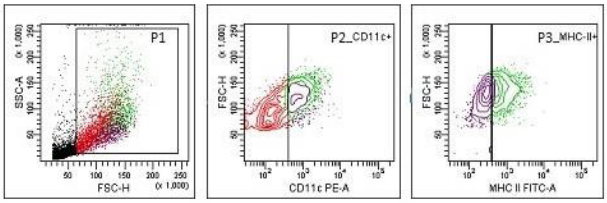

Figure 2. Characterization of generated bone marrow derived dendritic cell (BMDC). BMDC was stained with CD11c-Phycoeryhtrin (PE) and MHC II- Fluorescein isothiocyanate (FITC) and analysed with BD FACS Canto™ cell analyser. The contour plots were generated using BD FACS DIVA™ software.

Discussion

The current study discovered 17 volatile compounds that were detected using GC-MS, which may have an anti-atherogenic effect. The detection of these volatile

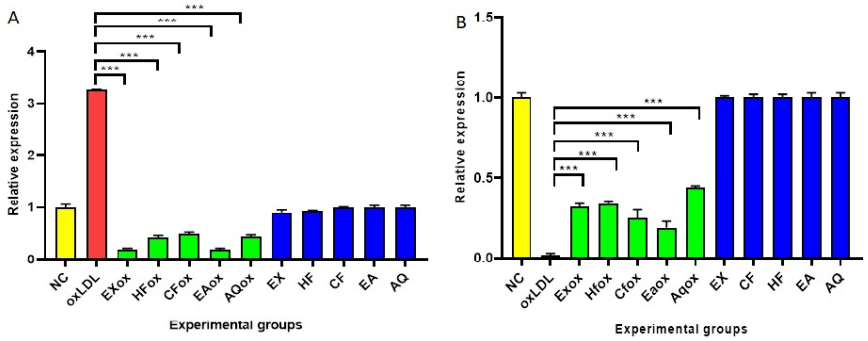

Figure 3. GP ethanol extract and its fractions reduced Jagged-1 gene expression but increased DLL-3 gene expression in oxLDL-treated BMDC after 72 hours. Total RNA was extracted from untreated and treated BMDC at 72 hours and synthesized into cDNA. Jagged-1 (A) and DLL-3 (B) gene expressions were quantitated using qRT-CR. Values are expressed as mean ± SD from three independent experiments (n=3). ***P<0.001 compared to oxLDL group vs. combination of oxLDL + extract/ fractions treated groups. (NC=untreated, oxLDL=oxLDL, Exox=GP extract+oxLDL, HFox=Hexane fraction+oxLDL, CFox=Chloroform fraction+oxLDL, EAox=Ethyl acetate fraction+oxLDL, AQox=Aqueous fraction+oxLDL, EX= GP extract, HF=Hexane fraction, CF=chloroform fraction, EA=Ethyl acetate fraction, AQ=Aqueous fraction).

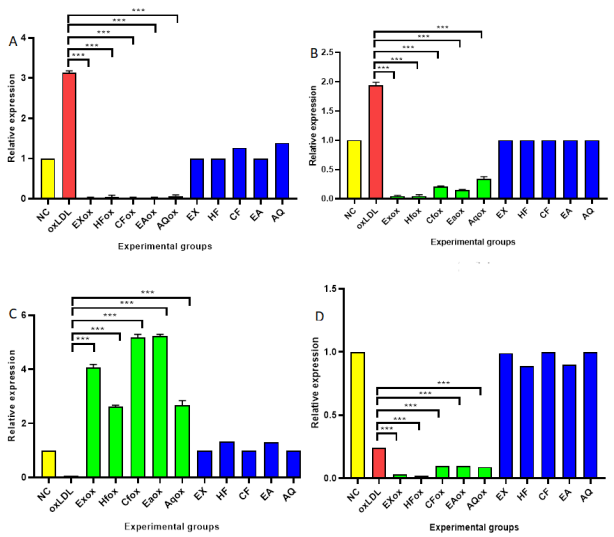

Figure 4. CD4+ T cells, that were co-cultured with the combination of oxLDL and GP ethanol extract and its fractions treated BMDC reduced T-bet, GATA-3, and RORγt, but induced Foxp3 gene expressions. CD4+ T cells were co-cultured with treated BMDC for 72 hours. Total RNA was extracted from proliferated CD4+ T cells and synthesized into cDNA. T-bet (A), GATA-3 (B), Foxp3 (C), and RORγt (D) expressions were quantitated using qRT-PCR. Values are expressed as mean ± SD from three independent experiments (n=3). ***P<0.001 compared to oxLDL group vs. combination of oxLDL + extract/ fractions treated groups. (NC = untreated, oxLDL = oxLDL, Exox = GP extract+oxLDL, HFox = Hexane fraction+oxLDL, CFox = Chloroform fraction+oxLDL, EAox = Ethyl acetate fraction+oxLDL, AQox = Aqueous fraction+oxLDL, EX = GP extract, HF = Hexane fraction, CF=chloroform fraction, EA=Ethyl acetate fraction, AQ = Aqueous fraction).

compounds was done using a library with a consistency of over 90%, their retention time, and their molecular formula. 2,4-Bis (1,1-dimethylethyl) phenol, which was found in GP ethanol extract and chloroform fraction, has potent antioxidant activity because it can scavenge free oxygen radicals when compensating for reactive oxygen species (14). Palmitic acid compounds such as decanedioic acid, bis(2-ethylhexyl) ester, hexadecanoic acid, methyl ester, and hexanedioic acid, bis(2-ethylhexyl) ester have antioxidant activities and anti-hypocholesterolaemia effects (15). Furthermore, hexadecanoic acid methyl ester found in both the GP ethanol extract and the GP hexane fraction, had the ability to lower blood cholesterol while inhibiting the cyclooxygenase II enzymes from activating anti-inflammatory cytokines (16). Further, this phytol was found in the GP hexane fraction and has been shown to have a significant antioxidant function through reducing hydroxyl radicals and nitric oxide, as well as preventing the production of thiobarbituric acid reactive substances (17). Next, stigmasterol and its derivative, 22,23-dihydro-stigmasterol showed anti-hyperlipidemia, anti-hypercholesterolemia, antioxidant, and anti-inflammatory properties. (18). In addition, stigmasterol induced cholesterol efflux and inhibited the secretion of tumour necrosis alpha (TNFα), IL-6, and IL-1β to hinder the formation of macrophage derived foam cells (19). Phytosterols were found in the GP ethanol extract and the hexane fraction, with mixed results in a mouse model of atherosclerosis. Phytosterol treatment in LDL receptor-deficient mice reduced plasma cholesterol and vascular atherosclerotic plaque area (20). However, ApoE deficient mice treated with phytosterol accelerated endothelial dysfunction, induced atherosclerotic plaque formation in comparison to mice treated with ezetimibe, a cholesterol absorption inhibitor (21). Following that, linoleic acid ethyl ester was discovered in GP ethanol extract and was found to have hypocholesterolemic and anti-coronary properties (22). Finally, anti-cancer, antioxidant, and anti-microbial activities have been identified for 1- tetradecene and 1-octadene, which were found in GP ethanol extract and GP chloroform fraction, respectively (23). The volatile compounds found in GP ethanol extract, such as phytol, oleic acid, and phenol, were confirmed in a study by Abidin et al, who found five basic volatile compounds in GP ethanol extract, including 2-hexenal, phenol, oleic acid, copaene, and phytol (24).

As an important link between innate and adaptive immune responses, DCs play a critical role in the regulation of antigen-specific immune response. Antigen processing in DCs resulted in the presentation of a peptide-MHC class II complex on the surface of DCs, which was recognised by naive CD4+ T cells through T cell receptors. These engagements stimulate the differentiation of naïve CD4+ T cells into various CD4+ T cells subsets and concomitantly secreted a wide array of pro-inflammatory cytokines. The addition of granulocyte monocyte colony-stimulating factor (GM-CSF) induced the differentiation of murine BMDCs from their respective precursors (25). According to the analysis of Ly6C+ monocytes in GM-CSF cultures, bone marrow cells were cultured for two hours to enable monocytes to bind to the culture plate, and then the adherence cells were treated with GM-CSF for seven days to see whether GM-CSF-derived BMDCs were derived from monocytes (26). Furthermore, in many studies of inflammation, GM-CSF causes monocytes to convert into CD11c+ MHCII+ cells (27). Here, we showed that murine BMDCs expressed CD11c+ MHCII^{low} cells. Murine BM cells treated with GM-CSF consisted of neutrophils until day five before they were diminished and left only with loosely attached MHC II^{low} cells, although firmly attached MHC II^{neg} Mphs remained (25). The MHC II^{low} cells consist of immature DCs with the possibility to grow as mature DCs, whereby Mph progenitors expand as MHC II^{neg} Mphs (28).

Subsequently, after co-culturing of treated BMDC with CD4+ T cells for three days, the adherence cells, which served as activated BMDC, were evaluated by performing total RNA extraction and cDNA synthesis to determine the expression of notch ligands, Jagged-1, and DLL-3 genes using qRT-PCR. The notch signalling

pathway is a cell signalling system that coordinates the communication between each cell and regulates a variety of cellular behaviours such as cell proliferation, differentiation, and death (29). Four notch receptors (Notch 1-4) and five notch ligands (DLL-1, 3, 4 and Jagged-1 and 2) were discovered in mammals (29). The engagement of notch ligands on the surface of APCs and notch receptors expressed by CD4+ T cells play an important role in regulating the differentiation of CD4+ T-cell (30). Since these ligands have been confirmed to be expressed in BMDC, the current research focused on notch ligands Jagged-1 and DLL-3 (31). The Jagged-1 and DLL-3 genes were substantially overexpressed in both untreated and GP ethanol extract and fractions treated BMDC, according to our findings. A previous study also showed that unstimulated or untreated DCs exhibited low level of notch ligand, Delta and Jagged (31). However, the addition of GP ethanol extract and its fractions in oxLDL-treated BMDC for three days significantly decreased the expression of Jagged-1 gene but significantly increased DLL-3 gene expression. Our findings backed up a previous study that found that either Jagged or Delta expression could be aggravated and that the expression can change depending on the circumstances and stimuli (31). Furthermore, the interaction of Jagged-1 and DLL-3 with their respective receptors has an effect on the differentiation of CD4+ T cell subsets. LPS-stimulated DCs elicited Delta-4 and Jagged-1 expressions, which induced Th1-type responses, according to previous research (32). In addition, other studies found that notch ligands expressed by mouse DCs were linked to Th1/Th2 responses (32). Delta-1-expressing DCs elicited Th1-type responses, while Jagged-1-expressing DCs elicited Th2-type responses (30). Moreover, DLL-3 was shown to stimulate Th17 differentiation in vitro (33). Intense expression of Jagged-1 in splenic DC could stimulate the differentiation of CD4+ T cells into Treg cells (32). Therefore, we speculated that Jagged-1 elicited Th1, Th2 and, Treg cell responses whereby DLL-3 stimulated Th17 cells differentiation. However, no other studies reported the effect of GP ethanol extract and its fractions on Jagged-1 and DLL-3 expressions in oxLDL-treated BMDC.

The second-largest immune cells detected in the atherosclerotic plaque after macrophages are T lymphocytes, including CD4+ T cells. Each CD4+ T cell subset has been shown to play either pro-atherogenic or anti-atherogenic role. Therefore, we investigated the effect of GP ethanol extract and fractions on the differentiation of CD4+ T cells during atherosclerosis development. The current study showed that Th1 transcription factor, T-bet gene was increased when CD4+ T cells were co-cultured with oxLDL-treated BMDC for three days. However, T-bet gene expression was hindered when CD4+ T cells were co-cultured with the combination of oxLDL and GP ethanol extract and fractions treated with BMDC. Th1 cells expressed transcription factor T-bet and secreted a wide range of pro-inflammatory cytokines such as IFN-γ, TNF-α, IL-2, IL-1β, and IL-12 and accelerated atherosclerosis progression (34). It also has been revealed that low-density lipoprotein receptor-deficient (LDLr $^{-/-}$) mice, which lacks of T-bet gene could not differentiate into Th1 cells resulting a significant reduction of atherosclerosis lesion development (35). On the other hand, Th2 cells are differentiated from naïve CD4+ T cells via IL-4 stimulating STAT-6, increasing the expression of GATA-3 (36). The Th2 cells mainly secret IL-4, IL-5, and IL-13 (36). Similar to T-bet, the current study demonstrated that GATA-3 gene was highly expressed when CD4+ T cells were co-cultured with oxLDL-treated BMDC for three days. However, the expression of GATA-3 gene was inhibited when CD4+ T cells were co-cultured with the combination of oxLDL and GP ethanol extract and fractions treated with BMDC.

The role of Th2 cells in the formation of atherosclerotic plaques is still debated. IL-4, a Th2 cytokine, has been shown to suppress Th1 responses, implying that IL-4 in ApoE-/- mice has an atheroprotective impact (37). In contrast, stimulation of Th2 responses against apoB-100 did not have effect on atherosclerosis development in ApoE$^{-/-}$ mice despite obvious expression of Th2 related cytokines such as IL-4, IL-5, and IL-10 (38).

Treg cells play a critical role in immune homeostasis and the maintenance of the self-tolerance mechanism (39). Treg cells are classified into two types based on their origin: normal Treg cells (nTreg) and inducible Treg cells (iTreg) (39). nTregs are characterised by the expression of CD4, CD25 and the transcriptional factor Foxp3 (39). The current study found that CD4+ T cells co-cultured with oxLDL-treated BMDC for three days had low expression of the Foxp3 gene. In contrast, Foxp3 gene was increased when CD4+ T cells were co-cultured with the combination of oxLDL and GP ethanol extract and fractions treated with BMDC. The deficiency of Treg cells in hypercholesterolemic mice promoted atherosclerosis development and vascular inflammation (40). Administration of Foxp3 T cells in ApoE$^{-/-}$ mice led to the inhibition of atherosclerosis progression (41). Furthermore, a previous study found that vaccination against Treg cells accelerated the formation of atherosclerotic plaques (42). Th17 cells, on the other hand, are a subset of CD4+ T cells that are stimulated by IL-6 and TGF- and generate IL-17A, IL-17F, and IL-22 (43). RORγt is the transcriptional factor that influences the differentiation of Th17 cells (43). The current study showed that RORγt gene was highly expressed when CD4+ T cells were co-cultured with oxLDL and treated with BMDC for three days. However, the expression of RORγt gene was reduced when CD4+ T cells were co-cultured with the combination of oxLDL and GP ethanol extract

and its fractions treated with BMDC. A previous research has shown that proatherogenic conditions and atherogenic stimuli like oxLDL stimulate Th17 cells, which is similar to what we found in our study (44). Furthermore, a study by Taleb et al found that high IL-17 expression in carotid plaques was associated with a low macrophage count and a high number of smooth muscle cells, implying that IL-17A improved atherosclerotic plaque stability (45).

A previous study by Dwijayanti et al indicated that 1 µg/mL of GP ethanol extract elicited the activation of CD4+CD25+, CD4+CD62L-, CD4+CD62L+, CD8+CD62L-, and CD8+CD62L+ T cells. However, GP ethanol extract at the concentration of 10 µg/mL repressed the activation of CD4+CD62L-, CD4+CD62L+, CD8+CD62L, and CD8+CD62L+ T cells but augmented the B cells proliferation (46). A recent study by Takanashi et al. showed that a cold abstraction extract of GP known as Toleaf stimulated IFN-γ production in splenic cells and aggravated IL-2, IL-4, and IL-12-mRNA expression levels, which enhanced Th1 and Th2 type responses (47). Further research is required because the differentiation of naive CD4+ T cells into Th1, Th2, Th17, and Treg cells occur in a complex microenvironment that is not only influenced by the expression of notch ligands but also by other factors. These include a variety of pathogen-associated molecular patterns, type of APCs, stability of T cell receptor engagement, co-stimulation durability, cytokine concentration, type of cytokine-induced signal transducer and activator of transcription factor signalling, stimulation of lineage-specific transcription factors, and eliciting of lineage-associated transcription factors (48).

Conclusion

The findings showed that the GP ethanol extract and its fractions contained a broad range of volatile compounds that might have an anti-atherogenic effect by suppressing pro-atherogenic cells, including Th1, Th2, and Th17 cells while increasing the population of Treg cells, which are typically low in atherosclerosis lesions. The anti-atherogenic effects of GP ethanol extract and its fractions on certain CD4+ T cell subsets that promote atherogenesis revealed that they have a lot of potential as herbal products for preventing atherosclerosis.

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Authors' contributions

MM performed the experiment. LV, DS, and RM designed the research workflow. RM wrote the manuscripts and was the principal investigator of the FGRS grants that financed this project. All authors have read and confirmed the final version of the submitted manuscript for publication.

Conflict of interests

The authors declared that they had no conflicts of interest in any kind.

Ethical considerations

Animal ethical approval was obtained from the Institutional Animal Care and Use Committee (IACUC) of Universiti Sains Malaysia [USM/Animal Ethics Approval/2015/ (705)].

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References

1. Tan HL, Chan KG, Pusparajah P, Lee LH, Goh BH. *Gynura procumbens*: an overview of the biological activities. Front Pharmacol. 2016;7:52. doi: 10.3389/fphar.2016.00052.
2. Bodeker G, Jaenickle C, Gruenwald J, Salleh H, Hashim RS. Health and Beauty from the Rainforest: Malaysian Traditions of Ramuan. 1st ed. Kuala Lumpur: Editions Didier Millet; 2009.
3. Afandi A, Halimhilmi Zulkiffli M, Sadikun A, Ismail S. Antioxidant properties of *Gynura procumbens* extracts and their inhibitory effects on two major human recombinant cytochrome P450s using a high throughout luminescence assay. Asian J Pharm Clin Res. 2014;7(5):36-41.
4. Kaewseejan N, Puangpronpitag D, Nakornriab M. Evaluation of phytochemical composition and antibacterial property of *Gynura procumbens* extract. Asian J Plant Sci. 2012;11(2):77-82. doi: 10.3923/ajps.2012.77.82.
5. Zahra AA, Kadir FA, Mahmood AA, Al hadi AA, Suzy SM, Sabri SZ, et al. Acute toxicity study and wound healing potential of *Gynura procumbens* leaf extract in rats. J Med Plants Res. 2011;5(12):2551-8. doi: 10.5897/jmpr.9000042.
6. Manogaran M, Lim V, Mohamed R. Phytoconstituents of the *Gynura procumbens* ethanol leaf extract and its fractions and their effects on viability of macrophages. J Herbmed Pharmacol. 2019;8(3):224-30. doi: 10.15171/jhp.2019.33.
7. Libby P. Inflammation in atherosclerosis. Nature. 2002;420(6917):868-74. doi: 10.1038/nature01323.
8. Hansson GK, Hermansson A. The immune system in atherosclerosis. Nat Immunol. 2011;12(3):204-12. doi: 10.1038/ni.2001.
9. Weber C, Zernecke A, Libby P. The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. Nat Rev Immunol. 2008;8(10):802-15. doi: 10.1038/nri2415.
10. Herrero-Fernandez B, Gomez-Bris R, Somovilla-Crespo B, Gonzalez-Granado JM. Immunobiology of atherosclerosis: a complex net of interactions. Int J Mol Sci. 2019;20(21):5293. doi: 10.3390/ijms20215293.
11. Witztum JL, Lichtman AH. The influence of innate and adaptive immune responses on atherosclerosis. Annu Rev Pathol. 2014;9:73-102. doi: 10.1146/annurev-pathol-020712-163936.

12. Ahmad Nazri KA, Fauzi NM, Buang F, Mohd Saad QH, Husain K, Jantan I, et al. *Gynura procumbens* standardised extract reduces cholesterol levels and modulates oxidative status in postmenopausal rats fed with cholesterol diet enriched with repeatedly heated palm oil. Evid Based Complement Alternat Med. 2019;2019:7246756. doi: 10.1155/2019/7246756.
13. Manogaran M, Vuanghao L, Mohamed R. *Gynura procumbens* ethanol extract and its fractions inhibit macrophage derived foam cell formation. J Ethnopharmacol. 2020;249:112410. doi: 10.1016/j.jep.2019.112410.
14. Salini TS, Divakaran D, Shabanamol S, Rebello S, Jisha MS. Antimicrobial and immunomodulatory potential of endophytic fungus *Fusarium solani* isolated from *Withania somnifera*. World J Pharm Res. 2014;3(10):879-90.
15. Dineshkumar G, Rajakumar R. GC-MS evaluation of bioactive molecules from the methanolic leaf extract of *Azadirachta indica* (A.JUSS). Asian J Pharm Sci Technol. 2015;5(2):64-9.
16. Hema R, Kumaravel S, Alagusundaram K. GC/MS determination of bioactive components of *Murraya koenigii*. J Am Sci. 2011;7(1):80-3.
17. Santos CC, Salvadori MS, Mota VG, Costa LM, de Almeida AA, de Oliveira GA, et al. Antinociceptive and antioxidant activities of phytol in vivo and in vitro models. Neurosci J. 2013;2013:949452. doi: 10.1155/2013/949452.
18. Havyarimana L, Ndendoung ST, Tamokou JD, Atchadé AT, Tanyi JM. Chemical constituents of *Millettia barteri* and their antimicrobial and antioxidant activities. Pharm Biol. 2012;50(2):141-6. doi: 10.3109/13880209.2011.579618.
19. Sabeva NS, McPhaul CM, Li X, Cory TJ, Feola DJ, Graf GA. Phytosterols differentially influence ABC transporter expression, cholesterol efflux and inflammatory cytokine secretion in macrophage foam cells. J Nutr Biochem. 2011;22(8):777-83. doi: 10.1016/j.jnutbio.2010.07.002.
20. Plat J, Beugels I, Gijbels MJ, de Winther MP, Mensink RP. Plant sterol or stanol esters retard lesion formation in LDL receptor-deficient mice independent of changes in serum plant sterols. J Lipid Res. 2006;47(12):2762-71. doi: 10.1194/jlr.M600346-JLR200.
21. Weingärtner O, Lütjohann D, Ji S, Weisshoff N, List F, Sudhop T, et al. Vascular effects of diet supplementation with plant sterols. J Am Coll Cardiol. 2008;51(16):1553-61. doi: 10.1016/j.jacc.2007.09.074.
22. Tyagi T, Agarwal M. Phytochemical screening and GC-MS analysis of bioactive constituents in the ethanolic extract of *Pistia stratiotes* L. and *Eichhornia crassipes* (Mart.) solms. J Pharmacogn Phytochem. 2017;6(1):195-206.
23. Mishra PM, Sree A. Antibacterial activity and GC-MS analysis of the extract of leaves of *Finlaysonia obovata* (a mangrove plant). Asian J Plant Sci. 2007;6(1):168-72. doi: 10.3923/ajps.2007.168.172.
24. Abidin ZZ, Mohamad WN, Samadi M. Optimization of chlorophyll extraction from *Gynura procumbens*. Malaysian J Anal Sci. 2016;20(6):1421-8. doi: 10.17576/mjas-2016-2006-22.
25. Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, et al. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J Exp Med. 1992;176(6):1693-702. doi: 10.1084/jem.176.6.1693.
26. Mayer CT, Ghorbani P, Nandan A, Dudek M, Arnold-Schrauf C, Hesse C, et al. Selective and efficient generation of functional Batf3-dependent CD103+ dendritic cells from mouse bone marrow. Blood. 2014;124(20):3081-91. doi: 10.1182/blood-2013-12-545772.
27. Naik SH, Metcalf D, van Nieuwenhuijze A, Wicks I, Wu L, O'Keeffe M, et al. Intrasplenic steady-state dendritic cell precursors that are distinct from monocytes. Nat Immunol. 2006;7(6):663-71. doi: 10.1038/ni1340.
28. Helft J, Böttcher J, Chakravarty P, Zelenay S, Huotari J, Schraml BU, et al. GM-CSF mouse bone marrow cultures comprise a heterogeneous population of CD11c(+) MHCII(+) macrophages and dendritic cells. Immunity. 2015;42(6):1197-211. doi: 10.1016/j.immuni.2015.05.018.
29. Siebel C, Lendahl U. Notch signaling in development, tissue homeostasis, and disease. Physiol Rev. 2017;97(4):1235-94. doi: 10.1152/physrev.00005.2017.
30. Amsen D, Blander JM, Lee GR, Tanigaki K, Honjo T, Flavell RA. Instruction of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells. Cell. 2004;117(4):515-26. doi: 10.1016/s0092-8674(04)00451-9.
31. Cheng P, Gabrilovich D. Notch signaling in differentiation and function of dendritic cells. Immunol Res. 2008;41(1):1-14. doi: 10.1007/s12026-007-8011-z.
32. Auderset F, Schuster S, Coutaz M, Koch U, Desgranges F, Merck E, et al. Redundant Notch1 and Notch2 signaling is necessary for IFNγ secretion by T helper 1 cells during infection with *Leishmania major*. PLoS Pathog. 2012;8(3):e1002560. doi: 10.1371/journal.ppat.1002560.
33. Jiao Z, Wang W, Hua S, Liu M, Wang H, Wang X, et al. Blockade of Notch signaling ameliorates murine collagen-induced arthritis via suppressing Th1 and Th17 cell responses. Am J Pathol. 2014;184(4):1085-93. doi: 10.1016/j.ajpath.2013.12.010.
34. Yoo HJ, Choi KM. Hepatokines as a link between obesity and cardiovascular diseases. Diabetes Metab J. 2015;39(1):10-5. doi:10.4093/dmj.2015.39.1.10.
35. Buono C, Binder CJ, Stavrakis G, Witztum JL, Glimcher LH, Lichtman AH. T-bet deficiency reduces atherosclerosis and alters plaque antigen-specific immune responses. Proc Natl Acad Sci U S A. 2005;102(5):1596-601. doi: 10.1073/pnas.0409015102.
36. Ho IC, Tai TS, Pai SY. GATA3 and the T-cell lineage: essential functions before and after T-helper-2-cell differentiation. Nat Rev Immunol. 2009;9(2):125-35. doi: 10.1038/nri2476.
37. Davenport P, Tipping PG. The role of interleukin-4 and interleukin-12 in the progression of atherosclerosis in apolipoprotein E-deficient mice. Am J Pathol. 2003;163(3):1117-25. doi: 10.1016/s0002-9440(10)63471-2.
38. Engelbertsen D, Rattik S, Knutsson A, Björkbacka H, Bengtsson E, Nilsson J. Induction of T helper 2 responses against human apolipoprotein B100 does not affect atherosclerosis in ApoE-/- mice. Cardiovasc Res. 2014;103(2):304-12. doi: 10.1093/cvr/cvu131.
39. Sakaguchi S, Miyara M, Costantino CM, Hafler DA. FOXP3+ regulatory T cells in the human immune system. Nat Rev Immunol. 2010;10(7):490-500. doi: 10.1038/nri2785.
40. Grönberg C, Nilsson J, Wigren M. Recent advances on

CD4(+) T cells in atherosclerosis and its implications for therapy. Eur J Pharmacol. 2017;816:58-66. doi: 10.1016/j.ejphar.2017.04.029.

41. Mallat Z, Gojova A, Brun V, Esposito B, Fournier N, Cottrez F, et al. Induction of a regulatory T cell type 1 response reduces the development of atherosclerosis in apolipoprotein E-knockout mice. Circulation. 2003;108(10):1232-7. doi: 10.1161/01.cir.0000089083.61317.a1.
42. van Es T, van Puijvelde GH, Foks AC, Habets KL, Bot I, Gilboa E, et al. Vaccination against Foxp3(+) regulatory T cells aggravates atherosclerosis. Atherosclerosis. 2010;209(1):74-80. doi: 10.1016/j.atherosclerosis.2009.08.041.
43. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature. 2006;441(7090):235-8. doi: 10.1038/nature04753.
44. Lim H, Kim YU, Sun H, Lee JH, Reynolds JM, Hanabuchi S, et al. Proatherogenic conditions promote autoimmune T helper 17 cell responses in vivo. Immunity. 2014;40(1):153-65. doi: 10.1016/j.immuni.2013.11.021.
45. Taleb S, Tedgui A, Mallat Z. IL-17 and Th17 cells in atherosclerosis: subtle and contextual roles. Arterioscler Thromb Vasc Biol. 2015;35(2):258-64. doi: 10.1161/atvbaha.114.303567.
46. Dwijayanti DR, Rifa'i M. *Gynura procumbens* ethanolic extract promotes lymphocyte activation and regulatory T cell generation in vitro. J Trop Life Sci. 2015;5(1):14-9.
47. Takanashi K, Dan K, Matsuoka T, Torii K. The cancer cell killing effects of *Gynura procumbens*, Toleaf are associated with CD4+ T lymphocyte activation. Pharm Front. 2019;1:e190004. doi: 10.20900/pf20190004.
48. Bhaumik S, Basu R. Cellular and molecular dynamics of Th17 differentiation and its developmental plasticity in the intestinal immune response. Front Immunol. 2017;8:254. doi: 10.3389/fimmu.2017.00254.