

***In Vitro* Atheroprotective Effects of *Trigonella Foenum Graecum* (TFG) and its Saponins in LPS-Stimulated Human Coronary Artery Endothelial Cells**

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Received: 23 April 2021, Revised: 5 November 2021, Accepted: 12 November 2021

Abstract

There has been a shift towards utilizing natural products as an adjunct therapy to standard treatment in the prevention of coronary artery disease, and *Trigonella foenum graecum* (TFG) is one of the potential natural products of interest. In the present study, we attempted to determine the effects of TFG and its saponins on atherosclerosis related biomarkers *in vitro*. Protein expression of markers of inflammation, endothelial activation and transcription factors were measured by Procarta™ and ELISA assays. Gene expression of the same markers were determined by qPCR and the interaction between monocytes and HCAECs were evaluated through monocyte binding assay following 16 h of treatment with TFG and saponins. Both TFG and its saponins exhibited reducing effects on atherosclerosis-related markers. Based on the area under the curve (AUC) analysis, TFG reduced protein and gene expressions of ICAM-1 and VCAM-1 better than the saponins, while saponins reduced E-selectin expression better than TFG. Saponins showed a reduction of gene and protein expressions of IL-6, IL-8, NF-κB p50 and p65 better than TFG. TFG is more effective in reducing binding of monocytes to endothelial cells than saponins. TFG better reduced endothelial activation but exerted weaker anti-inflammatory effects than saponins, suggesting the possible synergism with other compounds in the crude extract which enhances attenuation of endothelial activation while inhibiting anti-inflammatory properties of saponins in the crude extract.

Keywords: *Trigonella foenum graecum*, Saponins, Atherosclerosis, Inflammation, Endothelial activation

Introduction

The World Health Organization (WHO) reported that about 30 from 71 % of total deaths in Malaysia during 2002 due to chronic diseases is mainly caused by cardiovascular diseases (CVD). In 2008, CVD was the cause of 32 of 67 % of total deaths due to non-communicable disease [1,2]. The number may be increased if no intervention or preventive measures are initiated to control the prevalence of CVD. The majority of CVD are caused by atherosclerosis, a chronic process involving key processes which include endothelial activation, inflammation, oxidative stress and prothrombogenesis, leading to lipid-rich plaque formation in the walls of arteries which can lead to obstruction and ischaemia of major organs. According to the American Heart Association, approximately 75 % of fatal CVD reported are caused by atherosclerosis [1].

The current approach in the management of the atherosclerotic-related complications such as coronary artery disease (CAD) is to prevent them by addressing risk factors such as hypertension,

diabetes mellitus and dyslipidaemia through lifestyle modifications and/or medications. Medications such as lipid lowering drugs, anti-platelets and anti-hypertensives are prescribed to curb these risk factors [3]. In the event atherosclerosis has led to CAD-related complications such as acute coronary syndrome, several treatment modalities are available to manage ischaemic events such as angioplasty, endarterectomy, thrombolytic therapy and bypass surgery [4]. Despite these methods to improve outcomes of CAD, they are not without setbacks. Medications to address risk factors for CAD such as hypertension, diabetes mellitus and dyslipidaemia, are costly and carry side-effects that may at times pose danger on patients, whereas procedures to treat CAD are invasive and not without risks [5]. These problems have redirected strategies to prevent CAD by way of lifestyle modification such as diet and exercise. However, there has also been increased research interest in identifying cardioprotective properties of natural-occurring compounds, which carry with it less side-effects than conventional therapy.

There are several major cytokines that may contribute to the development of atherosclerosis. Elevations of these cytokines reflect increased atherogenesis which increases the risk of the atherosclerotic lesion formation. IL-6 is a pro-inflammatory cytokine but can also become an anti-inflammatory myokine [6]. Commonly, once IL-6 is released, it will lead to endothelial activation, amplify inflammation and initiate procoagulant responses which are the main contributors to the development of atherosclerosis lesions [7]. IL-8 also acts as an attractant which is involved in inflammatory cell recruitment such as neutrophils, lymphocytes and basophils [8]. Thus, IL-8 may contribute in the accumulation of inflammatory cells in atherosclerosis plaques.

Migration of monocytes into the tunica media of arteries are mediated by several cellular adhesion molecules that are expressed on the surface of endothelial cells such as ICAM-1, VCAM-1 and E-selectin which play the most important roles in the firm attachment and transendothelial migration of monocytes [9]. Elevated expression of ICAM-1 can contribute to accumulation of macrophages in subendothelial region which may lead to development of foam cells and atherosclerotic plaques. VCAM-1 has been reported to promote monocyte adhesion and accumulation on endothelial cells which leads to the development of atherosclerosis lesion [10]. E-selectin promotes the recruitment of monocytes from axial stream and enabling them to roll along the endothelium [11]. Accumulation of monocytes increases the risk of atherosclerotic lesion development [11].

There has been recent advancement in identifying natural-occurring compounds that could be used in the prevention of CAD which is cheaper, safer and could provide synergistic effects to standard medications for CAD. *Trigonella foenum graecum* (TFG), commonly known as fenugreek has been shown to exhibit cardioprotective properties [12]. Researchers found that TFG has shown significant hypocholesterolaemic and hypoglycaemic activities as well as anti-oxidant effects which all contribute to cardioprotection [13,14]. In addition, TFG also exhibits anti-cancer, anti-inflammatory, antiseptic, aphrodisiac, astringent, demulcent, emollient, expectorant, anthelmintic, wound healing and gastro protective properties suggesting the potential use of TFG as a therapeutic agent [15]. 4-hydroxyisoleucine an amino acid isolated from TFG was determined to have anti-oxidant and anti-inflammatory effects by inhibiting production of reactive oxygen species and reduce activation of certain inflammatory related pathway including JNK1/2, p38 MAPK, and NF- κ B [16]. Previous study by Kawabata *et al.* [17] (2011) shows the anti-inflammatory effects of TFG saponin in reducing TNF- α in THP-1 cell lines and restrained synthesis of melanin in murine melanoma B16F-1 cells. Moreover, steroidal saponin from TFG was identified to have peroxyl radical scavenging effects and reduction of release of ROS from inflamed mucosa [18,19].

Despite several preliminary animal and human trials reporting the anti-atherogenesis properties of TFG, there are very limited molecular and cell culture researches to determine the mechanisms of its atheroprotection, as well as saponins, the bioactive compound of TFG. This study aims to address these knowledge gaps by determining the influences of TFG and saponins on the expressions of inflammatory and endothelial activation markers, effects on transcription factors and impact on monocyte binding, which represents atherogenesis.

Materials and methods

TFG crude extract (Sigma-Aldrich, USA) stocks of 3.0 g/mL and saponins (98.26 % purity) (Sciphaar, China) stocks of 0.2 g/mL were dissolved in ethanol. The stocks were further diluted with treatment medium containing a mixture of 89 % of RPMI-1640 (Cellgro, USA), 10 % fetal bovine serum (Caisson Labs, USA) and 1 % Streptomycin-penicillin (Sigma-Aldrich, USA) to obtain a working concentration of 3,000 μ g/mL TFG crude extract and 200 μ g/mL saponins with ethanol (Friendemann Schmidt, Australia) percentage less than 0.1 %.

Cell viability of human coronary artery endothelial cells (HCAECs) (Lonza, USA) against TFG crude extract and saponins were tested using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Invitrogen, USA). The HCAECs were seeded into 96 wells culture plate and treated with various concentrations of TFG crude extract and saponins ranging from 23.4 to 3,000.0 and 1.6 to 200.0 $\mu\text{g}/\text{mL}$, respectively. Following 16 h incubation (37 °C, 5 % CO_2), 20 μL of 5 mg/mL of MTT solution was added to each well and incubated for another 4 h. Finally, the reaction was stopped with 100 μL of DMSO solution and the absorbance was measured at 540 nm.

HCAECs were cultured to 85 % confluency and treated with TFG crude extract and saponins ranging from 46.9 to 375.0 and 3.1 and 25.0 $\mu\text{g}/\text{mL}$, respectively. The cells were then stimulated with 5 μL of 200 $\mu\text{g}/\text{mL}$ of *Escherichia coli* lipopolysaccharides (LPS) from serotype 055:B5 (Sigma-Aldrich, USA) and incubated (37 °C, 5 % CO_2) for 16 h. The supernatants were collected and cells detached using accutase solution (Innovative Cell Technologies, USA) for further analysis. Concentrations of IL-6, IL-8, sICAM-1, sVCAM 1 and E-selectin were determined by measuring the protein expression for each marker in the supernatant of the LPS-stimulated treated HCAECs following 16 h of incubation using the Procarta Immunoassay Kit (Affymatrix, USA), a bead-based multiplex assay kit. All procedures were performed according to the manufacturer's instruction. Activated NF- κ B p50 and p65 proteins concentration in the nucleus of LPS-stimulated HCAECs following 16 h incubation was measured using cell based enzyme linked immunoassay (ELISA). Harvested HCAECs underwent nuclear extraction with Nuclear Extraction Kit (Cayman, USA) for nuclear protein isolation and proceeded with NF- κ B p50 and p65 proteins determination using NF- κ B p50 and p65 Transcription Factor Assay Kits (Cayman, USA) respectively. All procedures were performed in accordance with the manufacturer's instruction. The absorbance of the samples was read at 450 nm. HCAECs were harvested and total RNA was extracted with RNeasy Mini Kit (Qiagen, USA). Concentration and purity of total RNA was determined by Nanodrop and Agilent 2100 Bioanalyzer (Agilent, USA). The RNA was then reverse transcribed and amplified to cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad, USA) and proceeded with gene analysis (IL-6, IL-8, ICAM-1, VCAM-1, E-selectin, NF- κ B p50 and p65) using iQ™ SYBR® Green Supermix (Bio-Rad, USA) on CFX96 Thermal cycler using recommended real-time PCR protocol depending on the melting temperature of the primers used. The CT values of the genes were normalized on the basis of the reference genes: Beta-actin, HPRT-1 and ATP6V1A and calculated as fold change to HCAECs incubated with LPS alone.

Monocyte-endothelial cells interaction was measured by colorimetry *in vitro*. HCAECs were seeded into a 96-well culture plate, stimulated with LPS and treated with different concentrations of TFG crude extract and saponins ranging from 0.7 to 375.0 and 0.05 to 25.00 $\mu\text{g}/\text{mL}$, respectively. Following 16 h of incubation, U937 cell line (ATCC, USA) was added into each well and incubated for an hour. The unbound cells were then removed prior to adding 0.25 % rose Bengal and the reaction was stop using 1:1 ethanol:PBS solution. The absorbance was read at 570 nm. Each data was normalized to HCAECs incubated with LPS alone and percent inhibitions were calculated.

All data were expressed as mean \pm SD. Each sample was composed of biological and technical triplicates: n = 9. Differences between groups were assessed by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc analysis with SPSS for Windows (version 20, USA). The level of statistical significance was at $p < 0.05$. The effectiveness between TFG and saponins were measured through the area under the curve analysis for further comparison. The percentage (%) inhibition against LPS controls for each biomarker was obtained from area under the curve (AUC) analysis for TFG and saponins using Graph Version 4.0.

Results and discussion

HCAECs was decidedly used to determine the effects of TFG and its saponins on the expression of inflammation, endothelial activation markers, and transcription factors due to its greater susceptibility to processes involved in atherogenesis. Therefore, it is the best representation of endothelial cells of the arterial walls [20]. Based on a previous report, the saponin constituents of crude TFG extract was approximately 4 - 8 % [21,22]. Taking this into account and obtaining the mean content percentage of saponins in TFG, we approximated the percentage of the saponins contained in the concentration of TFG crude extract which gave a cell viability of > 85 % which was 375 $\mu\text{g}/\text{mL}$ to be 6.7 % with a concentration of saponin at 25 $\mu\text{g}/\text{mL}$. This allowed us to do a crude head-to-head comparison between TFG and saponins. In this *in vitro* experimental study, LPS was applied to stimulate the inflammation response following treatment with TFG and saponins to measure the effects of both extracts on the expression of inflammatory biomarkers expressed by the endothelial cells. RIN values of the samples, as

measured on a 2100 Bioanalyzer ranged from 6.3 to 8.8 (mean, 8.06; median, 8.3) were proceed with molecular analysis.

Effects of TFG and saponins on pro-inflammatory cytokines

This study showed a dose-dependent reduction in protein and gene expressions of IL-6 following treatment with TFG and its saponins with statistical significance observed at the higher concentrations for both compounds (**Figure 1(a)**). Similarly, IL-8 protein and gene expressions also reduced following treatment with TFG and saponins but only reached statistical significance at higher concentrations (**Figure 1(b)**). When comparing between TFG and saponins, the latter showed better cytokine reduction with its percentage (%) inhibition against LPS controls obtained from area under the curve (AUC) analysis being higher than its crude extract (**Table 1**). These results are supported by several previous studies. IL-6 was shown to be reduced in Freund's adjuvant-induced arthritis in albino rats following treatment with 200 - 400 mg/kg of TFG where 400 mg/kg dose showed more prominent results compared to the 200 mg/kg dose of TFG [23]. In another study, IL-6 in the kidney homogenate of diabetic nephropathy in alloxan-diabetic rats also reduced following treatment with TFG [24]. There are scarce studies on saponins extracted from TFG on cytokines, but 1 study reported tea saponin with ovalbumin decreased the expression of IL-8 in T-lymphocytes. To the best of our knowledge, there have been several studies applying TFG and its saponins on animal models but there are limited human coronary artery cell *in vitro* experiments available to determine the influence of TFG and its saponins on IL-6 and IL-8 expressions, particularly using human coronary endothelial cells.

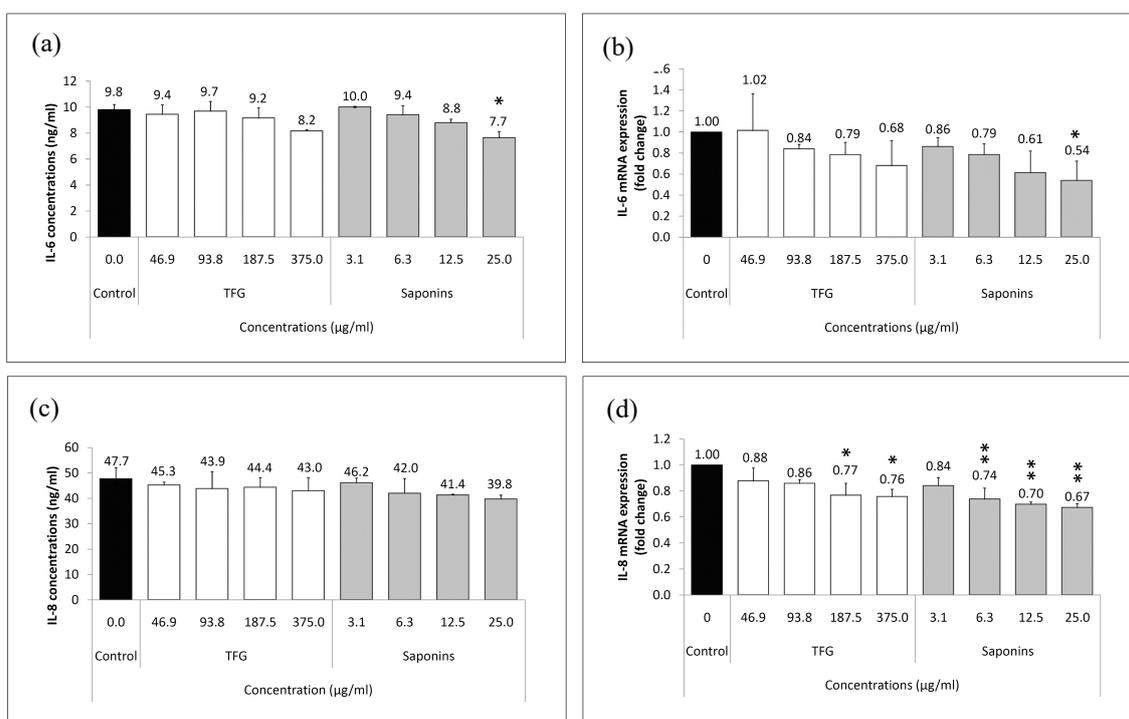


Figure 1 Effects of TFG (46.9 - 375.0 $\mu\text{g/mL}$) and its saponins (3.1 - 25.0 $\mu\text{g/mL}$) on IL-6 and IL-8 in LPS-stimulated HCAECs. (a) IL-6 protein expression, (b) IL-6 gene expression, (c) IL-8 protein expression, (d) IL-8 gene expression. Using ANOVA, post-hoc with Bonferroni correction; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to HCAECs incubated with LPS alone (control).

Effects of TFG and saponins on endothelial activation (adhesion molecules)

TFG crude extract demonstrated significant anti-atherogenesis effects by reducing ICAM-1 and VCAM-1 protein and gene expressions across all concentrations, while saponins only reduced the expressions of these biomarkers at higher concentrations (**Figure 2**). AUC analysis for both ICAM-1 and VCAM-1 protein and gene expressions also showed similar results where TFG were 81.0 and 31.9 %; 70.3 and 54 %, respectively while saponins were 31.7 and 7.0 %; 25.1 and 20.6 %, respectively (**Table 1**). On the other hand, saponins showed greater reduction in E-selectin protein expression compared to TFG with AUC analysis for protein expression doubled that of TFG (76.5 %) (**Table 1**). However, the results were contradicting with a previous study reported by Rafrat *et al.* where they found no significant changes in ICAM-1 level in type 2 diabetic patients after consuming 10 g per day of TFG for 8 weeks [25]. This could be due to the dose and bioavailability of TFG used which translates to a lower exposure of TFG at a cellular level to significantly reduce ICAM-1 concentration in the serum of these subjects. There are scarce reports on the effects of TFG and its saponins on endothelial markers especially *in vitro* and to the best of our knowledge, there is no study applying those extracts on HCAECs.

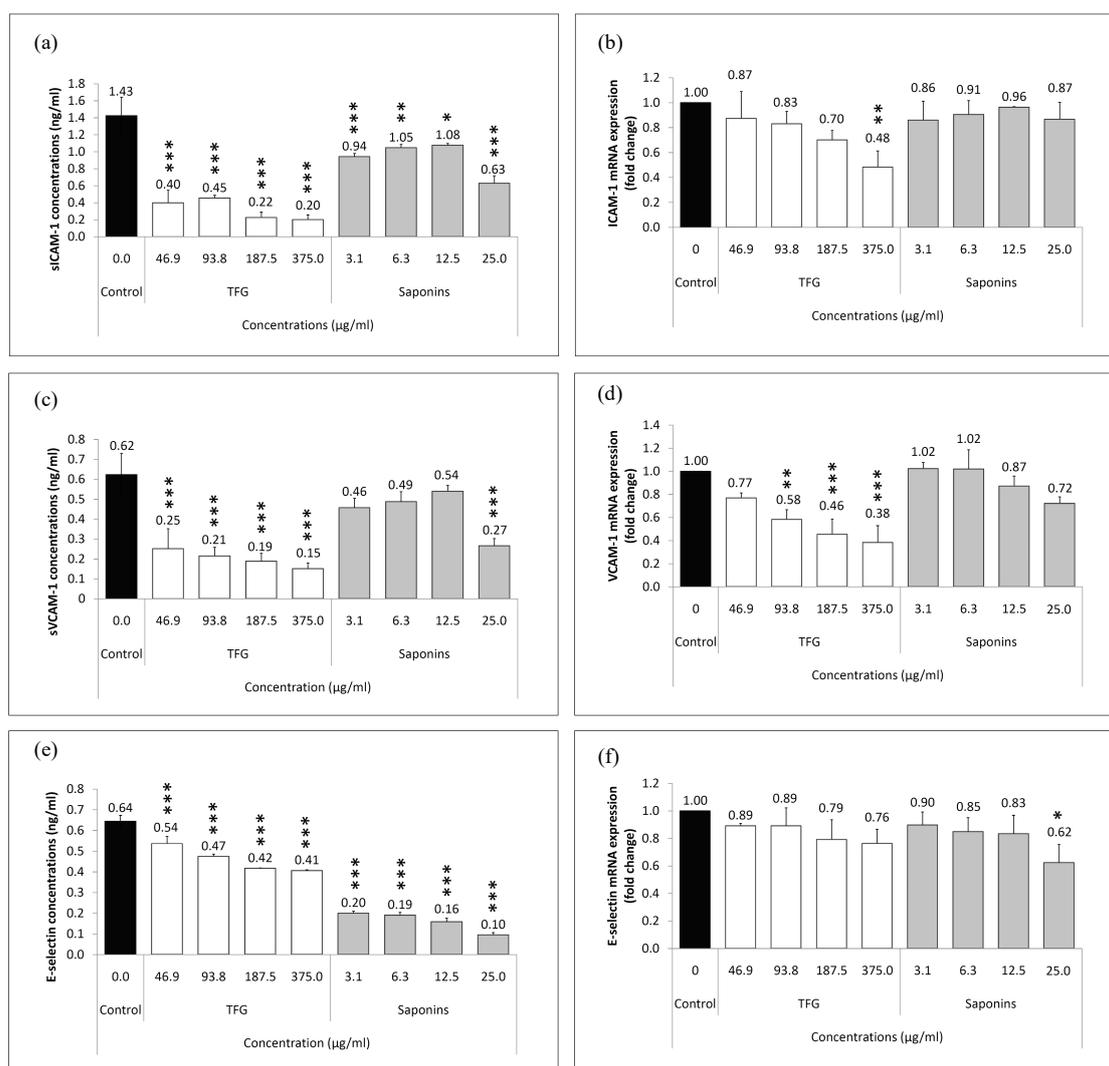


Figure 2 Effects of TFG (46.9 - 375.0 µg/mL) and its saponins (3.1 - 25.0 µg/mL) on sICAM-1, sVCAM-1 and E-selectin in LPS-stimulated HCAECs. (a) sICAM-1 protein expression, (b) sICAM-1 gene expression, (c) sVCAM-1 protein expression, (d) sVCAM-1 gene expression. Using ANOVA, post-hoc with Bonferroni correction; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to HCAECs incubated with LPS alone (control).

Effects of TFG and saponins on transcription factors (NF- κ B)

NF- κ B is a protein complex consisting of p50 - p65 heterodimer that controls transcription of DNA [26]. In its inactive form, NF- κ B is located in the cytosol, complexed with an inhibitory protein known as Inhibitory kappa-B (I κ B) [27]. In atherosclerosis, NF- κ B can be activated due to stress, cytokines, free radicals, and also ox-LDL causing the release of NF- κ B from I κ B and subsequently enters the nucleus [28]. Once within the nucleus, it will bind to the DNA, enabling the initiation of the transcription process, thus increasing the expression of other inflammatory markers and endothelial activation markers [27]. This study showed that saponins appear to reduce both NF- κ B p50 and p65 effectively compared to TFG crude extract (**Figure 3**). Saponins reduced both gene and protein expressions of NF- κ B p50 and p65 across all concentrations which may indicate that saponins can reduce NF- κ B p50 and p65 mRNA availability in the cytosol causing less production of NF- κ B p50 and p65 proteins, thus, less activated NF- κ B p50 and p65 proteins in the nucleus. However, TFG appears to reduce NF- κ B p50 protein with neutral effects on NF- κ B p50 gene expression with slightly decreased NF- κ B p65 (**Figure 3**), suggesting that TFG may affect either the I κ B protein by enhancing I κ B binding process or shutting down the entire p50 - p65 heterodimer function, causing less activated NF- κ B p50 - p65 heterodimer and less migration of NF- κ B p50 - p65 heterodimer from cytosol to nucleus. Those results were supported by the AUC analysis for both NF- κ B p50 and p65 protein and gene expression where the AUC analysis after being treated with saponins (59.5 and 58.7 %; 42.4 and 33.7 %, respectively) were higher than TFG (58.0 and 14.0 %; 26.0 and 24.4 %, respectively) (**Table 1**). A previous study reported that diosgenin, a type of saponin from TFG, suppressed TNF- α expression induced by NF- κ B activation through DNA binding, activation of I κ B α kinase, I κ B α phosphorylation, I κ B α degradation, p65 phosphorylation and p65 nuclear translocation through inhibition of Akt activation, and also abrogated the NF- κ B-dependent reporter gene expression [29].

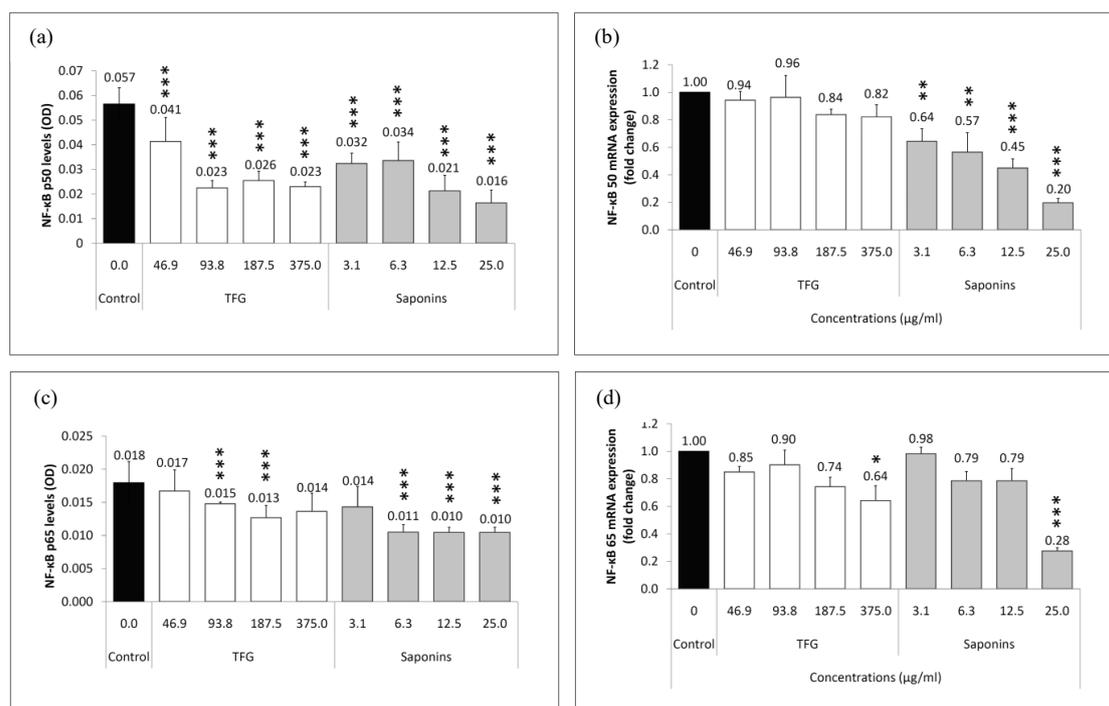


Figure 3 Effects of TFG (46.9 - 375.0 μ g/mL) and its saponins (3.1 - 25.0 μ g/mL) on NF- κ B p50 and p65 in LPS-stimulated HCAECs. (a) NF- κ B p50 protein expression, (b) NF- κ B p50 gene expression, (c) NF- κ B p65 protein expression, (d) NF- κ B p65 gene expression. Using ANOVA, post-hoc with Bonferroni correction; * p < 0.05, ** p < 0.01 and *** p < 0.001 compared to HCAECs incubated with LPS alone (control).

Effects of TFG and saponins on monocyte-endothelial cell interaction

The presence of ICAM-1, VCAM-1 and E-selectin can influence the interaction between monocytes and endothelial cells. This interaction was determined through monocyte binding assays. The results

showed that TFG was more effective than its bioactive compound, saponins, in reducing monocyte-endothelial cell interaction *in vitro* (**Figure 4**) and AUC analysis of reduction after treatment with TFG (32.0 %) were slightly higher than saponins (28.8 %) (**Table 1**). These findings are consistent with our previous results regarding gene and protein expressions of endothelial activation markers where TFG reduced ICAM-1 and VCAM-1 more effectively than saponins (**Figure 2**). Collectively, these observations highlight that perhaps ICAM-1 and VCAM-1 are better biomarkers reflecting endothelial activation than E-selectin as their lower concentrations following treatment with TFG also corresponds to reduced monocyte-endothelial cell interaction compared to saponins. These observed effects seen in the crude extract suggest that synergism of other compounds contained in TFG crude extract may be at play. This study highlights that TFG demonstrated better suppression on endothelial activation marker by reducing the mRNA levels of ICAM-1 and VCAM-1 thus inhibit its protein synthesis and reduce the expression of ICAM-1 and VCAM-1 protein on the surface of endothelial cells and supported by the reducing the *in vitro* interaction between monocytes and endothelial cells in monocyte-binding assays, suggesting that this could be due to synergism with other compounds present in the crude extract. Meanwhile, saponins show good anti-inflammatory properties by reducing pro-inflammatory cytokines (IL-6 and IL-8) and transcription factors (NF- κ B p50, and p65) in both protein and mRNA levels in its single form better than TFG suggesting the possibility of other compounds in TFG that may attenuate the anti-inflammatory properties of saponins in the crude extract. To the best of our knowledge, there is lack other studies have been done to determine the effects of TFG and its saponins on endothelial marker especially on HCAEC cell line. Most of the cellular studies were focusing on the potent anti-oxidant and anti-cancer properties of TFG. However, there is 1 study done by Liu *et al.* [30] (2012) using human umbilical vein endothelial cells (HUVECs) found that diosgenin that contains in TFG and saponins reduces endothelin-1 (ET-1) and plasminogen activator inhibitor-1 (PAI-1) production. Moreover, diosgenin also increase glutathione (GSH) and restores the eNOS mRNA expression level in chronic renal failure rats suggesting the potential of TFG as atheroprotective agent by protecting vasculature against oxidative stress and ameliorate vascular function [31].

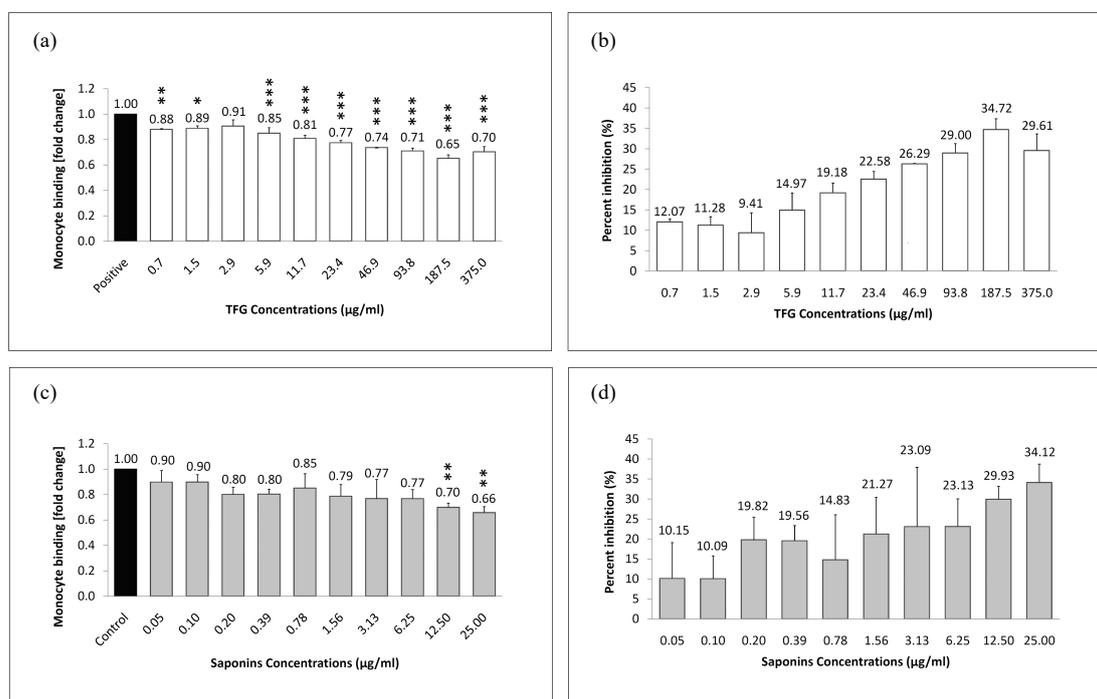


Figure 4 Effects of TFG (0.7 - 375.0 µg/mL) and saponins (0.05 - 25.0 µg/mL) on Monocytes-LPS-stimulated HCAECs Interaction. (a) Effect of TFG, (b) Percent inhibition by TFG, (c) Effect of saponins, (d) Percent inhibition by saponins. Data are expressed as means \pm SD. Using ANOVA, post-hoc with Bonferroni correction; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to HCAECs incubated with LPS alone (control).

Table 1 Percentage (%) inhibition of protein expression of pro-inflammatory cytokines, endothelial activation, transcription factors and monocytes-endothelial cells *in vitro* interaction by TFG and saponins based on area under the curve (AUC) analysis.

	IL-6	IL-8	ICAM-1	VCAM-1	E-selectin	NF-κB p50	NF-κB p65
Protein expression							
TFG	8.1	7.8	80.4*	69.8*	32.5	23.8	55.3
Saponins	11.4*	13.1*	34.5	28.7	76.6*	40.2*	58.8*
Gene expression							
TFG	23.3	18.2	31.9*	51.6*	21.5*	24.3	13.5
Saponins	36.4*	25.9*	8.5	21.9	20.3	34.7*	58.7*
Endothelial cells - Monocyte binding							
TFG	30.2*						
Saponins	28.0						

Note: Data expressed as percentage (%); IL-6: Interleukin 6; IL-8: Interleukin 8; ICAM-1: Intercellular Adhesion Molecule 1; VCAM-1: Vascular cell adhesion protein 1; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; EC: Endothelial cells; (*) indicate high percentage (%) inhibition of area under the curve against LPS control.

Conclusions

TFG can reduce endothelial activation better than its saponins suggesting that this could be due to synergism with other compounds present in the extract. However, saponins appears to exert better anti-inflammatory effects in its single form, suggesting the possibility of other compounds in TFG that may attenuate the suppression of endothelial activation of saponins alone. Thus, both extracts are potent anti-atherogenic agents but exert their effects through different mechanisms. Future studies to replicate cell culture results in *in vivo* and in clinical trials could provide further insight on how best to optimize this natural product as an atheroprotective supplement.

Acknowledgements

The authors would like to express their appreciation to the Centre for Pathology Diagnostic and Research Laboratories (CPDRL) and Institute for Medical Molecular Biotechnology of Faculty of Medicine, Universiti Teknologi MARA, Malaysia for providing the facilities to conduct this research. This research was funding by Ministry of High Education (MOHE) under Fundamental Research Grant Scheme (FRGS) [Grant code: 600-RMI/ST/FRGS 5/3/Fst (48/2011)], awarded to the Corresponding Author as the Project Leader.

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