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Anti-inflammatory and anti-oxidative activities of andrographolide determined using atherosclerosis induced mice

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Abstract: Atherosclerosis and relative cardiovascular complications remain the main reasons for death worldwide. This study stimulated atherosclerosis in C57BL/6J mice using P-407 via intraperitoneal injection, and treatment with Andrographolide (15, 30 and 45 mg/kg BW) was carried out for six weeks. The heart and aorta were harvested after six weeks and assessed using Enzyme-Linked Immunosorbent Assay (ELISA) and histological studies. The results demonstrated that the treatment with AGP reversed the effects of P-407 induced atherosclerosis. The doses of AGP correlated with the reduction of atherosclerosis biomarkers, and a high dose (45 mg/kg BW) was the most significant dose. The Low-Density Lipoprotein (LDL), Triglycerides (TG), and Atherogenic Index (AI) were significantly reduced by the AGP treatment. The histological results showed a reduction in inflammation, fibrosis and hypertrophy in the heart tissues of the groups treated with AGP compared to the disease control. In addition, AGP treatment significantly decreased Reactive Oxygen Species (ROS) and the inflammation marker (NF-kB). Furthermore, the AGP-treated groups showed typical morphological characteristics of the aorta, while the disease control cells were highly affected. The results demonstrated that AGP is highly recommended as a natural treatment to reduce the symptoms of atherosclerosis by reducing oxidative stress and inflammation.

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1. Introduction

Atherosclerosis and relative cardiovascular complications are the main leading causes of death globally, as shown in statistics from previous studies (Go *et al.*, 2013). Prolonged inflammations and oxidative stress contribute to increasing the threat of developing atherosclerosis and other heart diseases. In addition, cytokines responsible for the prolonged inflammation could lead to other diseases such as asthma, rheumatoid arthritis, and inflammatory bowel disease. There is now consensus that atherosclerosis is more common in individuals with elevated oxidative stress characterised by lipids and proteins in the vascular wall. The key mediator, Reactive Oxygen Species (ROS), is responsible for the signalling pathways that cause vascular inflammation in atherogenesis. Many studies reported that released ROS was causative in cardiovascular diseases like atherosclerosis (Bonomini *et al.*, 2008). Conversely, though, the activation of NF-κB was reported to be associated with the induction of several inflammations (Cheon *et al.*, 2015). These studies suggest that targeting oxidative stress and inflammation is a promising therapeutic strategy for preventing or treating atherosclerosis. However, the treatment of inflammatory conditions is carried out using tumour necrosis factor-alpha (TNF-α) inhibitors, Steroids and Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) (Low *et al.*, 2015). Using these medicines for the long term causes several side effects like increasing blood pressure, gastrointestinal inflammations and heart failure (Pirmohamed *et al.*, 2004).

The herbaceous plant *A. paniculata (Burma f*) Nees grows in tropical and semitropical regions. Previous studies on *A. paniculata* have uncovered the presence of a significant quantity of flavonoids, labdane diterpenoids, stigmasterol's and xanthones. The plant has been traditionally used in Asian countries such as China, Thailand, India and Malaysia to treat upper respiratory tract infections. The major constituents of this plant are Andrographolide (AGP) and 14-deoxy-11, 12-didehydroandrographolide (DDAGP) (Lim *et al.*, 2012). The combination of aqueous and methanol leaves extracts of *A. paniculata* exhibited significant improvement of lipopolysaccharide (LPS), causing the release of pro-inflammatory mediators (NO, IL-1β and IL-6), inflammatory mediators (TXB2 and PGE2) and allergic mediators (LTB4). Still, there were no signs of inhibition against histamine release. (Chandrasekaran *et al.*, 2010). Seven phytochemicals, namely, AGP, DDAGP neoandrographolide, isoandrographolide, andrograpanin, 7-O-methylwogonin, and skullcap flavone isolated from *A. paniculata* leaves underwent screened for *in vitro* anti-inflammatory and anti-allergic potential. The results of the study demonstrated that AGP can significantly extent inhibit inflammatory mediators NO, PGE2 and IL-1β release from LPS-stimulated cultured macrophages. The results of the study suggested the relationship between the mechanism of AGP and the downregulation of genes involved in the inflammatory cascade (Chandrasekaran *et al.*, 2011).

Numerous research studies have shown the medicinal properties of AGP. However, the effectiveness and the specific biological pathways through which AGP functions in treating cardiovascular conditions like atherosclerosis remain uncertain. Moreover, the high rate of atherosclerosis mortality and the limited existence of inexpensive natural medicines suggest that additional profound studies are needed for the development of anti-atherosclerosis treatments. Thus, the present study is aimed to investigate the efficacy and molecular mechanisms of AGP in attenuating P-407 induced atherosclerosis in C57BL/6J mice. Moreover, the biochemical profiles of the blood plasma were evaluated after six weeks of AGP

treatment at different concentrations. The histological changes in the heart and aortic tissues were observed to determine toxicity safety.

2. Materials and methods

2.1. Materials

Seven to nine-week-old male C57BL/6J mice were purchased from Monash University (Malaysia Campus), and the weighted average was 28.1 ± 0.08 g at the time of the experiment. AGP (99% purity as determined by HPLC) was obtained from the Department of Pharmaceutical Chemistry, School of Pharmacy, International Medical University (IMU), Malaysia. The P-407 (Sigma-Aldrich, St. Louis, Missouri, USA) was used for atherosclerosis induction. p 65-NF-κB ELISA kit was purchased from (Elabscience Biotechnology Inc., Houston, Texas).

2.2. Induction of hyperlipidaemia

The IMU Joint Committee (Research & Ethics Committee) of the International Medical University, Malaysia, accepted the animal experiment protocol with an acceptance letter (MAPC 1/2017). In brief, 35 male C57BL/6J mice were fed a regular, low-fat chow diet for two weeks to acclimate to their new surroundings.

The mice were then divided randomly into five groups. Group 1 (control) mice (n = 6) were fed a regular, low-fat chow diet devoid of cholic acid. Group 2 (disease control) mice (n = 10) were fed a regular, low-fat chow diet that did not include cholic acid. Mice in groups 3 (n = 6), 4 (n = 7), and 5 (n = 8) were given orally (p.o.) with 15, 30, and 45 mg/kg Body Weight (BW) of AGP along with normal. Mice in groups (2, 3, 4, and 5) received 0.5 g/kg BW of P-407 intraperitoneally every third day for six weeks. P-407 was given at least two hours before the mice were fed AGP. Palmer *et al.* (1998) devised the treatment regimen, finding that intraperitoneal injection of a single dosage of 0.5 g/kg BW of P-407 in C57BL/6J mice enhanced plasma TC and TG levels after 2 hours.

2.3. Animal sacrifice and tissue collection

After six weeks, the mice were fasted for 12 hours and anesthetised with an anaesthetic cocktail consisting of ketamine-tiletamine-xylazine (0.5 mL/kg BW) via intraperitoneal injection. The blood samples (2 mL) gathered by retro-orbital plexus puncturing were collected into Eppendorf tubes containing 2 µL EDTA. The tubes were immediately placed in an ice bath and centrifuged at 4000 rpm for 12 minutes to obtain the plasma. The plasma was stored at -80 °C until further analysis. Each mouse was sacrificed by bilateral thoracotomy, and blood samples were gathered via cardiac puncture. The heart, aorta, and adipose tissue organs were removed and weighed. The separated organs were cut into two pieces. The first component was soaked in a 10% neutral buffered formalin solution for histopathological examinations, while the second was snap-frozen in liquid nitrogen and kept at -80 °C for biomarker measurement.

All animal sacrifice and tissue collection methods were carried out in accordance with Kim *et al.* (2022) descriptions and the OECD Guidelines for Chemical Safety and Animal Welfare for the care and treatment of laboratory animals.

2.4. Biochemical analysis for blood samples

Biochemical analysis was carried out for the blood samples following standard methods and by authorised staff in the Hematology and Clinical Biochemistry Lab, Faculty of Veterinary Medicine, University Putra Malaysia. The liver and kidney function tests were analysed using the standard markers: Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST). Moreover, serum lipid profiles were assayed, including triglycerides (TG), total cholesterol (TC), low-density lipoprotein (LDL), and high-density lipoprotein (HDL). Further analyses were conducted to determine the Atherogenic Index (AI) and Very Low-Density Lipoprotein (VLDL). The average AI of P-407 induced atherosclerosis in C57BL/6J mice was determined using the formula shown in Equation (1) (Lama & Saikia, 2013).

$$\text{AI} = \frac{TC-HDL}{HDL} \quad (1)$$

The VLDL of P- 407 induced atherosclerosis in C57BL/6J mice was determined using the formula shown in Equation (2) (Friedewald *et al.*, 1972; Vujovic *et al.*, 2010).

$$\text{VLDL} = \frac{TG}{2.2} \quad (2)$$

2.5. Determination of NF-κB levels

Heart tissues were homogenised in ice-cold 1 x phosphate-buffered saline (PBS, pH 7.4) with a freshly added protease inhibitor cocktail (product number: 25955-11, Nacalai Tesque, INC, Japan) using a G50 model motor-driven tissue grinder. To 1 mg of tissue sample, 10 µL of protease inhibitor was applied. Heart homogenised samples underwent centrifugation at 14,000 rpm for 15 minutes at 4 degrees Celsius (Eppendorf chilled centrifuge 5920 R; Hamburg, Germany). Supernatants were collected and kept at -80°C until further examination. Yang *et al.* (2015) revealed how to detect NF-□B levels in supernatant samples. NF-κB expression was measured by using an ELISA kit purchased from Elabscience Biotechnology Inc., Houston, Texas. Generally, each sample (100 µL) was loaded into the wells of an NF-κB antibodies pre-coated 96-well plate. The samples were aspirated, and 100 µL of biotinylated detection antibodies and 100 µL Avidin-horseradish peroxidase complex were added, and 90 µL of TMB reagent was added and kept for 15 minutes. The absorbance readings solutions were determined at 450 nm using a Spectramax M3 multi-mode microplate reader (Molecular Devices; Sunnyvale, CA, USA). The NF-κB activity in samples was measured from a standard curve plotted using a given set of standard dilutions, and the experiment was repeated in triplicate. The NF-κB activity of AGP is expressed as mean (ng/mL) of mouse NF-κB p65 ± S.D.

2.6. Reactive Oxygen Species Detection (ROS)

ROS in heart tissue was determined as described in the literature (Sadagurski *et al.*, 2011) using a cell-permeable dye 5,6-carboxy-2',7'-dichlorofluorescein diacetate (Carboxy-DCFDA, Molecular Probes, Grand Island, NY, USA). Heart tissues were weighed before being thawed in a buffered medium consisting of 5 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) in PBS and soaked for 10 minutes. The buffered medium was decanted, and tissue samples were treated with eight µM Carboxy-DCFDA in fresh buffered medium and were incubated at 37 °C for 45 minutes. The surplus medium was subsequently poured off, and

samples were further incubated in 0.1% SDS lysis buffer in the presence of Tris-HCl to regulate acidity and osmolarity of the lysate and adjusted to pH 7.4 for 15 minutes at 4 °C. Samples were homogenised using G50 model motor-driven tissue grinder as mentioned above and centrifuged at 14,000 rpm for 20 minutes at 4°C. Supernatants were collected, transferred into a solid black microplate, and then analysed for fluorescence at a wavelength of 530 nm excited at 485 nm using a Spectramax M3 Multi-Mode microplate reader (Molecular Devices, Sunnyvale, CA, USA). Upon contact with the fluorescent probe to the samples, the protocol was performed entirely in a strict dark environment with very minimal backlight to prevent photodegradation of Carboxy-DCFDA.

2.7. Histopathological studies

Histopathology studies followed standard procedures at the Hematology and Clinical Biochemistry Lab, Faculty of Veterinary Medicine, University Putra Malaysia. The stained pictures of tissue samples were seen at 100X to 400X total magnification and taken with a Nikon Ni-U camera fitted with a Digital Sight DS-Ri2. The images were analysed using Nikon-Instrument software (NIS)-Elements Basic Research software. Briefly, tissue samples (heart and aorta) were thoroughly cleaned with PBS (pH 7.4) and preserved in 10% neutral-buffered formalin (Richard-Allan Scientific) for 12-24 hours at room temperature before being embedded in paraffin. A rotary microtome cut the paraffin blocks into 4 (heart) and 5 (aorta) µm thick pieces. Hematoxylin and eosin staining were carried out to detect inflammation and hypertrophy in the heart tissues (Myou *et al.*, 2003). Moreover, Masson's trichrome was performed to detect fibrosis in the heart tissues following the method described by (Bauman *et al.*, 2014). Oil-Red-O staining was also conducted on aortic tissue to assess the atherosclerotic lesion formation as described previously (Andrés-Manzano, 2015).

3. Results and discussion

Atherosclerosis and its associated risks are a significant concern as they contribute to the development of various diseases, including coronary heart disease, chronic kidney disease, and peripheral arterial disease (Reyes-Soffer *et al.*, 2022). Atherosclerosis is recognised as a life-threatening disease which contributes to three out of the top ten causes of death worldwide, namely, stroke, heart disease and type II diabetes (Horváth *et al.*, 2018). With that worrying concern, new and potent bioactive compounds are constantly being searched and tested to develop novel compounds combating this epidemic. *A. paniculata* is a well-known herbaceous plant belonging to the Acanthaceae family that has consistently shown its efficacy in treating various diseases, from leprosy to dysentery (Okhuarobo *et al.*, 2014). *A. paniculata* bioactive molecules were reported in several studies. The main bioactive molecule of interest is AGP due to the effects of preventing oxygen radical production and subsequently anti-inflammatory diseases (Azlan *et al.*, 2013).

Previous studies suggested that AGP blocks the cytochrome C released from the mitochondria and inhibits caspase -3 and -9 activation to prevent atherosclerosis by promoting endothelial cell survival (Azlan *et al.*, 2013). Azlan *et al.* (2013) have reported that the impairment of endothelial cells induced the pathogenesis of atherosclerosis. The mechanism of action suggests that the ability of AGP to promote endothelial cell survival and protection against apoptosis can assist in the suppression of apoptotic endothelial cells and subsequently alleviate atherosclerosis. The efficacy of AGP against atherosclerosis was identified, which was carried

out by a current study, which provides substantial scientific evidence to support the claim that AGP is effective as an anti-atherosclerosis agent.

The *in vivo* experimental model comprising C57BL/6J mice is designed to induce atherosclerosis and mimic the atherosclerosis conditions in an organism. Previous studies showed the utilisation of P-407 in successfully inducing atherosclerosis in mice models (Palmer *et al.*, 1998). The treatment of P-407 in mice models displayed significant hyperlipidemia, mid-blood pressure elevation, liver lipidosis cells, and contractile-type changes in cardiomyocytes (Korolenko *et al.*, 2016). Previous researchers reported that P-407 induced atherosclerosis C57BL/6J mice models demonstrated successful results, stimulating atherosclerosis lesions with the aid of high fat, high cholesterol cholate-containing diet (Korolenko et al., 2016; Palmer *et al.*, 1998).

AST and ALT levels were determined to obtain information on liver functions in an *in vivo* model after the diseases were induced and the treatments. The results showed significant differences ($p < 0.05$) in the ALT and AST levels between the standard control and disease control groups (Table-1). On the other hand, the AGP treatment groups showed low AST and ALT levels that were slightly lower than the control normal group. The AST and ALT concentrations strongly correlated to the dose of AGP, and the higher dose (45 mg) had the lowest values. AST and ALT are common liver enzymes that are used as indicators for liver function monitoring whilst under the treatment of drugs. The results for both liver enzymes in Group 5 (AGP-45 mg/kg BW) demonstrated the most significant difference from the disease control ($p < 0.05$). Results indicated the efficacy of AGP as anti-atherosclerosis in this P-407 induced atherosclerosis C57BL/6J mice model. In a previous study, *Porphyromonas gingivalis* caused liver damage in rabbits and was reversed with 20 mg/kg BW of AGP (Al-Batran *et al.*, 2013). A previous study found that a low ALT/AST ratio predicts coronary atherosclerosis (Adibi *et al.*, 2007). The AST and ALT increased levels are indicators of liver damage, as observed for the disease control that showed very high levels, while the normal group and AGP treated groups showed low levels. These findings demonstrate the efficacy of AGP for hepatoprotective effects.

Table-1: Average plasma liver enzyme levels in C57BL/6J mice at the end of the experiment (6 weeks)

Group	AST (U/L)	ALT (U/L)
Group 1 (normal)	289.67 ± 40.48	72.00 ± 9.24
Group 2 (disease control)	379.25 ± 54.99*	129.00 ± 32.19*
Group 3 (AGP 15 mg/kg)	279.67 ± 130.14[a]	75.75 ± 26.56[a]
Group 4 (AGP 30 mg/kg)	227.20 ± 67.99[b]	72.75 ± 9.35[a]
Group 5 (AGP 45 mg/kg)	219.75 ± 10[b]	59.33 ± 7.68[b]

Note: * Shows a substantial ($P < 0.05$) difference between normal and illness control groups. The presence of a letter implies a significant ($P < 0.05$) difference between illness control and treatment groups. The absence of the letter implies that there is no significant difference ($P > 0.05$) between the illness control and treatment groups. The same alphabet implies no significant difference ($P > 0.05$) between treatment groups. A different alphabet ($P < 0.05$) indicates a significant difference between treatment groups.

The average plasma lipid levels of TC, LDL, TG and HDL demonstrated significant variations among the groups at the end of the experiment (Table-2). The normal group lipid profile, which is the baseline was 44 ± 0.16, 0.63 ± 0.07, 0.12 ± 0.01, and 1.34±0.16 mmol/L for TC, LDL, TG and HDL, respectively. In comparison, the disease control group showed a highly significant ($P < 0.05$) increase for the TC, TG and LDL values, while the HDL was significantly

reduced. The AGP treatments reversed the effects of atherosclerosis, which affects lipid profiles based on dose-dependent response. The most significant TC, TG and LDL reduction was observed for the group subjected to treatment with 45 mg/kg BW AGP, and the HDL content was higher than the normal group. The disease control group's lipid profile, including TC, TG, LDL, and HDL, showed a significant increase in TC, TG, and LDL. However, the low levels of LDL in the mice group treated with AGP15, AGP30, and AGP45 showed a strong effect of pure AGP in treating atherosclerosis. Group 5 were subjected to a dosage of 45 mg/kg BW AGP, which showed the most significant reduction in the biochemical parameter levels ($p < 0.05$). The association between LDL and TG could be observed progressively. One of the isoforms of the apolipoprotein B (apoB) produced in the liver is apoB100, which happens to be the core structural apoliprotein of LDL.

In this study, the normal group had VLDL and AI compared to the disease control, which exhibited very high values. The AGP treated groups showed reduced VLDL and AI and the most significant reduction was observed for the high dose group. The AGP treatment reduced the VLDL and AI in correlation with the doses. In contrast, HDL levels, which are inversely associated with the risk of development of atherosclerosis (Barter, 2005), appear to be present relatively uniformly in all study groups but significantly raised in Group 5. HDL possess cardioprotective effect thus, it was known to reverse cholesterol transport (Assmann & GottoJr, 2004). A mere 1% decrement in HDL level was linked to a 3-4% increase in risks associated with heart diseases (Dhandapani, 2007). This coupled with the lowered TC, TG and LDL levels across the groups exhibits a dose - dependent effect of AGP in P-407 induced atherosclerosis C57BL/6J mice experimental model. Also, the AI serves as an indication of increased TC, LDL, TG in combination with a decrease of HDL; and AI is often used to assess the susceptibility to cardiovascular and its associated diseases (Popa *et al.*, 2012).

Table-2: Average of plasma lipid levels (TC, TG, LDL, & HDL) in C57BL/6J mice at end of experiment

Group	TC (mmol/L)	TG (mmol/L)	LDL (mmol/L)	HDL (mmol/L)
Group 1 (normal)	1.44 ± 0.16	0.63 ± 0.07	0.12 ± 0.01	1.34 ± 0.16
Group 2 (disease control)	9.65 ± 1.27*	13.78 ± 0.54*	4.96 ± 1.05*	0.97 ± 0.13*
Group 3 (AGP 15 mg/kg)	8.98 ± 0.87	12.17 ± 1.99^{a}	3.33 ± 0.22^{a}	1.20 ± 0.12
Group 4 (AGP 30 mg/kg)	7.58 ± 0.82^{a}	9.79 ± 0.86^{b}	3.30 ± 0.43^{a}	0.84 ± 0.07
Group 5 (AGP 45 mg/kg)	6.15 ± 0.59^{b}	8.13 ± 2.68^{c}	2.35 ± 0.27^{b}	1.67 ± 0.22^{b}

Note: * Shows a substantial ($P < 0.05$) difference between normal and illness control groups. The presence of a letter implies a significant ($P < 0.05$) difference between illness control and treatment groups. The absence of the letter implies that there is no significant difference ($P > 0.05$) between the illness control and treatment groups. The same alphabet implies no significant difference ($P > 0.05$) between treatment groups. A different alphabet ($P < 0.05$) indicates a significant difference between treatment groups.

NF-κB was determined in this study because it is responsible for the expression of pro-inflammatory cytokines and chemokines, thus is highly regarded as a proinflammatory signalling pathway (Su *et al.*, 2023). The results for the NF-κB demonstrated significant differences between the normal and disease groups due to the effects of induced atherosclerosis (Table-3). In contrast, a significant ($P < 0.05$) reduction in NF-κB was observed for the groups treated with the AGP at different concentrations. The group subjected to treatment using AGP 45 mg/kg BW exhibited a very low value for NF-κB (0.52 ± 0.05) compared to the normal group. NF-κB falls in the protein complex category that is responsible for a primary transcription factor of detrimental stimuli on a cellular level, including inflammation (Pateras *et al.*, 2014). NF-κB controls many genes involved in inflammation, and it is found to be active

in many inflammatory diseases, for example, bowel inflammations, arthritis, asthma and atherosclerosis (Monaco *et al.*, 2004). Moreover, elevation of NF-κB activators can cause high mortality, especially for cardiovascular disease patients (Venuraju *et al.*, 2010). The NF-κB found in heart tissue homogenates showed a significant elevation of NF-κB concentrations in Group 2 (disease control group). Tran *et al.* (2015) suggested that blocking inflammatory mediators such as NF-κB on the transcriptional level is a novel strategy to reduce inflammation. In this study, the therapeutic effect of AGP regarding atherosclerosis in P-407 induced C57BL/6J mice showed a significant reduction of the NF-κB concentration compared to the disease control group ($p < 0.05$). It indicated the anti-atherosclerosis effect posed by AGP. Several plant extracts were reported to reduce active NF-κB such as grape seed extract, thus very limited data is available about the bioactive compounds that limit their use as medicinal extracts (Dhanalakshmi *et al.*, 2003).

Table-3: NF-κB concentration and ROS level for C57BL/6J mice groups at the end of the experiment

Group	NF-κB concentration	ROS levels
Group 1 (normal)	0.77 ± 0.02	50.47 ± 7.60
Group 2 (disease control)	0.98 ± 0.03*	218.94 ± 38.55*
Group 3 (AGP 15 mg/kg)	0.65 ± 0.00[a]	202.55 ± 34.63
Group 4 (AGP 30 mg/kg)	0.57 ± 0.09[b]	169.00 ±42.65
Group 5 (AGP 45 mg/kg)	0.52 ± 0.05[b]	117.01 ± 14.28[a]

Note: * Shows a substantial ($P < 0.05$) difference between normal and illness control groups. The presence of a letter implies a significant ($P < 0.05$) difference between illness control and treatment groups. The absence of the letter implies that there is no significant difference ($P > 0.05$) between the illness control and treatment groups. The same alphabet implies no significant difference ($P > 0.05$) between treatment groups. A different alphabet ($P < 0.05$) indicates a significant difference between treatment groups.

The Reactive Oxygen Species (ROS) levels were determined to evaluate the cell damage caused by the induction of atherosclerosis in the rat groups compared to the normal group. The disease group demonstrated very high ROS (218.94 ± 38.55) compared to the normal group (50.47 ± 7.60) (Table-3). On the other hand, AGP dose-dependently decreased ROS levels, and the best results were observed in the rat group fed with AGP 45 mg/kg BW (117.01 ± 14.28). The rat groups subjected to feeding with AGP 15 and AGP 30 showed slight ROS reductions of 218.94 ± 38.55 and 202.55 ± 34.63, while 45 mg/kg BW demonstrated significant ($p < 0.05$) reduction. ROS elevated concentrations can cause elevated active NF-κB, enhancing the risk of atherosclerosis. Therefore, the inhibition of ROS by AGP was determined to evaluate the efficacy of reducing external stimuli and blocking NF-κB.

Mice heart tissue treated with P-407 showed increased infiltration of inflammatory cells and enhanced cardiomyocyte size compared to the normal control group of mice. The cells are predominantly fibroblasts, macrophages and lymphocytes. However, AGP treatment significantly reduced inflammatory cells and cardiomyocyte size dose-dependently compared to the untreated group. The effects of AGP at 15 mg/kg BW and 30 mg/kg/ BW were insignificant, whereas the effect at a high dose (45 mg/kg BW) was very significant. However, the untreated group had a high HW to BW ratio, an indicator of hypertrophy, which was reversed with AGP therapy. These results suggest that AGP attenuated inflammation and hypertrophic effect against P-407 induced atherosclerosis in mice. It was reported that P-407 treated mice manifest hyperlipidemia and exhibited increased fibrotic marker such as matrix metalloproteinase activity in the heart tissue, which was associated with myocardial injury. Hence, the measurement of fibrosis from the P-407 heart tissue of treated mice demonstrated

an increased area of fibrosis at the site of inflammation compared to the normal mice group. The level of fibrosis is marginal in normal mice. However, treatment with AGP dose-dependently and significantly decreased the area of fibrosis in comparison to the untreated mice. The effects of AGP at 15 mg/kg BW and 30 mg/kg BW were not significant, in contrast to the high dose (45 mg/kg BW), which was significant. The findings are reported for the first time that andrographolide treatment reduced cardiac fibrosis over the course of disease development in the P-407 treated mice.

Sections from the thoracic aorta of P-407 treated mice showed focal areas of pathological intimal thickening and areas of Diffuse Intimal Thickening (DIT). The spaces were seen within the aortic wall, suggesting lipid laden macrophages. Para-aortic adipose tissue shows a dense collection of brown fat. Sections from the P-407 treated mice showed extensive micro-vesicular fat, vacuolations, and scattered inflammatory cells. The findings suggest an inflammatory response within the adipose tissue of diseased mice. On the other hand, sections from AGP treated mice groups showed significant reduction of DIT in correlation with the AGP doses. Moreover, sections from the aorta and para-aortic adipose tissue stained with Oil-Red-O stain demonstrate evidence of foamy macrophages and lipids within the intimal layers. Sections from the disease group showed diffuse aggregation of Oil-Red-O positive elements within the aortic wall. Similar scattered stained elements were seen with low AGP dose, while the effect of high doses was significant compared to those in diseased mice. All other treated mice showed no evidence of aortic wall lipids. The continuous generation of oxidative stress can cause chronic inflammation (Lugrin *et al.*, 2014). The fluorescence intensity/mg of tissue is proportional to the levels of oxidative stress. A dose-dependent trend of AGP was observed to be significant for the reduction in ROS levels compared to the control disease. The results corroborated the presence of atherosclerosis induction and suggested the correlation between atherosclerosis and inflammation. The histopathological results were in agreement with biochemical parameters and ELISA. AGP dose-dependently decreased inflammation, cardiac hypertrophy, fibrosis, aortic thickening and lipid accumulation compared to the disease group. Overall, study of the Group 5 showed a significant difference in comparison with Group 2 (disease control) in all parameters investigated, and 45 mg/kg BW AGP was the most promising dose for the anti-atherosclerosis effects.

4. Conclusion

This study showed that atherosclerosis was successfully induced in C57BL/6J mice via intraperitoneal administration of P-407. The disease control mice (Group 2) demonstrated a high lipid profile, inflammation and oxidative stress compared to the normal group. The mice groups subjected to the treatment of AGP with different doses reversed the symptoms of atherosclerosis as determined by the blood biochemical analysis and histological examination. The trends in the data powerfully demonstrate that the activity of AGP was found to be dose-dependent. The highest AGP dose (45 mg/kg BW) produced the most significant results and is extremely promising for the treatment of atherosclerosis. Further study should be carried out to determine the AGP mechanisms involved in reducing oxidative stress and pro-inflammation cytokines. An in-depth analysis of precise cytokines and chemokines driving inflammation and oxidative stress pathways related to atherosclerosis might be an exploratory option for future AGP research. The effects of AGP treatment should be determined for other biomarkers, including different cytokines that indicate the damage caused by atherosclerosis.

Declaration of conflict of interest

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