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Original Article

Piper sarmentosum attenuates TNF-α-induced VCAM-1 and ICAM-1 expression in human umbilical vein endothelial cells

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الملخص

أهداف البحث: يلعب الالتهاب دورا رئيسا في التسبب في تصلب الشرايين. يعتبر بيبرسارمنتوسوم عشب له أنشطة كمضاد للأكسدة ومكافحة تصلب الشرايين. تهدف هذه الدراسة إلى تقييم الخصائص المضادة للالتهابات من المستخلص المائي لبيبر سارمنتوسوم في الخلايا البطانية للوريد السري البشري.

طرق البحث: تم تقسيم الخلايا البطانية للوريد السري البشري إلى ست مجموعات؛ التحكم، والعلاج بواسطة ١٠ نانوجرام/مل لعامل نخر الورم –ألفا، والعلاج المشترك ١٠ نانوجرام/مل لعامل نخر الورم –ألفا بأربعة تراكيز مختلفة من المستخلص المائي لبيبر سارمنتوسوم (١٠٠, ١٥٠, ٢٥٠ و٣٠٠ نانوجرام/مل) لمدة ٢٤ ساعة. بعد ذلك، تم استخراج البروتين من المركب-١ لالتصاق الخلايا الوعائية، والمركب-١ للالتصاق بين الخلايا، وتم قياس ي ٩٣٧ لالتصاق الخلايا الأحادية والعامل النووي-كابا ب والبروتين ٦٥ في الخلايا البطانية للوريد السري البشري.

النتائج: حفز العلاج بعامل نخر الورم-ألفا الخلايا البطانية للوريد السري البشري مع المستخلص المائي لبيبر سارمنتوسوم في تركيزات مختلفة خفض استخراج البروتين من المركب-١ لالتصاق الخلايا الوعائية والمركب-١ للالتصاق بين الخلايا بطريقة تعتمد على –الجرعة. علاوة على ذلك، منع المستخلص المائي لبيبر سارمنتوسوم أيضا عامل نخر الورم –ألفا ي ٩٣٧ تحفيز التصاق الخلايا الأحادية إلى الخلايا البطانية للوريد السري البشري. بالإضافة إلى ذلك، خفض المستخلص المائي لبيبر سارمنتوسوم عامل نخر الورم-ألفا من تحفيز العامل النووي –كابا ب والبروتين ٦٥ معتمدا على الجرعة.

الاستنتاجات: أظهرت النتائج أن المستخلص المائي لبيبر سارمنتوسوم حظر عامل نخر الورم- ألفا الناجم عن المركب-١ لالتصاق الخلايا الوعائية والمركب-١ للالتصاق بين الخلايا من خلال آلية تنطوي على العامل النووي –كابا ب.

الكلمات المفتاحية: الخلايا البطانية للوريد السري البشري؛ الالتهاب؛ المركب-١ الالتصاق بين الخلايا؛ بيبر سارمنتوسوم؛ المركب-١ التصاق الخلايا الوعائية

Abstract

Objectives: Inflammation plays a key role in the pathogenesis of atherosclerosis. *Piper sarmentosum* is an herb with antioxidant and anti-atherosclerotic activities. The aim of this study was to evaluate the anti-inflammatory properties of an aqueous extract of *P. sarmentosum* (AEPS) in human umbilical vein endothelial cells (HUVECs).

Methods: HUVECs were divided into six groups: control, treatment with 10 ng/ml TNF-α, and co-treatment of 10 ng/ml TNF-α with four different concentrations of AEPS (100, 150, 250, and 300 μg/ml) for 24 h. Subsequently, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) protein expression, U937 monocyte cells adhesion, and nuclear factor-kappaB (NF-κB) p65 expression in HUVECs were measured.

Results: Treatment of TNF-α-stimulated HUVECs with AEPS at different concentrations resulted in decreased VCAM-1 and ICAM-1 protein expression in a dose-dependent manner. Furthermore, AEPS also inhibited TNF-α-stimulated U937 monocyte cells adhesion to HUVECs. In addition, AEPS reduced TNF-α-induced NF-κB p65 expression in a dose-dependent manner.

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Conclusions: The results indicated that AEPS suppressed TNF-α-induced VCAM-1 and ICAM-1 expression NF-κB signaling.

Keywords: Human umbilical vein endothelial cells; Inflammation; Intercellular adhesion molecule-1; *Piper sarmentosum*; Vascular cell adhesion molecule-1

Introduction

Atherosclerosis is a progressive vascular disease that is characterized by endothelial dysfunction, elevated and modified low-density lipoprotein (LDL), and the proliferation of smooth muscle cells and fibrous tissue in the arterial wall.[1] Endothelial dysfunction will trigger inflammatory reactions, leading to development of atherosclerotic plaques.[2] During the early stages of atherosclerosis development, injury to the endothelium will trigger the endothelial cells to express cell adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin on its surface.[3] The adhesion molecules enable the circulating monocytes to adhere to the endothelium followed by their infiltration and differentiation into macrophages.[4]

Pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), increased the expression of chemotactic factors, other cytokines, and cell adhesion molecules, all of which contribute to the inflammatory process.[5,6] TNF-α also stimulates nuclear factor-kappaB (NF-κB) which is an important transcription factor involved in endothelial dysfunction, expression of adhesion molecules, and inflammatory responses.[7] TNF-α is one of the pleiotropic cytokines involved in most cases of inflammation and other immune response inductions.[8,9] Inducing Rat-2 fibroblasts with TNF-α led to the generation of reactive oxygen species.[8] TNF-α was also able to upregulate the expression of adhesion molecules including intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) as well as induce the adhesion of monocytes in cultured endothelial cells.[10,11]

Piper sarmentosum (PS) is an herbaceous plant that belongs to the Piperaceae family and is widely distributed in the tropical and subtropical regions of the world.[12] Recent studies have reported that an aqueous extract of *P. sarmentosum* (AEPS) exhibited multiple cardiovascular protective effects such as antioxidant,[13] anti-hypertensive,[14] and anti-inflammatory effects.[15] Besides, AEPS is effective in reducing atherosclerotic lesions in hypercholesterolemic animals.[16]

Although the precise mechanisms by which AEPS reduces atherosclerosis have not been completely elucidated, it has been hypothesized that the anti-atherosclerotic activity of AEPS relies on its antioxidant potential and its ability to promote endothelial nitric oxide production to prevent endothelial dysfunction.[7,17] However, the effect of AEPS on the expression of adhesion molecules as well as monocyte adhesion to endothelial cells in response to inflammation has not been thoroughly studied yet. Therefore, this study investigated whether AEPS can modulate TNF-α-induced expression of VCAM-1 and ICAM-1 as well as the subsequent monocyte adhesion to human umbilical vein endothelial cells (HUVECs). Furthermore, the activation of NF-κB was studied, as it is the key molecule that regulates the expression of adhesion molecules.

Materials and Methods

Materials

Basal culture medium 200 (M200) (Cascade Biologics, Portland, Oregon, USA), low serum growth supplement (LSGS) (Cascade Biologics), phosphate buffered saline (PBS), TNF-α (Sigma–Aldrich Co, St. Louis, Missouri, USA), Human Soluble VCAM-1 and ICAM-1 ELISA kits (Chemicon® International Inc, Temecula, California, USA), Nuclear extraction kit (Chemicon® International Inc), NF-κB p65 UPSTATE® EZ-TFA Universal Transcription Factor Assay (Millipore Inc, Bedford, Massachusetts, USA), radioimmunoprecipitation assay (RIPA) buffer, (Sigma–Aldrich Co), and U937 monocytes (ATCC® CRL-1593.2™) (American Type Culture Collection, Manassas, Virginia, USA) were used for the experiments.

Preparation of the AEPS

Fresh leaves of PS were obtained from the Ethnobotanical Garden, Forest Research Institute Malaysia (FRIM) (voucher specimen: FRI 45870). The AEPS was prepared according to previous methods.[17] The fresh leaves were sun-dried and grinded into the powder form. The powder was mixed with water at 80 °C for 3 h (10%, w/v). The AEPS was then freeze-dried and kept at 4 °C.

HUVEC isolation and culture

This study was approved by the Ethical Research Committee of Universiti Kebangsaan Malaysia Medical Centre (approval number: FF-138-2007). Human umbilical cords were obtained with informed consent from healthy subjects in the labor room of Hospital Kuala Lumpur. HUVECs were isolated from human umbilical cords via a collagenase perfusion technique as described previously.[17] Briefly, the cells were isolated from umbilical cords using 0.1% collagenase and cultured in M200 supplemented with LSGS at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. M200 is a basal medium regularly used with LSGS to support the growth of human large vessel endothelial cells.[18]

HUVEC treatment protocol

HUVECs at passage 3 were cultured in 6-well plates at the density of 1×10^5 cells per well. At 80% confluency, the cultured HUVEC were treated with AEPS at four different concentrations (100, 150, 250, and 300 μg/ml) concomitantly with TNF-α (10 ng/ml) for 24 h. The treatment protocol was adopted from a previous study.[19] These four concentrations of AEPS were chosen as they were able to reduce oxidative

stress markers in HUVECs, and AEPS concentrations up to 1000 μg/ml were not cytotoxic to HUVECs after 72 h of incubation.[19] Furthermore, 10 ng/ml TNF-α was used as it had been shown to induce VCAM-1 and ICAM-1 expression in HUVECs[20] as well as causing a significant reduction in HUVEC viability.[21]

VCAM-1 and ICAM-1 protein expressions

The expressions of VCAM-1 and ICAM-1 in HUVECs were determined using Human Soluble VCAM-1 and ICAM-1 enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions. Following the 24 h treatment, HUVECs were washed with cold PBS, and RIPA buffer was added to lyse the cells. The cell suspension was centrifuged and the supernatant containing the HUVEC lysates were collected. HUVEC lysates were pipetted into a 96-well plate coated with human anti-VCAM-1 and -ICAM-1 antibodies. A streptavidin-horseradish peroxidase (HRP) mixture was added to the wells and the plate was washed with wash buffer to remove any unbound substances. This was followed by the addition of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate and stop solution. The optical density of each well was measured at 450 nm using a microplate reader.

Monocyte adhesion to HUVECs

HUVEC were cultured in 6-well plates and treated with different concentrations of AEPS (100, 150, 250, and 300 μg/ml) and TNF-α (10 ng/ml) for 24 h. After incubation, cells were washed with PBS. U937 monocytes were then added to the well and incubated for 1 h at 37 °C. Unattached U937 cells were then removed by washing. The amount of adherent U937 cells on the HUVEC surface were identified and counted under an inverted light microscope.

NF-κB p65 expression

NF-κB p65 expression in the nuclear extract of HUVECs was determined using the NF-κB p65 UPSTATE® EZ-TFA Universal Transcription Factor Assay kit according to the manufacturer's instructions. Briefly, HUVECs were sonicated and diluted in lysis buffer A (10 mM HEPES pH 7.9, 1.5 mM, 10 mM KCl, 0.5−5 mM DTT, 0.1% Triton X-100, and protease inhibitor mixture). The nucleus was separated from the cytosol by centrifugation at 8000 rpm for 20 min. Next, the mixture was diluted with lysis buffer B (20 mM HEPES pH 7.9, 1.5 mM, 0.42 NaCl, 0.22 mM EDTA, 0.5−5 mM DTT, 0.1% Igepal CA-630, 25% (v/v) glycerol, and protease inhibitor mixture) and incubated for 30−60 min at 4 °C in a rotator. The mixture was centrifuged at 16,000 rpm at 4 °C for 5 min and the supernatant was then aliquoted and kept at −80 °C. Protein concentration in the extract was measured using the Bradford technique.[22] Samples of nuclear extract were pipetted into transcription factor-coated plate. The plate was incubated for 2 h at room temperature and followed by washing using trifluoroacetic (TFA) buffer. The p65 antibody was pipetted into the wells of the plate and incubated for 60 min at room temperature. IgG secondary antibody conjugated to HRP was added and incubated for 30 min before the final wash. Finally, TMB substrate and stop solution was added to stop the reaction. The optical density of each well was measured at 450 nm wavelength using a microplate reader.

Statistical analysis

The results are expressed as mean ± standard error for mean (SEM). The Statistical Package for Social Sciences (SPSS) version 20.0 was used to analyze the data. All data sets were tested for normal distributions using the Kolmogorov−Smirnov test, and were normally distributed. The difference between the groups were analyzed using two-way analysis of variance (ANOVA) with post-hoc Tukey test. A value of $P < 0.05$ was considered significant.

Results

Effects of AEPS on VCAM-1 and ICAM-1 expression in TNF-α-treated HUVECs

Figures 1 and 2 show the effects of AEPS on VCAM-1 and ICAM-1 levels in TNF-α-treated HUVECs, respectively. Treatment of HUVECs with TNF-α markedly increased the level of VCAM-1 from 2.5 ± 0.71 ng/ml to 127.5 ± 10.61 ng/ml (Figure 1), and ICAM-1 from 10.3 ± 0.35 ng/ml to 82.3 ± 17.32 ng/ml compared to the control (Figure 2). Treatment of HUVECs with all four doses of AEPS dose-dependently reduced VCAM-1 levels by 2.16- to 10.63-fold compared to the TNF-α group. As for ICAM-1, AEPS at concentrations of 150, 250, and 300 μg/ml dose-dependently reduced ICAM-1 by 2.13- to 8.17-fold compared to the TNF-α group.

Inhibition of monocyte adhesion to HUVECs by AEPS

Inducing HUVECs with TNF-α increased the number of adherent monocytes by 3-fold compared to the control (Figure 3). Treatment with all four doses of AEPS dose-dependently reduced monocytes adhesion to HUVECs by 1.48- to 2.59-fold as compared to the TNF-α group.

Effects of AEPS on TNF-α-induced NF-κB p65 expression

To determine the effect of AEPS on the transcriptional regulation of VCAM-1 and ICAM-1 expression, NF-κB p65 expression levels in the nuclear extract of TNF-α-treated HUVECs were measured. TNF-α-treated HUVECs showed a marked increase in the expression of NF-κB p65 by 3.73-fold compared to the control (Figure 4). Treatment of HUVECs with all four doses of AEPS dose-dependently reduced NF-κB p65 expression by 0.22- to 0.59-fold when compared to the TNF-α group.

Discussion

During the early stages of atherosclerosis, inflammatory mediators such as cell adhesion molecules VCAM-1 and ICAM-1 and other cytokines play important roles in the acute inflammation process.[6] VCAM-1 and ICAM-1 are

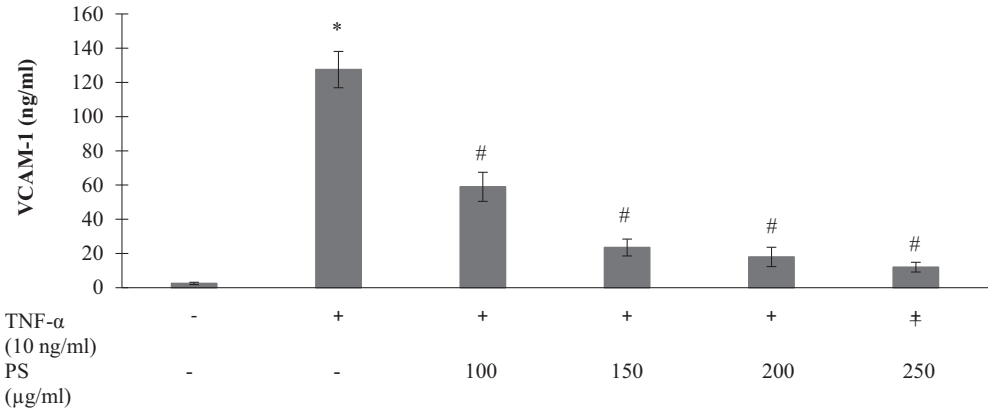

Figure 1: Effect of aqueous extract of *Piper sarmentosum* on VCAM-I level in HUVECs treated with TNF-α. Values are expressed as mean ± SEM, n = 6. $^{*}P < 0.05$ compared with control. $P < 0.05$ compared with the TNF-α group. VCAM-1, vascular cell adhesion molecule-1; TNF-α, tumor necrosis factor-α; PS, *Piper sarmentosum*.

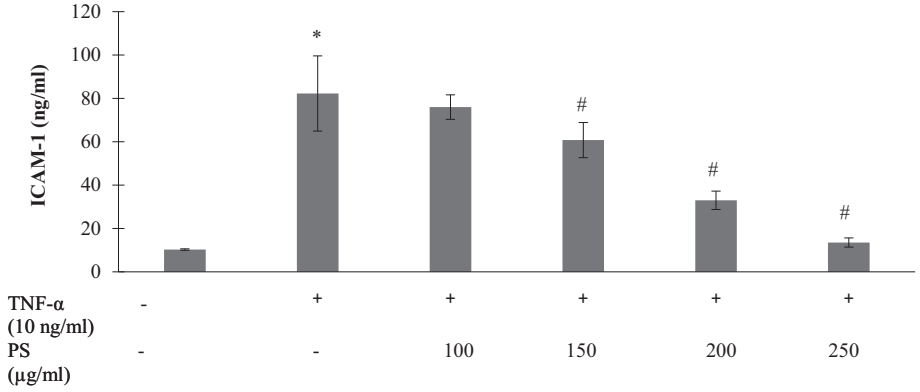

Figure 2: Effect of aqueous extract of *Piper sarmentosum* on ICAM-1 expression in HUVEC treated with TNF-α. Values are expressed as mean ± SEM, n = 6. $*P < 0.05$ compared with control. $\#P < 0.05$ compared with the TNF-α group. ICAM-1, intercellular adhesion molecule-1; TNF-α, tumor necrosis factor-α; PS, *Piper sarmentosum*.

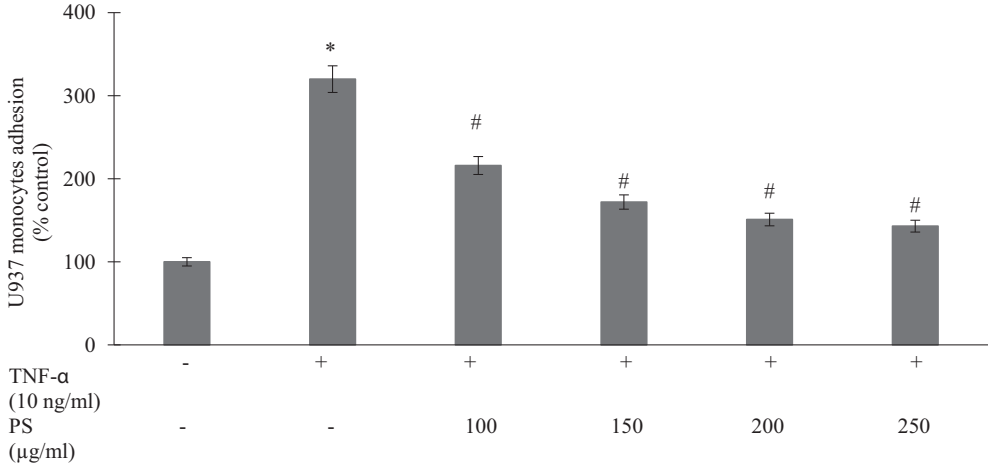

Figure 3: Effect of aqueous extract of *Piper sarmentosum* on monocyte adhesion to HUVEC treated with TNF-α. Values are expressed as mean ± SEM, n = 6. $*P < 0.05$ compared with control. $\#P < 0.05$ compared with the TNF-α group. TNF-α, tumor necrosis factor-α; PS, *Piper sarmentosum*.

immunoglobulin glycoproteins that are closely related in structure, function, and expression patterns.[23] Expression of VCAM-1 and ICAM-1 in the aortic endothelium results in the accumulation of monocytes in the arterial intima.[24] This reaction is one of the processes involved in initiating atherosclerotic lesions.[1]

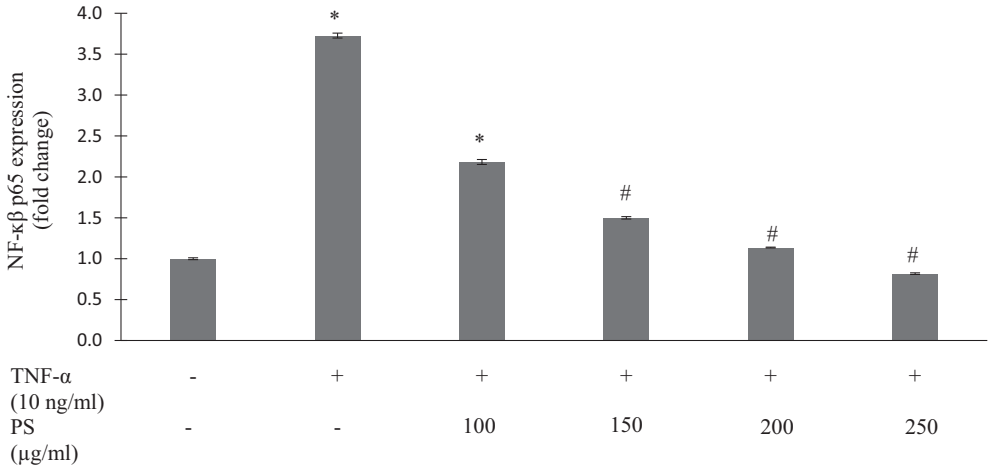

Figure 4: Effect of aqueous extract of *Piper sarmentosum* on NF-κB p65 expression in HUVEC treated with TNF-α. Values are expressed as mean ± SEM, n = 6. $^{*}P < 0.05$ compared with control. $^{\#}P < 0.05$ compared with the TNF-α group. NF-κβ, nuclear factor-kappaB; TNF-α, tumor necrosis factor-α; PS, *Piper sarmentosum*.

In the present study, we investigated the effects of AEPS on the expression of cell adhesion molecules (VCAM-1 and ICAM-1) and monocyte adhesion in TNF-α-treated HUVECs. In response to TNF-α treatment, the expression of VCAM-1, ICAM-1, and number of adherent monocytes increased by 29-, 8.4-, and 3-fold, respectively. These findings were in accordance with the results from a previous study whereby TNF-α stimulated the expression of cell adhesion molecules such as VCAM-1, ICAM-1, and E-selectin in HUVECs.[4] The increase in expression of adhesion molecules in TNF-α-stimulated cells is a useful marker for the activation of local and systemic inflammation.[25]

We found that TNF-α-induced VCAM-1 and ICAM-1 expressions were attenuated by AEPS treatment in a concentration-dependent manner. The expression suppressions of VCAM-1 and ICAM-1 by AEPS were supported with findings from previous studies whereby AEPS reduced the levels of VCAM-1 and ICAM-1 in the plasma of hypercholesterolemic rabbits[26] and reduced ICAM-1 mRNA expression in hydrogen peroxide-treated HUVECs.[27] The results also showed that AEPS suppressed VCAM-1 better than ICAM-1. For instance, 100 μg/ml AEPS was able to reduce VCAM-1 expression but not ICAM-1. VCAM-1 plays a major role in the initiation of atherosclerosis in mice.[23] As a result of the reduced expression of VCAM-1 and ICAM-1, AEPS inhibited the adhesion of monocytes to HUVEC. The adhesion of monocytes to endothelial cells is an important step in atherosclerosis. Monocytes need to adhere to endothelial cells before migrating to the subendothelial layer to form macrophages and subsequently foam cells, which are part of an atherosclerotic plaque.[23] As AEPS is able to inhibit the adhesion of monocytes to endothelial cells, the result further supports an anti-atherosclerotic effect of AEPS as shown previously.[16]

NF-κB is an important transcription factor involved in the early stage of atherosclerosis and endothelial dysfunction.[20] NF-κB is involved in the signal transduction pathway for TNF-α-induced expression of adhesion molecules including VCAM-1 and ICAM-1[5, 7, 20]. The NF-κB family consists of p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), c-Rel (Rel), and RelB, with p50 and p65 subunits being important dimers for nuclear transcription.[23] The NF-κB dimers are held inactive through an association with IκB proteins in the cytoplasm. Upon stimulation by inflammatory cytokines such as TNF-α, IκB will be phosphorylated and degraded by IκB kinase. This leads to the translocation of released NF-κB dimers to the nucleus where they bind to specific DNA sequences to promote the transcription of target genes.[23] The p50 subunit helps in DNA binding whereas the p65 subunit is responsible for initiating transcription.[28]

Expression of the NF-κB complex on specific genes leads to regulations in physiological responses such as inflammation and cellular proliferation.[6] The results showed that NF-κB p65 expression increased by 3.73-fold compared to the control when stimulated with TNF-α. Expression of TNF-α-stimulated NF-κB p65 subunit was attenuated by AEPS. This suggests that the reductions in VCAM-1 and ICAM-1 expressions by AEPS were achieved partly through an inhibition of the NF-κB pathway. In another study, AEPS reduced NF-κB mRNA expression in hydrogen peroxide-treated HUVEC.[27] Apart from NF-κB, TNF-α also stimulated other transcription factors such as activator protein-1 (AP-1), which is important in facilitating VCAM-1 and ICAM-1 expressions.[29] However, this study did not measure the effects of AEPS on AP-1 activity. Further study is needed to confirm whether the suppression of VCAM-1 and ICAM-1 by AEPS is also achieved through an inhibition of the AP-1 pathway.

Phytochemically, AEPS contains high amounts of flavonoids, which are a group of phenolic compounds with antioxidant and anti-inflammatory activities.[30] Among the most important flavonoids found in AEPS are rutin (quercetin rhamnosil-3-glucoside) and vitexin (apigenin-8-C-β-D-glucopyranoside).[30] AEPS contains 75.70 ± 0.50 ppm (0.757%) rutin and 51.93 ± 0.55 ppm (0.5193%) vitexin.[30] Additionally, PS contains other flavonoids such as naringenin, hesperetin, taxifolin and quercetin.[13] Rutin and vitexin have high antioxidant activities and cytoprotective effects against oxidative stress in HUVECs.[30,31] Rutin possess anti-inflammatory activities by suppressing VCAM-1, ICAM-1, E-selectin, and NF-κB expressions in

lipopolysaccharide (LPS)-stimulated cells.[32] Rutin also inhibits LPS-stimulated TNF-α, which has been implicated in many cases of inflammatory lesions.[32]

This study provides new findings on the anti-inflammatory action of PS that may have a potential therapeutic use for vascular diseases, such as atherosclerosis, through mechanisms involving the inhibition of VCAM-1 and ICAM-1 expression as well as NF-κB activation in endothelial cells. However, this study used crude AEPS, which was not a purified compound. Therefore, the study was unable to specify which compound contributed to the observed effects, but we hypothesized it was due to the action of flavonoids mentioned above.

Conclusion

AEPS attenuates TNF-α-induced expressions of VCAM-1 and ICAM-1, thereby reducing adhesion of monocytes to the endothelial cells. This effect of AEPS was partly due to its inhibitory action on the NF-κB activation induced by TNF-α. Our findings suggest that AEPS could be a potential therapeutic agent for endothelial protection against early progression of atherosclerosis. Anti-inflammatory properties of AEPS in the work presented here could be further explored in future studies in diseased states.

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Conflict of interest

The authors have no conflict of interest to declare.

Ethical approval

This study was approved by Ethical Research Committee of Universiti Kebangsaan Malaysia Medical Centre (approval number: FF-138-2007).

Authors' contributions

SMI: Performing the study, analyzing the data, and preparing the manuscript. UMS: Performing the study, analyzing the data, and preparing the manuscript. CKH: Evaluating the data, correcting the manuscript, and coordinating the study. AA: Providing the grant for the study, correcting the manuscript, and coordinating the study. AU: Supervising the work, providing the grant for the study, evaluating the data, correcting the manuscript, and coordinating the study. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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