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Research Article

Agext and Its Active Compounds as Potential Therapeutic Prevention against Atherosclerosis and Cardiovascular Disease

Liu K[1], Mi Y[2], Bao Y[3], Luan Q[1], Liu Y[4], Zhang M[4], Ammar AB[5], Ali K[6], Elosta A[7], Lin M[4], Ahmed N[7], Slevin M[7,8], Liu F[4]* and Liu D[7]*

[1]The Affiliated Hospital of Weifang Medical University, Shandong, China

[2]Department of Pharmacy, The First Affiliated Hospital, Weifang Medical University, Shandong, China

[3]No.4 People's Hospital of Zibo, Shandong, China

[4]Faculty of Medical Images, Weifang Medical University, Shandong, China

[5]College of Applied Medical Science, University of Hail, Saudi Arabia

[6]Yanbu College of Applied Medical Sciences, Taibah University, Saudi Arabia

[7]Department of Life Sciences, Manchester Metropolitan University, Manchester, M1 5GD, UK

[8]The University of Medicine and Pharmacy of Targu Mures, 541039, Romania

***Corresponding authors:** Donghui Liu, Department of Life Sciences, Manchester Metropolitan University, Manchester, M1 5GD, UK

Feng Liu, Faculty of Medical Images, Weifang Medical University, Shandong, 261053, China

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Abstract

Advanced Glycation End-Products (AGEs) have been implicated in the chronic complications of Diabetes Mellitus (DM) and play an important role in the pathogenesis of atherosclerosis in human arteries. The main active compounds found in Aged Garlic Extract (AGExt) are S-Allyl Cysteine (SAC), N-Acetylcysteine (NAC) and S-allyl Mercaptocysteine (SAMC) and have powerful protective effects against oxidative stress and inhibit cellular damage by AGEs.

Aims: To explore the effect of AGExt on the development of atherosclerosis also investigated the endothelial protective effects of AGExt, using Human Coronary Artery-Derived Endothelial Cells (HCAEC). The use of an optimised mixture of the above compounds required to obtain and provide maximal beneficial effects against oxidative stress, cell apoptosis and protein glycation. Results: AGExt has protective effects against the cellular damage of HCAEC. In addition, the *in vivo* study accessed the activity of SAC and NAC to reduce the severity of atherosclerosis formation in Apolipoprotein (ApoE-/-) DM model mouse. The compounds of AGExt *in vitro* had strong protective actions against the AGEs induced damage to ECs even at low concentrations (100 ng/ml). Such low concentrations may have therapeutic usefulness in patients with diabetes.

Keywords: AGExt; AGEs; Atherosclerosis; Diabetes mellitus; Glycation; Oxidative stress

Introduction

Hyperglycaemia has an important role in the development of long-term complications of Diabetes Mellitus (DM) such as retinopathy, nephropathy, neuropathy, cataract, impaired wound healing and atherosclerosis [1,2]. However, the relationship between tissue damage and hyperglycaemia is not fully understood [3,4]. Several mechanisms have been proposed but the most interesting one is the role of protein glycation and the formation and accumulation of Advanced Glycation End-Products (AGEs) [5,6]. During chronic hyperglycaemia, body proteins undergo a process called glycation where covalent binding between carbonyl groups from reducing sugars and free amino groups from proteins forms a labile Schiff base [6,7]. This unstable Schiff base rearranges to a more stable compound called an Amadori product. These glycated proteins undergo further oxidative reactions, involving dicarbonyl intermediates, forming AGEs. The process of protein glycation is usually accompanied by the generation of Reactive Oxygen Species (ROS) [8,9]. Furthermore, glycation-derived free radicals can cause protein oxidation and the fragmentation of lipids and nucleic acids [10,11].

It is commonly accepted that the accumulation of tissue AGEs and associated oxidative stress are responsible for the molecular and cellular damage which have been implicated in diabetic complications [12-16]. The accumulation of AGEs has also been implicated in the development of insulin resistance as well as in the pathogenesis of diabetic complication [17-19]. AGEs play important role in the pathogenesis of atherosclerosis in human arteries as well as cancer, chronic kidney disease, cardiovascular and various neurodegenerative diseases including Alzheimer's disease [20-23].

Cardiovascular disease remains the foremost cause of death worldwide though a steady decline in mortality and morbidity has been recognised over the past few decades [24,25]. Prolonged exposure to hyperglycaemia is now considered as a major factor in the pathogenesis of atherosclerosis in patients with DM [26].

Atherosclerosis is a disease characterised by dysfunction and inflammations of arterial endothelial cells [27-29]. However, atherosclerosis is a complex and multifactorial process, the key initiating process in atherogenesis is the subendothelial cholesterol retention which is both necessary and sufficient to provoke lesion initiation [30]. It has been shown that cellular lipidosis is the principal event in the pathogenesis of the atherosclerotic lesion [31,32]. Retention of cholesterol, transported by Low-Density Lipoprotein (LDL) in subendothelial space of arterial wall, is an absolute requirement for lesion development. According to Tabas et al. [33], the molecular basis of lipoprotein retention is associated with the

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interaction of lipoprotein and extracellular matrix molecules. Local responses to these retained lipoproteins include an inflammatory response with subsequent lesion development [34]. Specific focus is placed on the potential of these innate immune targets for therapeutic interventions to retard the progression of atherosclerosis or to induce its regression [34,35]. The response-to-retention model considers only the retention of cholesterol on the extracellular matrix, completely ignoring the retention of intracellular cholesterol [36].

Natural plant products have been claimed to possess beneficial effects for the prevention of various aspects of cardiovascular disease [37]. AGExt is one of these products which is formulated by soaking sliced raw garlic cloves in 15 - 20% aqueous ethanolic solution for up to 20 months at room temperature. The extract is then filtered and concentrated under pressure at low temperature. The ageing process of garlic converts the odorous and harsh irritating compounds to odourless and non-irritating water-soluble organic sulphur compounds, such as SAC, NAC, SAMC, allicin and selenium [38]. These compounds are exceptionally powerful antioxidant phytochemicals with increasing antioxidant activities [39]. On the other hand, Black-AGExt (b-AGExt) is another processed food that is produced by heating raw garlic at highly controlled temperature and humidity for approximately one month. It contains proteins, high amounts of polysaccharides, melanoidins, phenolic and sulphur compounds. The antioxidant activities of b-AGExt are mainly related to presence of high amounts of polyphenols as well as the transformation of unstable compounds in raw garlic into more stable organosulphur compounds [40].

There is growing evidence that AGExt has moderate cholesterol-lowering and blood pressure-reducing effects [41] and has significant vascular protective effects against the AGEs induced damage to the cardio/cerebrovascular system [17,42-44]. Several studies have shown the protective effects of AGExt against atherosclerosis by preventing hypertension, decreasing serum cholesterol and triglycerides levels, and inhibiting platelet aggregation and LDL oxidation [45-50]. Currently, a novel approach is under phase 1 and phase 2 clinical trial in diabetes type 1 and type 2 patients (our and Don et al's unpublished data).

The present study investigates and compares the ability of potent cysteine-derived compounds found in AGExt or b-AGExt, to provide potentially anti-atherogenic/endothelial protective effects and provide justification for their consideration as main-line additional supplements or therapeutics for patients at risk of cardio/cerebrovascular disease. *In vitro* studies were also carried out to assess their endothelial protective effects using Human Coronary Artery-Derived Endothelial Cell (HCAEC) and produce an optimised mixture providing maximal beneficial effects against oxidative stress, cell apoptosis (induction by hypoxia and re-oxygenation) and protein glycation (induction by exposure to high glucose levels and methylglyoxal). This would allow us to optimise the prevention of Endothelial Cell (EC) damage (key to the initiation and progression of atherosclerosis) and EC activation (associated with inflammation and plaque progression). Furthermore, the effects of AGExt, b-AGExt and their active components on EC's vascularisation, and the EC angiogenesis in the presence of AGEs following pre-incubation of the EC cells with varying AGExt components was also investigated. In addition, the combination of SAC and NAC has been conducted *in vitro* and has also been assessed in the Apolipoprotein (ApoE-/-) DM model mouse *in vivo*.

Materials and Methods

Reagents

Unless stated, otherwise, all chemicals were purchased from Sigma-Aldrich at the highest purity. AGExt and other bio-active components, such as SAC, NAC and SAMC were kindly provided by the Wakunaga Pharmaceutical Company, Japan). B-AGExt was supplied by SINO BNP COM (Qingdao, Shandong, China). The compounds were tested alone and in combinations and varying concentrations to identify the maximal potential therapeutic effects.

In vitro glycation of protein

Lysozyme (10 mg/ml) was glycated by methylglyoxal (0.1 M) with and/or without AGExt components in 0.1 M sodium phosphate buffer, pH 7.4, containing 3 mM sodium azide, at 37°C in dark for one week. After incubation, unreacted sugars were removed by extensive dialysis against distilled water for 2 days at 4°C. The endotoxin content of all protein solutions was below the detection limit (<0.125 EU/ml). The aliquoted samples were stored at -20°C until needed for analysis.

Measurement of cross-linked AGEs

Cross-linked AGEs were assessed by using 12% Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS–PAGE). The gels were stained with Coomassie Brilliant Blue, de-stained, photographed and analysed using Image-Lab software (Bio-Rad, Watford, UK). The integrated density was measured to analyse the cross-linked AGEs on one-dimensional electrophoretic gels and the relative intensity after the addition of AGExt, b-AGExt and their components SAC, NAC and SAMC. Furthermore, the effects of combinations of SAC+NAC, SAC+SAMC and NAC+SAMC were also tested.

Cell culture

Human Coronary Artery-Derived Endothelial Cells (HCAECs) were purchased from Health Protection Agency Culture Collections (HPACC, UK), and maintained in a T-25 flask and in MV endothelial cell growth medium (HPACC, UK) supplemented with 10% de-complemented Foetal Bovine Serum (FBS) under water humidified 5% CO_2 air at 37°C. The media were changed every 3 days and used between passage 2 and 5. Cell viability was optimised by the Alamar blue assay [51,52]. Cells were seeded in 96 well plates ($1.6 - 1.8 \times 10^4$ cells per well) for 72 hours, with or without tested compounds. The absorbance of each well was measured at 490 nm using a microplate reader (Multiskan Ascent, Thermo Life Sciences, Hampshire, UK).

Cell migration assay

HCAECs migration assay was performed using our standard wound scratch assay as described previously [53] and the HCAECs were tested in the presence and absence of AGEs. Briefly, HCAECs were seeded on 1 cm × 1 cm glass coverslips at a concentration of 4×10^5 cells/ml in 1 ml of completed MV medium in each well of 12-well plates and incubated in a water humidified and 5% CO_2 air incubator at 37°C. When cells reached about 90% confluence, the growth medium was replaced with Serum Poor Medium [SPM, basal growth medium containing 0.5% (v/v) FBS] and incubated for a further 24 hours. After the 24 hours incubation, each well of the 12-well plates was washed with warm Phosphate Buffer Saline (PBS)

three times and the adherent cells were then scratched in one single continuous line across the glass coverslip using a razor blade and the wells were then washed carefully with warm PBS three times to remove any floating cells. Then the AGExt and its components were added to the wells (at 0.1 μg/ml) and SPM [basal medium contains 0.5% (v/v) FBS] was applied to the cells, and cells were incubated under the same conditions as mentioned above for 24 hours. HCAECs incubated in SPM were used as control. Fibroblast Growth Factor-2 (FGF-2) at a concentration of 25 ng/ml acted as a positive control.

After the 24 hours incubation, 100 μl of 4% (w/v) of Paraformaldehyde (PFA) was added to each well at Room Temperature (RT) for 15 min to fix the cells. The medium was then removed, and the wells were washed with PBS, followed by 200 μl of 100% ethanol fixation for 5 min. The wells were left to dry before staining the cells with methylene blue for 5 min. The stain was removed, and the wells were washed with distilled water (dH_2O). Finally, the cell migration was assessed by phase-contrast microscopy and images were taken using a digital camera (ZEISS, Fisher Scientific, Loughborough, UK). Cells were treated in triplicate and pictures of five areas of each well were taken. The image analysis was performed using Image-J. Both migration distance and the number of migrated cells were measured and the mean ± SD were calculated. Each experiment was performed at least twice and a representative example is given in Figure 3.

Matrigel™ endothelial cell tube formation assay

The preparation of HCAECs was performed as described above. Briefly, 50 μl of Matrigel™ basement membrane matrix reduced in growth factors (BD Bioscience, Berkshire, UK) was added to each well in 96-well plates and incubated at 37°C for 1 hour to let the Matrigel™ polymerise. After that, 9×10^3/50 μl cells were added to each well including those compounds in low serum (0.5% FBS) MV media with 0.1μg/ml or without the test compounds. Both the test and controls were incubated for 24 hours at 37°C. After the 24 hours-incubation, HCAECs migrated and aligned to form tubes (defined by the enclosure of circumscribed areas). The counts of closed tube-like areas were made in five fields by microscopy (ZEISS, Fisher Scientific, Loughborough, UK) using the ×10 objective. FGF-2 (25 ng/ml) were used as positive control. All the experiments were done in triplicate, repeated three times, and results shown as mean ± SD.

Apoptosis assays

Apoptosis and oxidative stress were generated using a hypoxia chamber (Stemcell Technologies, Cambridge, UK) and cells subjected to low oxygen levels (0.5%) for 24 hours. HCAEC cells were seeded in 48-well plates with complete growth media for overnight and then treated with AGExt, b-AGExt, SAC+NAC, SAC+SAMC, and NAC+SAMC at 0.1 μg/ml for 24 hours. The cells were then incubated in hypoxia chamber for further 24 hours. Then HCAECs were incubated with Propidium Iodide (PI) for one hour and fixed with 4% PFA for 15 minutes at room temperature. The HCAEC cells stained with PI were quantified with a fluorescence microscope (ZEISS, Fisher Scientific, Loughborough, UK). Untreated HCAEC cells and those in normoxia acted as control.

Nuclear membrane damage and generation of oxidative stress

Nuclear membrane damage and generation of oxidative stress were evaluated by nuclear inclusion of PI counterstained with Hoechst 33258 and by assessing the levels of active p53 (phospho-p53, Cell Signalling, London, UK) to determine the activation rate of apoptosis and protection of the selected active compounds. HCAECs cells lysates were prepared from HCAECs which have undergone apoptosis. Total proteins (30 μg/well) were applied to 10% SDS-PAGE followed by Western blotting.

The HCAECs stained with PI (nuclear positivity = apoptosis) were quantified using a fluorescence microscope. HCAECs untreated with AGExt, b-AGExt and other components were used as controls and the HCAECs with normoxia conditions also acted as a control.

Endothelial cell permeability assay

HCAEC cells permeability assay was performed by using an *in vitro* Vascular Permeability assay kit (Millipore, Burlington, USA). HCAECs were cultured to confluence (at about 90% confluence they formed an endothelial monolayer) on transwell collagen pre-coated permeable (pored) support in the insert well of a 24-well plate. Then the HCAEC cells monolayer was pre-incubated with AGExt, b-AGExt, and the mixed compounds (SAC+NAC, SAC+SAMC and NAC+SAMC) at 0.1 μg/ml for 24 hours. The monolayer was treated with AGEs (250.0 μg/ml) alone or together with SAC+NAC, SAC+SAMC and NAC+SAMC at 0.1 μg/ml for a further 24 hours. After treatment, a high molecular weight Fluorescein Isothiocyanate-Dextran (FITC-Dextran) was added on top of the cells (insert well), allowing the fluorescent molecules to pass through the ECs monolayer at a rate proportional to the monolayer's permeability. The extents of permeability were determined by measuring the fluorescence as per the manufacture's protocol of the receiver plate well solution.

Western-blot analysis

After cell culture, a general Radioimmunoprecipitation Assay Buffer (RIPA buffer) containing a protease and phosphatase inhibitor cocktail was used to prepare the cell lysates. These cell lysates were incubated on ice for 20 minutes, and then were sonicated for 20 sec and centrifuged for 10 min at 10×10^3 ×g at 4°C. The supernatants containing protein were collected, their protein concentrations were estimated using the Bicinchoninic Acid (BCA) protein assay and were stored at -80°C for later use. Equal quantities of proteins (30 μg) were mixed with 2× Laemmli sample buffer, boiled in a water bath for 15 min and then centrifuged briefly. Samples were separated along with pre-stained molecular weight markers (32,000 - 200,000 kDa) by 10% SDS-PAGE. Proteins were electro-transferred (Hoefer, Bucks, UK) onto nitrocellulose filters for 1 hr (Whatman, Protran BA85, Germany) and the filters were blocked for 1 hour at room temperature in tris buffered saline with tween (TBS-Tween, pH 7.4) containing 1% Bovine Serum Albumin (BSA). Filters were stained with the primary antibodies diluted in the blocking buffer, overnight at 4°C on a rotating shaker. Primary antibody was applied at 1:1,000 dilution: p-p53 (Cell Signalling, London, UK). The following day, the filters were washed (5×5 minutes in TBS-Tween at room temperature), filters were stained with goat anti-rabbit HRP-conjugated secondary antibodies diluted in TBS-Tween containing 5% de-fatted milk (1:2,000 dilution for 1 hr at room temperature) with continuous mixing. After a further five washes in TBS-tween, proteins were visualized using enhanced chemiluminescence detection (ECL, Thermo Scientific, Cambridge, UK), and semi-quantitatively identified fold differences compared with house-keeping controls (α-tubulin, Abcam, Cambridge, UK)

were determined using Image-Lab software (Bio-rad, Watford, UK). The Western blot experiments were repeated twice and a representative example is shown in Figure 6.

Diabetes mellitus mice model

Streptozotocin (STZ, Solarbio Co Ltd, Beijing, China) treatment was done as described [54,55]. Briefly, after 12-14 hours of fasting, diabetes in mice was induced by intraperitoneal injection of 140 mg/kg STZ. Mice were considered diabetic when glucose levels exceeded 11.1 mmol/L (monitored after 24 hours of fasting) at 72 hours after STZ injection. Control mice were injected with saline only.

More than 95% of STZ (140 mg/kg) treated mice developed diabetes within 1 week, and the loss of body weight was relatively low (mean ± 0.3 g). Blood sugar was measured in tail blood samples using a glucometer (Siemens, Munich, Germany).

Healthy male mice (6 week old) were used for the study. Six-week-old ApoE-/- mice were purchased from Nanjing Biomedical Research Institute of Nanjing University, Nanjing, China. They were maintained in a temperature-controlled room with a 12:12 hour light: dark schedule and provided with a standard mouse pellet diet (Biopike, Shanghai, China) and water ad libitum. They were kept in ventilated cages with free access to water and food for experimental use. No obvious adverse events were seen in any of the experimental group.

Drug intervention

The AGExt concentrations were optimised by our lab; we had found that 0.1 μg/ml was the lowest concentration that had any effect *in vitro* for cell culture. The compound dosages were defined at 200 mg/kg and were used *in vivo* in this study. The mice were divided into 6 groups: a: ApoE-/- mice fed with normal chow; b: ApoE-/- mice fed with high-fat chow diet; c: DM model mice fed with high-fat chow; d: ApoE-/- mice fed with normal chow plus SAC+NAC mixture; e: ApoE-/- mice fed with high-fat chow plus SAC+NAC mixture; f: DM model mice fed with high-fat chow plus SAC+NAC mixture.

Tissue preparation

Animals were anaesthetised with pentobarbital sodium ($C_{11}H_{17}N_2NaO_3$) solution (50 mg/kg; Xinhua Pharmaceutical Co Ltd, Shandong, China) and sacrificed by cervical dislocation. The blood samples were collected and the aortic artery was separated and removed immediately, and photographed. For histology study, diced 2mm tissues were resected quickly, fixed, and embedded in paraffin.

Histochemistry staining

Histological analysis was carried out on mice aortic artery following feeding with a high-fat diet with/without AGExt compounds according to standard histological protocols (n=5 mice were used; 10 sections from each analysed). Briefly, the paraffin-embedded tissues were deparaffinised by sequential washes in xylene (2×), rehydrated in descending ethanol from 100% (2×) to 70% (1×), then into tap water. Sections were stained followed by standard Haematoxylin & Eosin (H&E) staining procedure.

Image analysis

The stained slides were examined microscopically (ZEISS, Fisher Scientific, Loughborough, UK) and digital images of whole cross-sections were taken. The Figure 10 shows representative images of the H&E-stained aortic artery.

Statistical analysis

All the *in vitro* data are presented as the mean ± SD for One-way ANOVA analysis from 3 independent experiments, each experiment was done in triplicate, and the data were analysed using GraphPad Prism software version 7.0 for Windows (GraphPad Software, USA). The values were compared using a paired Student's t-test. The *in vivo* data were obtained from 5 mice in each group and are shown as mean±SD. The statistical differences: * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$) were considered statistically significant as compared with the control.

Results

This study has examined the potential benefits of AGExt, b-AGExt and their active components to inhibit AGEs cross-links. As shown in Figure 1, lysozyme glycated by methylglyoxal for one week produces sufficient cross-linked AGEs that present as dimers with an approximate molecular weight of 28 kDa. Glycated lysozyme (Figure 1, lane 2) showed reduced electrophoretic mobility for the lysozyme monomer compared to native lysozyme (Figure 1, lane 1). AGEs-induced dimerization of lysozyme was inhibited by the addition of

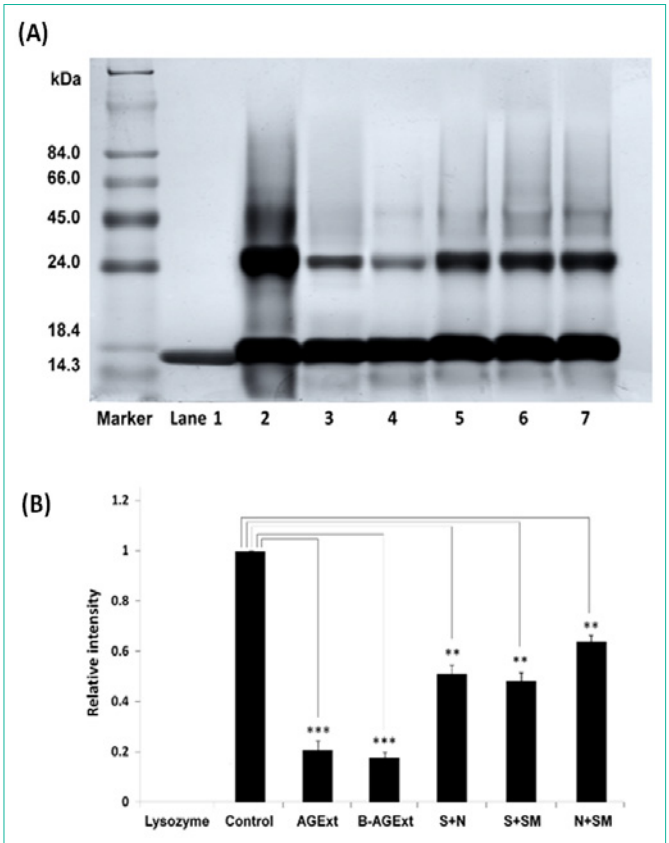

Figure 1: (A) The effect of AGExt, Black-AGExt, SAC, NAC and SAMC on the formation of AGEs using 12% SDS-PAGE. Lysozyme (10 mg/mL) was glycated with methylglyoxal (0.1 M) in 0.1M phosphate buffer, pH 7.4 for one week at 37°C. Unmodified lysozyme (lane 1), glycated lysozyme (lane 2) and glycated lysozyme in the presence of AGExt (lane 3), B-AGExt (lane 4), SAC (lane 5), NAC (lane 6) and SAMC (lane 7).
(B) The image analysis of gels showing the relative intensity of cross linked AGEs formation in the presence or absence of 0.1 μg/ml of AGExt, b-AGExt, SAC, NAC and SAMC respectively. (**: P<0.01, ***: P<0.001).

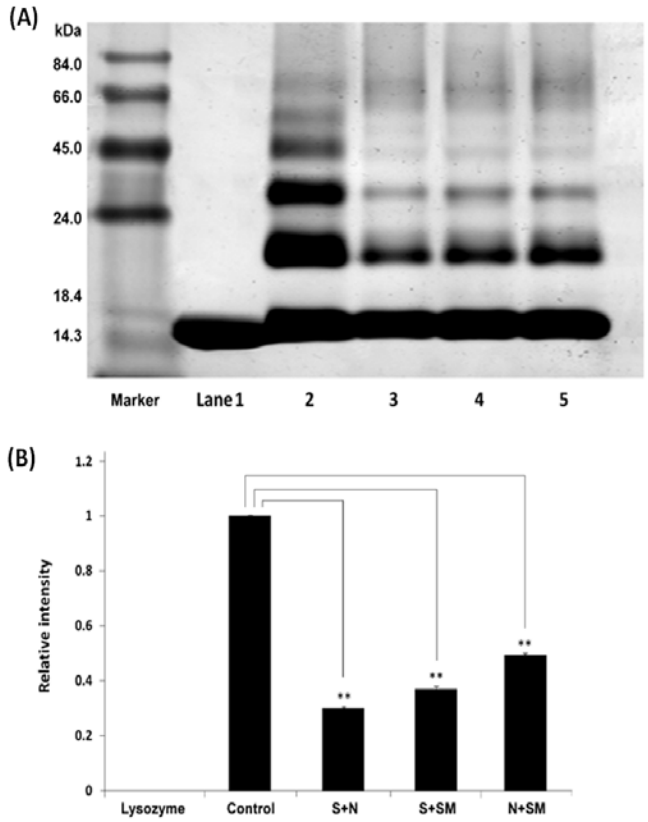

Figure 2: (A) The image shows the differences in anti-glycation effects between the aged garlic extract compounds combinations. SAC+NAC gave the most effective inhibition of Lysozyme glycation.
The positive control: Lysozyme glycated by Methylglyoxal; S+N: SAC+NAC, S+SM: SAC+SAMC and N+SM: SAC+SAMC (at the concentration of 0.1 μg/ml respectively). The control's intensity was set to 1, so others were issued relatively.
The effect of SAC+NAC, SAC+SAMC or NAC+SAMC on the formation of AGEs using 12% SDS-PAGE. Lysozyme (10 mg/mL) was glycated with Methylglyoxal (0.1 M) in 0.1M phosphate buffer, pH 7.4 for one week at 37°C. Unmodified lysozyme (lane 1), glycated lysozyme (lane 2) or glycated lysozyme in the presence of SAC+NAC (lane 3), SAC+SAMC (lane 4), NAC+SAMC (lane 5).
(B)The bar chart shows the relative intensity of cross linked AGEs formation in the presence or absence of 0.1 μg/ml of SAC+NAC, SAC+SAMC or NAC+SAMC. SAC+NAC, SAC+SAMC and NAC+SAMC inhibit the AGEs formation at the concentration at 0.1 μg/ml with reduced the lysozyme glycation compare to the positive control. The control's intensity was set to 1, so others were issued relatively (**: P<0.01).

0.1 μg/ml of AGExt, b-AGExt, SAC, NAC and SAMC (Figure 1, lanes 3-7) respectively. All inhibitors showed significant effects on the formation of AGEs. However, b-AGExt showed the most significant ($p<0.001$) effect on cross-linked AGEs formation. B-AGExt > AGExt > SAC > NAC > SAMC (Figure 1).

Further experiments were designed to compare in parallel the combined effects of SAC, NAC and SAMC on the *in vitro* formation of cross-linked AGEs (Figure 2). Incubation of lysozyme with methylglyoxal for one week produced sufficient cross-linked AGEs, which causes the formation of dimers is ~28 kDa. Glycated lysozyme (Figure 2, lane 2) was used as the control and clearly showed reduced electrophoretic mobility with higher relative molecular weight (Mr) as compared to native lysozyme (Figure 2A, lane 1). The Mr of glycated lysozyme was approximately twice that of the original lysozyme as determined by molecular weight markers (Figure 2, lane M). AGEs-induced dimerization of lysozyme was inhibited by the combined addition of 0.1μg/ml of SAC+NAC, SAC+SAMC or NAC+SAMC (Figure 2, lanes 3-5) respectively. The bar chart showed that the combined effect of all inhibitors was statistically significant ($p<0.01$) effective in reducing cross-linked AGEs formation *in vitro* as compared to the glycated lysozyme, in which, the SAC and NAC combination gave the most significant ($p<0.001$) inhibition on AGEs formation (Figure 2).

AGExt, b-AGExt and the mixture of compounds were tested by HCAEC proliferation assay showed there were no differences between the cells with and without AGExt, b-AGExt, SAC, NAC and SAMC on cell growth (data not shown).

HCAEC cells migration assay showed that b-AGExt provided the most effective protection for cell migration in the presence of AGEs

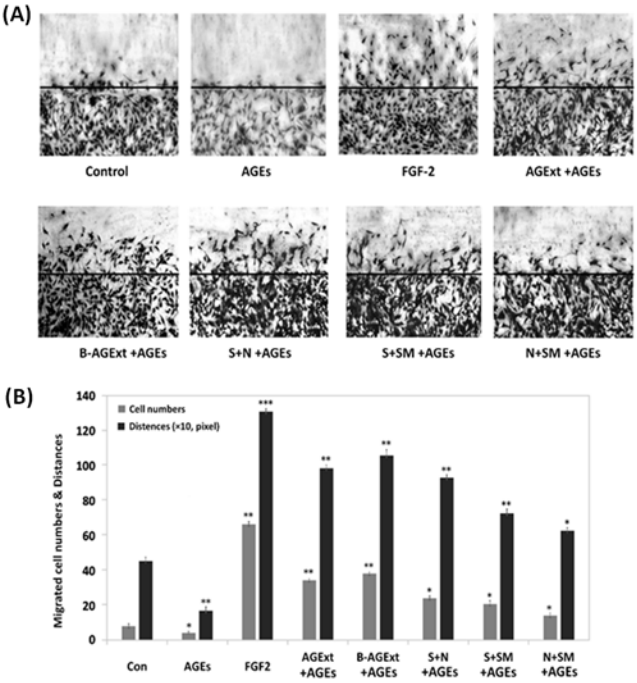

Figure 3: A) The images show the effects of AGExt, black-AGExt, and the compounds that affect the HCAECs cell migration in the presence of AGEs. The Black-AGExt provided the most effective protection for cell migration in the presence of AGEs.
Control: HCAECs grown in 0.5% FBS media was as a control; AGEs: HCAECs seeded in completed growth media plus AGEs (250 μg/ml) were also as a control; FGF-2: HCAECs with FGF-2 (25 ng/ml) grown in 0.5% FBS media were as positive control; AGExt: HCAECs with 0.5% FBS media plus AGExt (0.1 μg/ml) and AGEs (250 μg/ml); B-AGExt: HCAECs with 0.5% FBS media plus Black-AGExt (0.1 μg/ml) and AGEs (250 μg/ml); N+S: HCAECs with 0.5% FBS media plus SAC+NAC (0.1 μg/ml) and AGEs (250 μg/ml); S+SM: HCAECs with 0.5% FBS media plus SAC+SAMC (0.1 μg/ml) and AGEs (250 μg/ml); N+SM: HCAECs with 0.5% FBS media plus NAC+SAMC (0.1 μg/ml) and AGEs (250 μg/ml).
(B) Bar graph shows the significant difference between the control and the tests, with which black-AGExt produced the strongest protection against cell damage from AGEs (250.0 μg/ml). And black-AGExt also produced the protective effect, but the results from the individual compounds (NAC, SAC and SAMC) showed them to be significantly less effective. (*: P<0.05, **: P<0.01, ***: P<0.001).

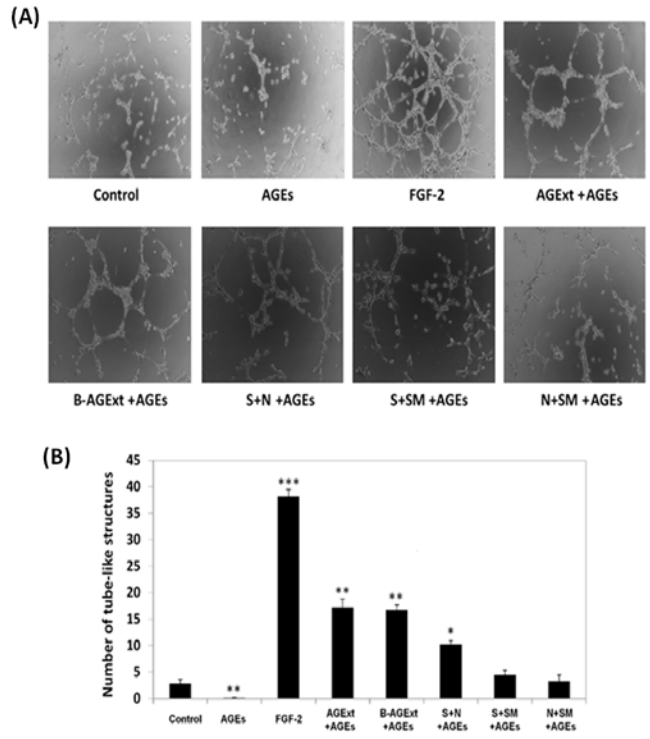

Figure 4: A) Image shows significant differences in tube-like areas counted using a phase-contrast microscope (×10) between the controls and the cells pre-incubated with AGExt, b-AGExt and compound combinations (SAC+NAC, SAC+SAMC and NAC+SAMC) respectively.
Control: Cells grown in 0.5% FBS medium; AGEs: cells grown in 0.5% FBS medium plus AGEs (250.0 µg/ml) was a negative control; FGF-2: Cells seeded in 0.5% FBS plus FGF-2 (25 ng/ml) was a positive control; AGExt+AGEs: medium contains 0.5% FBS plus AGExt 0.1 µg/ml and AGEs 250.0 µg/ml; B-AGExt+AGEs: Medium contains 10% FBS plus Black-AGExt 0.1 µg/ml and AGEs 250.0 µg/ml; S+N+AGEs: Medium contains 0.5% FBS plus SAC+NAC (0.1 µg/ml) and AGEs 250.0 µg/ml, S+SM+AGEs: Medium contains 0.5% FBS plus SAC+SAMC (0.1µg/ml) and AGEs 250.0µg/ml, and NAC+SAMC+AGEs: Medium contains 10% FBS plus NAC+SAMC 0.1µg/ml and AGEs 250.0µg/ml
(B) The Bar chart shows the results from the tested ECs and the control. AGExt and Black-AGExt provided the most effective protection for cell migration in the presence of AGEs and significantly higher compared to the control. (*: P<0.05, **: P<0.01, ***: P<0.001).

and significantly higher compared to the control and cells incubated with AGEs alone. For the mixtures, the combination of SAC+NAC gave the strongest protection (both by the cell numbers and migrated distances) against cell damage amongst the three groups (SAC+NAC, SAC+SAMC and NAC+SAMC). Both the cells number and the migrated distances were measured using 10×object (Figure 3).

In the HCAEC cell tube formation assay, AGExt, b-AGExt and the compounds showed protection against the monolayer damage by the high AGEs concentration (250.0 µg/ml). The combination of compounds, SAC+NAC shows greater protection than the other two combinations SAC+SAMC and NAC+SAMC (Figure 4).

The bar graph in Figure 4 depicts the significant difference between the control and the test conditions, whereas AGExt produced the strongest protection against cell damage from AGEs (250.0 µg/ml) and b-AGExt also had a protective effect. The combination of SAC+NAC gave better protection than the other two compound

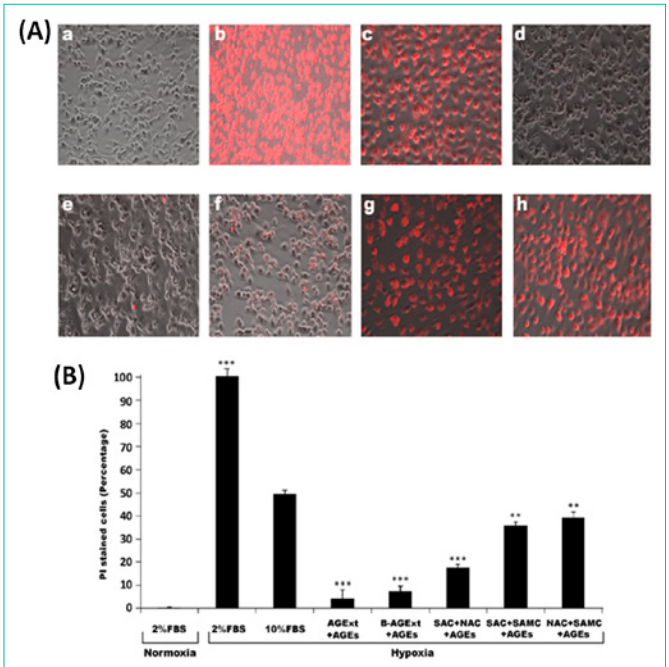

Figure 5: (A) HCAEC cells incubated with AGEs (250.0 µg/ml) together with AGExt, b-AGExt and the compounds mixtures under hypoxia for 24 hours and then stained with PI for 1 hour.
a: 2% FBS media Normoxia control; **b**: 2% FBS media Hypoxia control; **c**: 10% FBS media Hypoxia control; **d**: 2% FBS media + AGExt (0.1 µg/ml); **e**: 2% FBS media + b-AGExt (0.1 µg/ml); **f**: 2% FBS media plus SAC+NAC (0.1 µg/ml); **g**: 2% FBS media plus SAC+SAMC (0.1 µg/ml); **h**: 2% FBS media plus NAC+SAMC (0.1 µg/ml).
The HCAECs stained with propidium iodide (PI nuclear positivity = apoptosis) were quantified with a fluorescence microscope. Un-treated HCAECs was used as a control and the HCAECs with normoxia was also as a control.
The images show the PI stained positive HCAEC cells pre-incubated with AGExt, b-AGExt and its compounds under normal condition and/or hypoxia.
(B) The bar graph shows the percentage of apoptosis cells.
The image (×10) indicates the significant differences in the effects of AGExt, Black-AGExt and the compounds combinations protected HCAEC cells under hypoxia. (*: P<0.05, **: P<0.01, ***: P<0.001).

mixtures (NAC+SAMC and NAC+SAMC) which were less effective.

Apoptosis assay showed HCAEC cells stained with propidium iodide (nuclear positivity= apoptosis). HCAEC cells incubated with AGExt, b-AGExt and the mixed compounds showed a protective effect from the AGEs damage in hypoxia. AGExt and the mixed compounds depicted significant differences between control and the HCAEC incubated with AGEs (Figure 5).

Western blot study confirmed that there were differences between the positive controls (hypoxia) and the cells incubated with the activated ingredients SAC, NAC and SAMC in mixture under hypoxic conditions. These results indicated the mixed ingredients from AGExt protected the HCAEC from hypoxia induced damage (Figure 6).

The HCAEC cells monolayer permeability assay detects the passage of fluorescent molecules through the HCAEC monolayer. AGExt had the strongest protection ($p<0.001$) of ECs' damage against high concentration of AGEs (250.0 µg/ml), but the NAC+SAMC mixture gave the least protection (Figure 7). There was no significant difference between SAC+SAMC and NAC+SAMC,

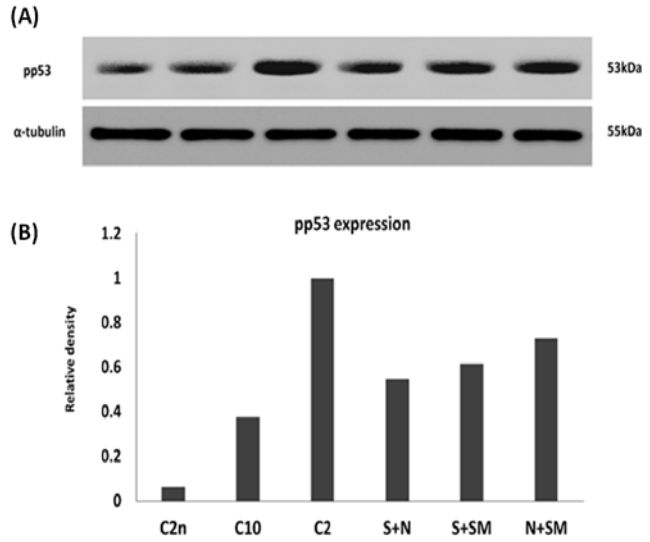

Figure 6: A) Shows the phospho-p-53 expression on the HCAEC cells undergone apoptosis.
Cell lysates were made from HCAEC cells undergone apoptosis. 30 µg/well of total proteins were applied to 10% SDS-PAGE and Western blot were employed.
C2n: HCAECs incubated in 2% FBS medium as control (Normoxia); C10: HCAECs incubated in 10% FBS medium as control (Hypoxia); C2: HCAECs incubated in 2% FBS medium as control (Hypoxia); S+N: HCAECs incubated in 2% FBS medium plus SAC+NAC (0.1 µg/ml, Hypoxia); S+SM: HCAECs incubated in 2% FBS medium plus SAC+SAMC (0.1 µg/ml, Hypoxia); N+SM: HCAECs incubated in 2% FBS medium plus NAC+SAMC (0.1 µg/ml, Hypoxia).
(B) Bar chart shows the activated ingredients SAC, NAC and SAMC in mixture protect the HCAEC cells' damage from hypoxia.

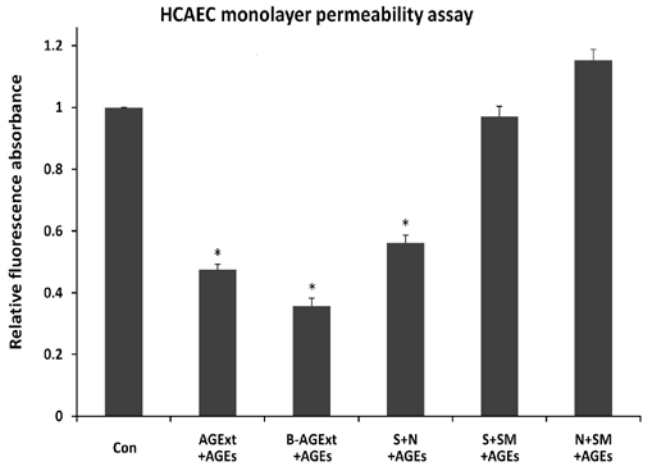

Figure 7: After the HCAEC cells monolayer was incubated with AGExt, b-AGExt, SAC+NAC, SAC+SAMC and NAC+SAMC as well as AGEs for 24 hours, a high molecular weight FITC-Dextran was added on top of the cells (insert well), allowing the fluorescent molecules to pass through the endothelial cell monolayer at a rate proportional to the monolayer's permeability. These results showed that the AGExt, b-AGExt and the active ingredients in AGExt could protect the ECs' damage from AGEs.

while the SAC+NAC has a stronger protective effect and thus it could be therapeutic value. The bar graph in Figure 7 shows the AGExt compounds that give the most significant protection (intact monolayer) against the AGEs-disruption. The combination of SAC and NAC, had better protection than other mixtures (SAC+SAMC, and NAC+SAMC). AGExt showed the most protection against the monolayer damage from the high AGEs concentration (250.0 µg/ml), and the NAC+SAMC produced the least effect.

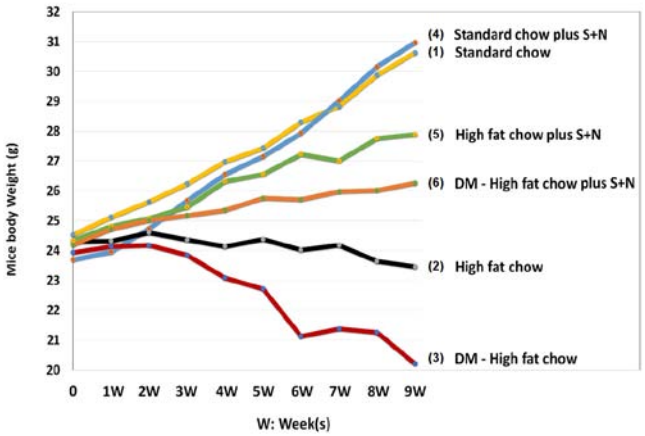

Figure 8: Body weight of ApoE-/- mice (5 of each group) was records over 9 weeks.
*S+N: SAC and NAC mixtures.
(1) ApoE-/- mice fed with standard chow; (2) ApoE-/- mice fed with high-fat chow diet; (3); DM mice fed with high-fat chow; (4) ApoE-/- mice fed with standard chow plus mixture; (5) ApoE-/- mice fed with high-fat chow plus mixture; (6) ApoE-/- DM mice fed with high-fat chow plus mixture. Atherosclerosis was induced in ApoE-/- DM model mouse fed a high-fat chow diet; fatty streak plaque was also developed in the ApoE-/- mice, fed with high-fat chow, but not in the ApoE-/- mice fed with high-fat chow plus mixture. In addition, SAC plus NAC mixture reduced superoxide production in the aortic walls, as detected by H&E staining.

In the *in vivo* mouse diabetic model study, DM group showed a significant loss of weight, but the DM plus SAC+NAC group showed negligible changes in weight. The weight of mice fed with high-fat chow group shows slow loss of weight. In the ApoE-/- mice control group (normal chow), the mice in the normal chow plus SAC+NAC compounds and on high-fat chow plus SAC+NAC compounds groups steadily gained weight (Figure 8).

The pathology showed that the aorta of the DM model mouse had a narrowing lumen and huge lipid plaque with lipid vacuoles on the intima while the mice of DM plus SAC+NAC group had a slightly curly intima only and the other areas were smooth. The biochemical results showed: the levels of blood glucose, LDL, cholesterol and triglyceride in the DM model mouse fed with high-fat chow group were statistically significantly ($P<0.01$) increased compared with that in ApoE-/- mice fed with standard (normal) chow group, and normal chow plus SAC+NAC group (Figure 9). The most interesting finding is that the levels of blood glucose, LDL, cholesterol and triglyceride in the DM model mouse fed with high-fat chow plus SAC+NAC mixture group were significantly lower than those fed with high-fat chow group ($P<0.01$). The levels of LDL, cholesterol and triglyceride in the ApoE-/- mice fed with high-fat chow group were statistically significantly higher compared with mice fed with high-fat chow plus SAC+NAC group ($P<0.01$). There was no significant difference of blood glucose level between the two high-fat chow group, though the level of ApoE-/- mice fed with high-fat chow group was higher than that in ApoE-/- mice fed with high-fat chow plus SAC+NAC

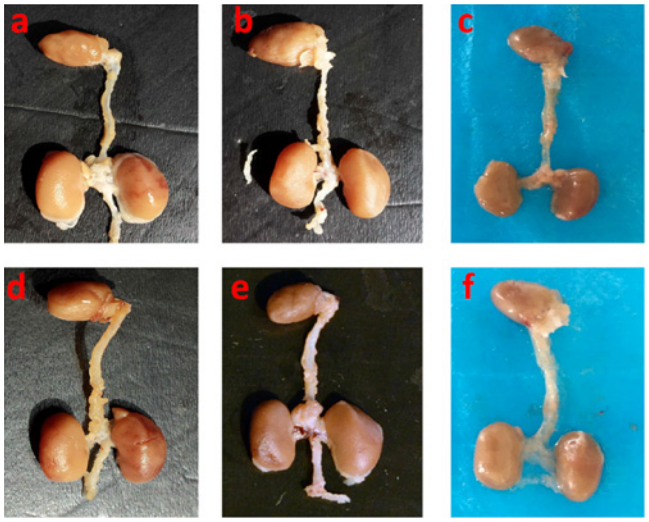

Figure 9: AGExt compounds greatly inhibited the plaque formation in the ApoE-/- mice fed with high-fat chow.
a: ApoE-/- mice fed with standard chow; **b**: ApoE-/- mice fed with high-fat chow diet; **c**: DM model mice fed with high-fat chow; **d**: ApoE-/- mice fed with normal chow plus SAC+NAC mixture; **e**: ApoE-/- mice fed with high-fat chow plus SAC+NAC mixture; **f**: DM model mice fed with high-fat chow plus SAC+NAC mixture.
The pathology result showed that the aorta of mice in the DM group had a narrowing lumen and huge lipid plaque with lipid vacuoles on the intima while the DM model (**: P<0.01 fed with high-fat chow plus SAC+NAC group had a slightly curly intima only and the other area was still smooth.
There was no atherosclerosis in the ApoE-/- group fed with standard chow, standard chow plus AGExt compounds and high-fat chow plus compounds mixture group. There were atherosclerosis plaques formed in mice fed with high-fat chow plus AGExt compounds, but the plaques were much smaller than those in mice fed with high-fat chow without the addition of AGExt compounds, the atherosclerosis plaque was formed in DM mice fed with high-fat chow without AGExt compounds.

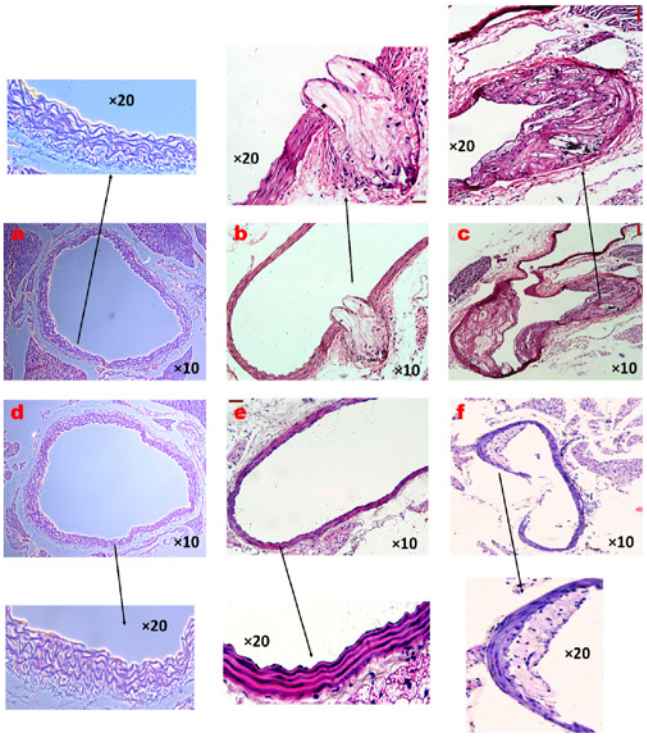

Figure 10: The AGExt compounds reduce the formation of atherosclerosis plaques in DM mice.
The AGExt compounds reduced the formation of atherosclerosis plaques in DM mice SAC+NAC mixture significantly reduces the formation of atherosclerotic plaque in aortic artery wall in ApoE-/- mice and DM model mouse.
a: ApoE-/- mice fed with standard chow; **b**: ApoE-/- mice fed with high-fat chow diet; **c**: DM model mice fed with high-fat chow; **d**: ApoE-/- mice fed with standard chow plus SAC+NAC mixture; **e**: ApoE-/- mice fed with high-fat chow plus SAC+NAC mixture; **f**: DM model mouse fed with high-fat chow plus SAC+NAC mixture.
Mice abdominal aortas were collected from each group and the tissues were then sectioned and stained for H&E.
Atherosclerosis was induced in ApoE-/- DM model mouse fed a high-fat chow diet **(c)**. Fatty streak plaque was also developed in the ApoE-/- mice fed with high-fat chow **(b)**, and the ApoE-/- DM model mouse fed with high-fat chow plus SAC+NAC mixture **(f)**, but no plaque was found neither in the ApoE-/- mice fed with high-fat chow plus SAC and NAC mixture group **(e)** nor in the ApoE-/- mice fed with standard chow group **(a)** and the mice fed with standard chow plus SAC and NAC mixture group **(d)**. In addition, the SAC+NAC mixture reduced superoxide production in the aortic walls, as detected by H&E staining.

group. The levels of LDL and triglyceride in the ApoE-/- mice on high-fat chow group were statistically higher than the ApoE-/- mice fed with normal chow group and ApoE-/- mice fed normal chow plus SAC+NAC group, but there was no significant difference (P>0.05) on levels of Cholesterol among these groups (Table 1).

There were no plaques in the standard chow (control) group or amongst the standard chow plus SAC+NAC compounds group. Curiously there were no obvious plaques in the high-fat chow plus SAC+NAC compounds group. Plaques were observed in ApoE-/- mice fed with the high-fat chow group and numerous plaques appeared in the DM model mouse group. The aortic vascular endometrial lining was irregular, the vessel volumes became smaller, large lipid plaques had formed and lipid cavity accompanied with atherosclerosis in the DM model group (Figure 10). However, in the DM mice fed with high-fat chow plus SAC+NAC group, the aortic wall layering was clearly seen, the inner membrane was slightly curly but smooth, except for the small plaque. There was no noticeable pathologic change in the control group, the standard chow plus SAC+NAC compounds group and the high-fat chow plus SAC+NAC compounds group. SAC+NAC mixture treatment significantly modified the serum lipid profiles.

Discussion

The evidence for involvement of AGEs in long-term complications of DM is growing. AGExt may have a role as a nutritional supplement that protects against the harmful effects of treatment on DM, inflammatory diseases and cancer [45,56-58]. Atherosclerosis occurs more readily in DM and is characterised by endothelial dysfunction and inflammation as a result of the accumulation of lipids in the vascular internal wall [29,59,60]. Atherosclerotic plaque in the aortic artery is an indicator of generalised atherosclerosis [27,29]. Lipid metabolism is a risk factor and it has been associated with inflammatory atherosclerosis in humans [61-63].

Diabetes is a major public health problem and atherosclerosis that results from damage to the macro-vasculature. AGExt has several bioactivities such as anti-glycation, anti-ageing, anti-cancer, anti-oxidation and lowering of lipid [14,64]. It is considered as a

Table 1: A list of blood test results (mean±SD) of ApoE-/- and null mice.

Group	BG (mmol/L)	LDL (mmol/L)	HDL (mmol/L)	Chol (mmol/L)	TG (mmol/L)
a, S.C	3.76±1.32	2.21±0.79	2.85±1.25	8.35±0.63	0.74±0.20
b, S.C plus S+N	4.07±1.63	1.86±0.41	2.55±0.45	8.34±1.74	0.66±0.08
c, HF	5.25±1.64	6.61±1.42*	2.02±0.70	11.02±1.44*	1.33±0.62*
d, HF plus S+N	4.05±1.37	2.08±0.58†	2.31±0.31	6.02±0.50†	0.52±0.04
e, DM	17.32±4.16**	16.21±2.00**	5.95±1.67*	30.03±4.25**	1.75±0.57*
f, DM plus S+N	8.33±2.34*	8.12±1.96*	3.78±1.19	19.16±3.80*	0.51±0.11

BG: Blood Sugar; LDL: Low-Density Lipoprotein; HDL: High-Density Lipoprotein; Chol: Cholesterol and TG: Triglycerides; S.C: Standard Chow; HF: High-Fat Chow. **a**: Standard Chow; **b**: Standard Chow Plus SAC and NAC Mixture; **c**: High-Fat Chow; **d**: High-Fat Chow Plus SAC and NAC Mixture; **e**: High-Fat Chow; **f**: High-Fat Chow Plus SAC and NAC Mixture. *: P<0.05, **: P<0.01

promising nutraceutical for anti-atherosclerotic therapy and also protects against protein glycation in diabetic patients [31,36].

Extensive studies have concluded that atherosclerosis has a complex pathogenesis, the main components of which are lipid accumulation and chronic inflammation in the arterial wall [14,65]. Dyslipidaemia, high blood glucose and other metabolic alterations that accompany diabetes development are critically involved in the pathogenesis of atherosclerosis at virtually all the steps leading to progression of atherosclerosis [60,65-67]. In this study, we have demonstrated that AGExt and in particular its most active components, including the combination of water-soluble cysteinyl moieties, S-Allyl Cysteine (SAC) and N-Acetylcysteine (NAC) are exceptionally powerful anti-oxidant phytochemicals, protective against oxidative stress and inhibiting subsequent cellular damage *in vitro* and the formation of atherosclerotic plaques in the artery of DM model mouse *in vivo*. Therefore, AGExt could be of therapeutic importance in the treatment of diabetes and cardiovascular disease in patients.

Epidemiological studies have confirmed that insulin resistance is presented in pre-diabetic conditions, which lasts for many years prior to the onset of diabetes [68,69]. Oxidative stress, activation of the congenital immune system and abnormal energy metabolism are considered the key mechanisms in induction of insulin resistance. Nevertheless, the specific pathogenesis of insulin resistance has not been fully elucidated. AGExt has various biochemical activities and has proved to be beneficial for DM due to its antimicrobial, antioxidant effects and reduction of blood pressure. It also plays roles in the prevention of colorectal cancer and cardiovascular mortality and improvement of insulin tolerance in patients with DM [70-72]. However, some of its effectiveness should be confirmed in further study [73,74]. SAC is a sulphur-containing amino acid from garlic with antioxidant, anti-cancer, neurotrophic and other properties. Animal experiments have provided evidence that SAC can improve plasma insulin and reduce blood sugar levels, repair protein metabolism and to a certain extent, act to reduce hyperlipidemia. NAC is an antioxidant based on its sulfydryl group supply, which has been used for the relief and the treatment of a variety of conditions because of its anti-oxidaent, anti-inflammation and anti-apoptosis. The use of SAC and NAC in combination may have synergy in therapy of DM. Hence the b-AGExt has potent protective properties but needs further work.

To our knowledge this is the first study to investigate the AGExt activated ingredients in the mixture protecting against atherosclerosis and vascular dysfunction *in vivo*. These important findings suggest that the AGExt ingredients could potentially reduce AGEs formation, prevent diabetic complications and improve the lipid metabolism in DM model mouse.

Protein glycation is also accompanied by oxidation which leads to the formation of protein carbonyl groups and loss of protein thiols [75], which suggests that AGExt and its ingredients may prevent oxidation induced protein damage.

In this study, we demonstrated that lipid metabolism contributes towards microvascular dysfunctions. SAC and NAC could be the primary building blocks for lipid metabolic and fatty acyl-chain remodelling. We have demonstrated that the ApoE-/- mice were protected from aortic artery damage by the high lipid. In contrast, the DM model mouse and the mice with high-fat chow developed atherosclerosis plaques.

The increasing prevalence of DM makes it imperative that research should focus on both its prevention and treatment. This study has improved the understanding of the mechanism linking inflammation to diabetes and related complications, has provided novel strategies for preventing diabetic complications [64,76,77]. However, how the high-fat chow related to lipoprotein metabolism still needs to be further clarified.

Both *in vitro* and *in vivo* studies elucidated the mechanisms by which AGExt and its individual compounds affect protein glycation *in vitro* and the prohibition of atherosclerotic plaque formation in DM model mouse *in vivo*. These findings have enhanced our understanding as to how the SAC and NAC protect endothelial cells dysfunction, damage caused by AGEs both *in vitro* and *in vivo*. An important finding to emerge is that the combination of SAC and NAC can reduce atherosclerotic plaque formation induced by high-fat chow in ApoE-/- DM model mouse *in vivo*.

We have established that AGExt and the activated ingredients SAC and NAC showed potent antioxidant and antiglycation effects against AGEs formation *in vitro*. There were statistically significant differences between the ApoE-/- mice and the DM model mouse fed with or without SAC and NAC mixture. It supports the concept that the application of AGExt in diabetes could protect against the development of atherosclerosis and complications of diabetes. We have illustrated the potential of this approach using SAC and NAC mixture in diabetic and control groups. The SAC+NAC mixture improved the metabolism of lipid in the mice fed with high-fat chow. The blood levels of LDL and cholesterol in ApoE-/- mice fed with high-fat chow plus SAC+NAC were significantly lower than the

ApoE-/- mice fed with high-fat chow alone. Moreover, the cholesterol levels of ApoE-/- mice fed with high-fat chow plus SAC+NAC mixture were lower than the control groups, i.e. ApoE-/- mice fed with standard chow with or without SAC+NAC. The reason for it could be that the SAC+NAC mixture improves/or promotes lipid metabolism, and the supplement of SAC+NAC mixture regulates hepatic glucose production when taken in high-fat food. Furthermore, the SAC+NAC mixture may work well when the diet is of high-fat, it could be stimulated to improve the glucose and/or lipid tolerance in the mice taking high-fat food. This combinatorial approach could identify mechanistic and potentially pathogenic mechanisms in the pathophysiology and biochemical underlying mechanisms in DM, and this is agree with other studies of lipid metabolic pathways in disease [30,78]. Therefore, this approach could provide a potential therapeutic usefulness in DM.

Conclusions

We have produced strong and novel evidence that AGExt can protect against the damage from AGEs on the cardiovascular system, resulting in DM and atherosclerosis disease. The active compounds SAC and NAC could reduce atherosclerosis plaque formation. SAC+NAC treatment protected against experimental atherosclerosis in ApoE-/- DM model mouse by suppressing superoxide production in the atherosclerotic lesions. The combined treatment of SAC and NAC significantly reduced blood sugar levels in DM model mouse with STZ induced ApoE-/- diabetic atherosclerosis. These data warrant further study to ascertain their usefulness in patients with DM. Our results suggest that anti-oxidative agents, food and antioxidant behaviour may be beneficial to protect from atherosclerosis in the clinical setting.

The therapy of SAC combined with NAC could significantly reduce the blood lipoprotein level of STZ-ApoE-/- mice and may to some degree ease the trend of weight loss and the aorta atheromatous but it requires further work to establish its therapeutic potential in humans.

Declaration

Ethical approval for animal experiments: Ethics Committee at Weifang Medical University, Shandong, China, approved the use of tissue samples, animal studies and the experimental procedures, and full ethical approval was granted by them.

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Conflict of interest: The authors wish to confirm that there are no known conflicts of interest associated with this publication and declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Consent for publication: Has been obtained from all authors.

References

1. Aldini G, et al. Molecular strategies to prevent, inhibit, and degrade advanced glycoxidation and advanced lipoxidation end products. Free Radic Re. 2013; 47: 93-137.
2. Singh Grewal A, Bhardwaj S, Pandita D, Lather V, Singh Sekhon B. Updates on aldose reductase inhibitors for management of diabetic complications and non-diabetic diseases. Mini reviews in medicinal chemistry. 2016: 16: 120-162.
3. Hsiung S, et al. Hyperglycemia does not affect tissue repair responses in shear stress-induced atherosclerotic plaques in ApoE−/− mice. Scientific reports. 2018; 8: 1-11.
4. Rask-Madsen C, King GL. Vascular complications of diabetes: mechanisms of injury and protective factors. Cell metabolism. 2013; 17: 20-33.
5. Xu J, et al. Involvement of Advanced Glycation End Products in the Pathogenesis of Diabetic Retinopathy. Cell Physiol Biochem. 2018; 48: 705-717.
6. Chaudhuri J, et al. The Role of Advanced Glycation End Products in Aging and Metabolic Diseases: Bridging Association and Causality. Cell Metab. 2018; 28: 337-352.
7. Perrone A, Giovino A, Benny J, Martinelli F. Advanced Glycation End Products (AGEs): Biochemistry, Signaling, Analytical Methods, and Epigenetic Effects. Oxid Med Cell Longev. 2020; 2020: 3818196.
8. Moldogazieva NT, Mokhosoev IM, Mel'nikova TI, Porozov YB, Terentiev AA. Oxidative stress and advanced lipoxidation and glycation end products (ALEs and AGEs) in aging and age-related diseases. Oxidative medicine and cellular longevity. 2019; 2019: 3085756.
9. Nowotny K, Jung T, Hohn A, Weber D, Grune T. Advanced glycation end products and oxidative stress in type 2 diabetes mellitus. Biomolecules. 2015; 5: 194-222.
10. Ahmed N. Advanced glycation endproducts—role in pathology of diabetic complications. Diabetes research and clinical practice. 2005; 67: 3-21.
11. Cripps MJ, et al. Carnosine scavenging of glucolipotoxic free radicals enhances insulin secretion and glucose uptake. Scientific reports. 2017; 7: 1-7.
12. Mashilipa C, Wang Q, Slevin M, Ahmed N. Antiglycation and antioxidant properties of soy sauces. J Med Food. 2011; 14: 1647-1653.
13. Papachristoforou E, Lambadiari V, Maratou E, Makrilakis, K. Association of glycemic indices (hyperglycemia, glucose variability, and hypoglycemia) with oxidative stress and diabetic complications. Journal of Diabetes Research. 2020; 2020: 7489795.
14. Kang Q, Yang C. Oxidative stress and diabetic retinopathy: Molecular mechanisms, pathogenetic role and therapeutic implications. Redox Biology. 2020; 37: 101799.
15. Pickering RJ, et al. Recent novel approaches to limit oxidative stress and inflammation in diabetic complications. Clinical & translational immunology. 2018; 7: e1016.
16. Pasupuleti VR, et al. A Review on Oxidative Stress, Diabetic Complications, and the Roles of Honey Polyphenols. Oxidative medicine and cellular longevity. 2020; 2020: 8878172.
17. Slevin M, Ahmed N, Wang Q, McDowell G, Badimon L. Unique vascular protective properties of natural products: supplements or future main-line drugs with significant anti-atherosclerotic potential? Vasc Cell. 2012; 4: 9.
18. Song F, Schmidt AM. Glycation and insulin resistance: novel mechanisms and unique targets? Arterioscler Thromb Vasc Biol. 2012; 32: 1760-1765.
19. Ormazabal V, et al. Association between insulin resistance and the development of cardiovascular disease. Cardiovascular Diabetology. 2018; 17: 122.

20. Sasaki N, et al. Advanced glycation end products in Alzheimer's disease and other neurodegenerative diseases. Am J Pathol. 1998; 153: 1149-1155.

21. Tokunaga M, et al. Diet, nutrients and noncommunicable diseases. The Open Nutraceuticals Journal. 2012; 5: 146-159.

22. Vistoli G, et al. Advanced glycoxidation and lipoxidation end products (AGEs and ALEs): an overview of their mechanisms of formation. Free Radical Research. 2013; 47: 3-27.

23. Ko S-Y, et al. The possible mechanism of advanced glycation end products (AGEs) for Alzheimer's disease. PLoS One. 2015; 10: e0143345.

24. Mensah GA, et al. Decline in cardiovascular mortality: possible causes and implications. Circulation research. 2017; 120: 366-380.

25. Fadini GP, et al. Circulating stem cells and cardiovascular outcomes: from basic science to the clinic. European heart journal. 2020; 41: 4271-4282.

26. Hsieh CL, et al. Inhibitory effect of some selected nutraceutic herbs on LDL glycation induced by glucose and glyoxal. Journal of Ethnopharmacology. 2005; 102: 357-363.

27. Kronzon I, Tunick PA. Aortic atherosclerotic disease and stroke. Circulation. 2006; 114: 63-75.

28. Puri R, et al. C-reactive protein, but not low-density lipoprotein cholesterol levels, associate with coronary atheroma regression and cardiovascular events after maximally intensive statin therapy. Circulation. 2013; 128: 2395-2403.

29. Kramer, CD. et al. Distinct gene signatures in aortic tissue from ApoE(-/-) mice exposed to pathogens or Western diet. Bmc Genomics. 2014; 15: 1176.

30. Sas KM, et al. Shared and distinct lipid-lipid interactions in plasma and affected tissues in a diabetic mouse model. Journal of lipid research. 2018; 59: 173-183.

31. SELIM M. The Role of Glycation in Pathology of Diabetic Microvascular Complications. 2019.

32. Han X. Lipidomics for studying metabolism. Nature Reviews Endocrinology. 2016; 12: 668-679.

33. Tabas I, Williams KJ, Boren J. Subendothelial lipoprotein retention as the initiating process in atherosclerosis - Update and therapeutic implications. Circulation. 2007; 116: 1832-1844.

34. Bentzon JF, Otsuka F, Virmani R, Falk E. Mechanisms of plaque formation and rupture. Circ Res. 2014; 114: 1852-1866.

35. Insull Jr W. The pathology of atherosclerosis: plaque development and plaque responses to medical treatment. The American journal of medicine. 2009; 122: S3-S14.

36. N Orekhov A. Direct anti-atherosclerotic therapy; development of natural anti-atherosclerotic drugs preventing cellular cholesterol retention. Current Pharmaceutical Design. 2013; 19: 5909-5928.

37. Ekor M. The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. Frontiers in Pharmacology. 2014; 4: 177.

38. Shang A, et al. Bioactive compounds and biological functions of garlic (Allium sativum L.). Foods. 2019; 8: 246.

39. Elosta A, Slevin M, Rahman K, Ahmed N. Aged garlic has more potent antiglycation and antioxidant properties compared to fresh garlic extract in vitro. Scientific Reports. 2017; 7: 39613.

40. García-Villalón A, et al. In vitro studies of an aged black garlic extract enriched in S-allylcysteine and polyphenols with cardioprotective effects. Journal of functional foods. 2016; 27: 189-200.

41. Steiner M, Li W. Aged garlic extract, a modulator of cardiovascular risk factors: a dose-finding study on the effects of AGE on platelet functions. J Nutr. 2001; 131: 980S-984S.

42. Singh VP, Bali A, Singh N, Jaggi AS. Advanced glycation end products and diabetic complications. Korean J Physiol Pharmacol. 2014; 18: 1-14.

43. Bhat S, Mary S, Giri AP, Kulkarni MJ. Mechanisms of vascular defects in diabetes mellitus. 2017; 423-449.

44. Rhee SY, Kim YS. The Role of Advanced Glycation End Products in Diabetic Vascular Complications. Diabetes & Metabolism Journal. 2018; 42. 188-195.

45. Bayan L, Koulivand PH, Gorji A. Garlic: a review of potential therapeutic effects. Avicenna journal of phytomedicine. 2914; 4: 1-14.

46. Gruenwald J, Bongartz U, Bothe G, Uebelhack R. Effects of aged garlic extract on arterial elasticity in a placebo-controlled clinical trial using EndoPAT™ technology. Experimental and therapeutic medicine. 2020; 19: 1490-1499.

47. Sobenin IA, Myasoedova VA, Iltchuk MI, Zhang D-W, Orekhov AN. Therapeutic effects of garlic in cardiovascular atherosclerotic disease. Chinese journal of natural medicines. 2019; 17: 721-728.

48. Nabi R, Alvi SS, Saeed M, Ahmad S, Khan MS. Glycation and HMG-CoA reductase inhibitors: implication in diabetes and associated complications. Current diabetes reviews. 2019; 15: 213-223.

49. Ahmad S, et al. A glycation angle to look into the diabetic vasculopathy: cause and cure. Current vascular pharmacology. 2017; 15: 352-364.

50. Ahmad MS, Ahmed N. Antiglycation properties of aged garlic extract: possible role in prevention of diabetic complications. The Journal of nutrition. 2006; 136: 796S-799S.

51. Voytik-Harbin SL, Brightman AO, Waisner B, Lamar CH, Badylak SF. Application and evaluation of the alamarBlue assay for cell growth and survival of fibroblasts. In Vitro Cell Dev Biol Anim. 1998; 34: 239-246.

52. Kumar P, Nagarajan A, Uchil PD. Analysis of Cell Viability by the alamarBlue Assay. Cold Spring Harb Protoc. 2018; 2018: pdb. prot095489.

53. Liu D, et al. Low Concentration of Sodium Butyrate from Ultrabraid+NaBu suture, Promotes Angiogenesis and Tissue Remodelling in Tendon-bones Injury. Sci Rep. 2016; 6: 34649.

54. Franko A, et al. Liver adapts mitochondrial function to insulin resistant and diabetic states in mice. J Hepatol. 2014; 60: 816-823.

55. Franko A, et al. Bezafibrate Improves Insulin Sensitivity and Metabolic Flexibility in STZ-Induced Diabetic Mice. Diabetes. 2016; 65: 2540-2552.

56. Arreola R, et al. Immunomodulation and anti-inflammatory effects of garlic compounds. Journal of immunology research. 2015; 2015: 401630.

57. Ried K, Travica N, Sali A. The effect of Kyolic aged garlic extract on gut microbiota, inflammation, and cardiovascular markers in hypertensives: The GarGIC Trial. Frontiers in nutrition. 2018; 5: 122.

58. Petrovic V, et al. Anti-cancer potential of homemade fresh garlic extract is related to increased endoplasmic reticulum stress. Nutrients. 2018; 10: 450.

59. La Sala L, Prattichizzo F, Ceriello A. The link between diabetes and atherosclerosis. European journal of preventive cardiology. 2019; 26: 15-24.

60. Poznyak A, et al. The diabetes mellitus–atherosclerosis connection: The role of lipid and glucose metabolism and chronic inflammation. International journal of molecular sciences. 2020; 21: 1835.

61. Linton MF, et al. The role of lipids and lipoproteins in atherosclerosis. Endotext. 2019.

62. Rafieian-Kopaei M, Setorki M, Doudi M, Baradaran A, Nasri H. Atherosclerosis: process, indicators, risk factors and new hopes. International journal of preventive medicine. 2014; 5: 927-946.

63. Mauricio D, Castelblanco E, Alonso N. Multidisciplinary Digital Publishing Institute. 2020.

64. Boren J, et al. Low-density lipoproteins cause atherosclerotic cardiovascular disease: pathophysiological, genetic, and therapeutic insights: a consensus statement from the European Atherosclerosis Society Consensus Panel. European heart journal. 2020; 41: 2313-2330.

65. Miname MH, Santos RD. Reducing cardiovascular risk in patients with familial hypercholesterolemia: risk prediction and lipid management. Progress in cardiovascular diseases. 2019; 62: 414-422.

66. Summerhill VI, Grechko AV, Yet S-F, Sobenin IA, Orekhov AN. The atherogenic role of circulating modified lipids in atherosclerosis. International journal of molecular sciences. 2019; 20: 3561.

67. Maguire EM, Pearce SW, Xiao Q. Foam cell formation: A new target for fighting atherosclerosis and cardiovascular disease. Vascular pharmacology. 2019; 112: 54-71.

68. Park L, et al. Suppression of accelerated diabetic atherosclerosis by the soluble receptor for advanced glycation endproducts. Nature medicine. 1998; 4: 1025-1031.

69. Basta G, Schmidt AM, De Caterina R. Advanced glycation end products and vascular inflammation: implications for accelerated atherosclerosis in diabetes. Cardiovascular research. 2004; 63: 582-592.

70. Wang J, Zhang X, Lan H, Wang W. Effect of garlic supplement in the management of type 2 diabetes mellitus (T2DM): a meta-analysis of randomized controlled trials. Food & nutrition research. 2017; 61: 1377571.

71. Santhosha S, Jamuna P, Prabhavathi S. Bioactive components of garlic and their physiological role in health maintenance: A review. Food Bioscience. 2013; 3: 59-74.

72. Maeda T, Miki S, Morihara N, Kagawa, Y. Aged garlic extract ameliorates fatty liver and insulin resistance and improves the gut microbiota profile in a mouse model of insulin resistance. Experimental and therapeutic medicine. 2019; 18: 857-866.

73. Qian X. Garlic for the prevention of cardiovascular morbidity and mortality in hypertensive patients: summaries of nursing care-related systematic reviews from the cochrane library. International Journal of Evidence-Based Healthcare. 2013; 11: 83.

74. Chiavarini M, Minelli L, Fabiani R. Garlic consumption and colorectal cancer risk in man: a systematic review and meta-analysis. Public health nutrition. 2016; 19: 308-317.

75. Catalán V, Frühbeck G, Gómez-Ambrosi J. in Obesity. 2018: 163-189.

76. Everett BM, et al. Anti-inflammatory therapy with canakinumab for the prevention and management of diabetes. Journal of the American College of Cardiology. 2018; 71: 2392-2401.

77. Kosmas CE, et al. Anti-inflammatory therapy for cardiovascular disease. Annals of translational medicine. 2019; 7: 147.

78. Stöckli J, et al. Metabolomic analysis of insulin resistance across different mouse strains and diets. Journal of Biological Chemistry. 2017; 292: 19135-19145.