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# Chios Mastic Gum Extract and Isolated Phytosterol Tirucallol Exhibit Anti-Inflammatory Activity in Human Aortic Endothelial Cells

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**Chios mastic gum (CMG) is a white, semitransparent, natural resin that is obtained as a trunk exudate from mastic trees. Triterpenic compounds and phytosterols like tirucallol are among its major components. CMG has been associated with cardiovascular protection, exerting its effect mainly through increasing the antioxidant defense system, and effectively lowering the levels of serum cholesterol in human subjects. However, data on its anti-inflammatory effect on endothelium are scarce. Attachment of leukocytes to the vascular endothelium and the subsequent migration of cells into the vessel wall are early events in atherogenesis, and this process requires the expression of endothelial adhesion molecules. In this study, we examined the effect of CMG neutral extract (25–200 μg/ml) and tirucallol (0.1–100 μM) on the following: 1) the expression of adhesion molecules (VCAM-1 and ICAM-1) by Cell ELISA and 2) the attachment of monocytes (U937 cells) in TNF-α stimulated Human Aortic Endothelial Cells (HAEC) by Adhesion assay. The impact of treatment with CMG neutral extract and tirucallol in NFkB phosphorylation was also examined by a cell-based ELISA kit. Both CMG extract and tirucallol inhibit significantly VCAM-1 and ICAM-1 expression in TNF-α-stimulated HAEC. They also inhibit significantly the binding of U937 cells to TNF-α-stimulated HAEC and attenuate the phosphorylation of NFkB p65. This study extends existing data regarding the cardioprotective effect of CMG, expands the spectrum of known phytosterols with potent antiatheromatic activity, provides new insight into the mechanisms underlying the beneficial effect of CMG on endothelial function, and may aid in design of new therapy for intervention in atherosclerosis.** Exp Biol Med 234:553–561, 2009

**Key words:** adhesion molecules; atherosclerosis; Chios mastic gum; HAEC; tirucallol

## Introduction

*Pistacia lentiscus* var. *Chia* of the Anacardiaceae family is grown almost exclusively in the southern part of Chios island, a Greek island in the Aegean. Chios mastic gum (CMG) is a white, semitransparent, natural resin that is obtained as a trunk exudate from mastic trees. Mastic gum and essential oil are natural antimicrobial agents that have found extensive uses in Mediterranean and Middle Eastern countries, both as a dietary supplement and as an herbal remedy, since ancient times. They have been used in traditional Greek medicine by ancient Greek physicians, such as Hippocrates, Dioscorides and Galenos (1, 2), who mentioned their properties and recommended their use for various gastrointestinal disorders like gastralgia, dyspepsia, and peptic ulcer (3). Additionally, mastic gum is already known for its antioxidant capacity (4), as well as for its beneficial action against *Helicobacter pylori* (5, 6).

CMG possesses anti-bacterial activity (7, 8), cosmetic properties and actions beneficial for the teeth (2), as well as antioxidant properties (9, 10). Furthermore, *in vitro* and *in*

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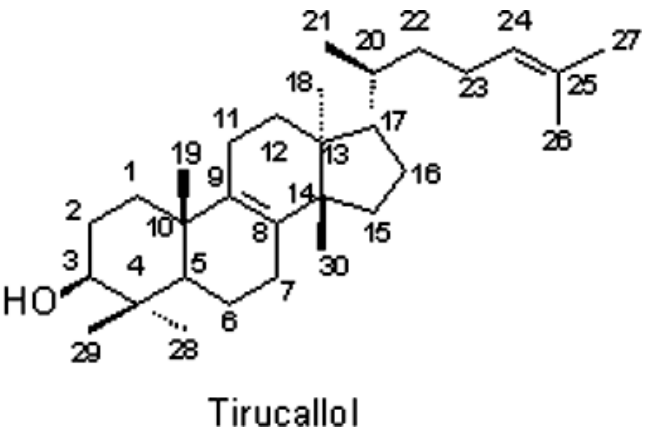


**Figure 1.** Chemical structure of isolated phytosterol from Chios mastic gum, tirucallol.

*vivo* data support that CMG extract and constituents are biologically active against *Helicobacter pylori* (11). Recent studies demonstrate that Chios mastic induces apoptosis (12) and possesses antiproliferative activity (13) in colon cancer cells. CMG mainly consists of triterpenes of the oleanane, euphane, and lupane types (10, 14) and α-tocopherol (15). Latest analysis indicated that the acidic fraction of CMG extract consists of triterpenic acids with major oleanonic acid content, while the neutral fraction consists of neutral triterpenic compounds (alcohols and aldehydes), as well as phytosterols like tirucallol (11). CMG has also been associated with cardiovascular protection, exerting its effect mainly through increasing the antioxidant defense system (9, 10) and effectively lowering the levels of serum cholesterol in human subjects (16). Moreover, CMG extract has been shown to inhibit human low-density lipoprotein (LDL) oxidation *in vitro* (10) and oxidized LDL cytotoxic effect on peripheral blood mononuclear cells (PBMC) (9).

Atherosclerosis is considered a chronic inflammatory process with increased oxidative stress, in which the adhesion of monocytes to the vascular endothelium and their subsequent migration into the vessel wall are the pivotal early events in atherogenesis (17). The interaction between monocytes and vascular endothelial cells (ECs) are mediated by adhesion molecules, including vascular adhesion molecule 1 (VCAM-1), intracellular adhesion molecule 1 (ICAM-1), and E-selectin on the surface of the vascular endothelium. The increased expression of adhesion molecules by ECs in human atherosclerotic lesions, activated by inflammatory cytokines, may lead to further recruitment of leukocytes to atherosclerotic sites leading thus to the formation of atherosclerotic lesion and endothelial dysfunction.

Although CMG's cardioprotective effect, via its antioxidant capacity and cholesterol lowering effect, is already known (9, 16), whether it can directly inhibit the inflammatory process leading to atherogenesis has not been clarified. In this study, we examined the hypothesis that CMG neutral extract and tirucallol (isolated phytosterol from neutral extract) could inhibit the expression of adhesion molecules (VCAM-1 and ICAM-1) and the attachment of monocytes in Human Aortic Endothelial cells (HAEC). The impact of treatment with CMG neutral extract and tirucallol on Tumor Necrosis Factor-α (TNF-α)–related signaling in HAEC, such as activation of Nuclear Factor-kB (NFkB) phosphorylation, was also examined.

## Materials and Methods

**Plant Material: Gum Extraction.** Commercial mastic gum was supplied by the Chios Mastic Growers Association, the exclusive worldwide producer of the resin. Preparation of CMG extract was carried out as described previously (11). Briefly, a mixture of ethyl acetate (500 ml) and methanol (1,500 ml) was added to 500 g of mastic gum and, after 2 days, a layer of poly-β-myrcene (150 g) was decanted. The clear supernatant solution was filtered and evaporated in a rotary evaporator at 45°C with an 80-kPa vacuum. The semisolid residue afforded was dried in a desiccator at 70°C and 1,000-mbar vacuum, giving a white powder (350 g), the total mastic extract without polymer (TMEWP). Further fragmentation of the TMEWP by extraction with aqueous 5% $Na_2CO_3$ (1 liter) and ether (3.5 liters), as described by Barton et al (18), and re-extraction of the organic phase three times with 5% $Na_2CO_3$ (1 liter each time) afforded the mastic neutral (phytosterolic) fraction (135 g) as the organic phase. Acidification of the two aqueous phases with 1N HCl (3 liters) and re-extraction with ether (6 liters) afforded the mastic acidic fraction (190 g) in the organic phase.

**Isolation of Neutral Compounds (Phytosterols).** A total of 17.2 g of the neutral fraction were submitted to column liquid chromatography over normal-phase silica gel with a cyclohexane-$CH_2Cl_2$ gradient (from cyclohexane at 100% to $CH_2Cl_2$ at 100%). Twenty-one liters of solvent were used in total, and 22 fractions were afforded. Fraction 5 (839 mg) was separated by medium pressure liquid chromatography (MPLC), which was performed on a Büchi model 688 apparatus with a column containing Si gel (type 60; 20–40 μm; Merck), and with a cyclohexane-$CH_2Cl_2$ gradient (from 90/10 to 100% $CH_2Cl_2$; total volume used, 2 liters). The column afforded tirucallol (110 mg) and butyrospermol (105 mg). Separation of other fractions led to several neutral, non-phytosterolic triterpenic compounds (dammaradienone, 28-norolean-12-en-3-one, oleanonic aldehyde and oleanolic aldehyde) (11). The molecular structure of tirucallol is displayed in Figure 1. The above constituents were identified by 1D and 2D NMR on Bruker DRX 400 and Bruker AC 200 spectrometers (1H [400 and 200 MHz] and 13C [50 MHz]), and MS on a Finnigan GCQ Plus mass spectrometer and by comparison with data in the published literature (19).

**Culture of HAEC.** HAEC were provided as cryopreserved cells by Clonetics (Cambrex Corporation, USA) and

were grown in culture flasks at 37°C in a humidified 95% air-5% $CO_2$ atmosphere in Endothelial Cell Basal Medium (EBM; Clonetics, Cambrex Corporation, USA) supplemented with fetal bovine serum (FBS, 2%), human epidermal growth factor (hEGF, 10 ng/ml), hydrocortisone (1.0 μg/ml), gentamicin (50 μg/ml), amphotericin B (50 ng/ml), and bovine brain extract (BBE, 3 mg/ml). The growth medium was changed every other day until confluence. Cells under passage 8 were used for this study.

**MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] Assay.** Cultures of HAEC were grown in endothelial cell growth medium (EGM, Clonetics, Cambrex Corporation, USA) supplemented with 2% FBS (Gibco, Invitrogen, USA), in T–75-$cm^2$ flasks at 37°C, 95% humidity and 5% $CO_2$ atmosphere. Subcultures were carried out every 3–4 days using a trypsin 0.025% and EDTA 0.01% solution (Gibco, Invitrogen, USA). Cell viability was estimated by a modification of the MTT assay (20), which determines the metabolically active mitochondria of cells. Briefly, cells were plated in their growth medium at density of 6,000 cells/well in 96 flat bottomed well plates. Twenty-four hours after plating, test substances were added at final concentrations, ranging from 25 to 200 μg/ml (for CMG extract) and from 1 to 100 μM (for tirucallol), in DMEM phenol red (PR)–free (Gibco, Invitrogen, USA). Cells in their growth medium, without test substances, were used as control samples (vehicle). After 48-h incubation, the medium was replaced with MTT (Sigma-Aldrich, Europe) and dissolved at a final concentration of 1 mg/ml in serum-free, PR-free medium, for a further 4 h incubation. Then, the MTT-formazan product was solubilised thoroughly in isopropanol, and the optical density was measured at a test wavelength of 550 nm and a reference wavelength of 690 nm.

**Cell ELISA.** To examine whether CMG neutral extract and tirucallol could modify the expression of VCAM-1 and ICAM-1, cell ELISA was conducted. Briefly, to measure the cell-surface expression of adhesion molecules, HAEC monolayers in flat-bottomed 96-well plates (at 80% confluence) were pretreated with CMG neutral extract (25, 50, 100, 200 μg/ml) or tirucallol (0.1, 1, 10, 100 μM) for 18 h, then were stimulated for 6 h at 37°C with 1 ng/ml TNF-α (Sigma-Aldrich, Europe); after that, the cells were fixed with 0.1% glutaraldehyde in PBS for 30 min at 4°C. Plates were blocked at 37°C for 1 h with 5% skimmed milk powder in PBS and an incubation at 4°C overnight with a primary monoclonal goat antibody against human ICAM-1 or VCAM-1, at a final concentration 2 μg/ml in 5% skimmed milk PBS, followed. Then, the plates were washed three times with 0.1% Tween-20 in PBS and incubated with a horseradish peroxidase-conjugated rabbit antimouse IgG secondary antibody at a dilution of 1:5,000 at room temperature for 1 h. Subsequently, the plates were washed three times with 0.1% Tween-20 in PBS, and, finally, the expression of cell adhesion molecules was quantified by the addition of the peroxides substrate *o*-phenylenediamine hydrochloride. As a positive control, we used vitamin E (α-tocopherol, 20 μM), an antioxidant known to exert its effect through modulation of cytokines, adhesion molecules, mobilization of NFkB transcription factor, and interaction of immune cells with endothelial cells (21, 22). The absorption of each well was measured at 450 nm using a microplate ELISA reader.

**Fluorescent Labelling of Monocytes.** U937 cells (human monocytic cell line which exhibits many characteristics of monocytes; ECACC, UK) were fluorescently labelled with 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM; Molecular Probes, Invitrogen, USA) for the quantitative cell adhesion assay. Nonfluorescent BCECF-AM is lipophilic, and its methylester is cleaved intercellularly and becomes a highly charged fluorescent BCECF, which is retained by viable cells. The BCECF-AM was obtained as a 1 g/L stock solution in anhydrous DMSO and was stored at –80°C. After labelling the U937 cells ($10^6$ cells/ml) with 10 μM BCECF-AM in RPMI-1640 PR-free (Gibco, Invitrogen, USA), FBS-free and L-glutamine–free medium for 1 h at 37°C and 5% $CO_2$, the cells were washed two times with PBS to remove the excess dye. Finally, the cells were resuspended in RPMI-1640 PR, 2% FBS medium at a density of $2\times10^4$ cells/well, according to manufacturer's instructions.

**U937 Cell Adhesion Assay.** HAEC were cultured until confluence in 96-well plates and were pretreated with either the CMG neutral extract (25, 50, 100, 200 μg/ml) or tirucallol (0.1, 1, 10, 100 μM), for 18 h. Then cells were stimulated for 6 h at 37°C with TNF-α (2 ng/ml). BCECF-labelled U937 cells ($2\times10^4$ cells/well), were incubated with HAEC for 1 h at 37°C and 5% $CO_2$. After incubation, nonadherent cells were removed by washing gently each well two times with PBS. The attached cells were lysed with 50 mmol/L Tris buffer (pH 7.6) containing 0.1% SDS. α-Tocopherol (20 μM) was used as a positive control. The fluorescent intensity of each well was measured with a fluorescence multi-well plate reader set at excitation and emission wavelengths of 485 nm and 530 nm, respectively.

**Measurement of NFkB p65 Phosphorylation.** To measure NFkB phosphorylation, confluent HAEC were starved in serum-free culture medium for 18 h and then pretreated without or with CMG neutral extract (25, 50, 100, 200 μg/ml) or tirucallol (0.1, 1, 10, 100 μM) for 18 h. Then, cells were stimulated for 5 min at 37°C with TNF-α (2 ng/ml). We performed a time course experiment by stimulating HAEC with TNF-α (2 ng/ml) for 5 min, 15 min, and 30 min. Finally, NFkB p65 phosphorylation was measured with CASE™ kit (SABiosciences, USA). The CASE™ kit can be used to monitor activation of a signal transduction pathway by measuring phosphorylation of a key mediator of a pathway of interest, without the need to prepare cell lysates and to perform a Western blot. In our study, NFkB (p50/p65) is a key transcription factor that is implicated in the regulation of a variety of genes participating in inflammatory responses, including genes encoding

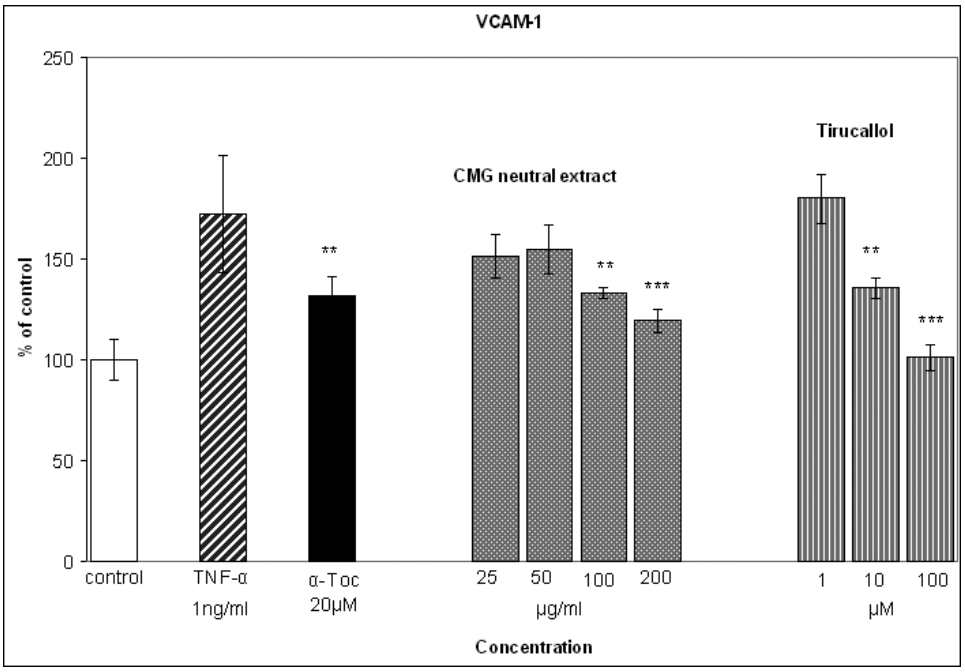


**Figure 2.** CMG neutral extract and tirucallol inhibit TNF-α–induced VCAM-1 protein expression in HAEC. HAEC were incubated as described in Methods, in absence of TNF-α or compounds (control), with α-tocopherol (α-Toc, 20 μM) or with different concentrations of CMG neutral extract (25–200 μg/ml) and tirucallol (1–100 μM) for 18 h, followed by stimulation with TNF-α (1 ng/ml) for up to 24 h. Adhesion molecules were measured by cell ELISA. Data are expressed as percentage of control and shown as means ± SD of three independent experiments (each conducted in triplicates). *$P < 0.05$ value was considered statistically significant when compared to TNF-α–treated cells (**$P < 0.01$, ***$P < 0.001$).

VCAM-1 and ICAM-1. Moreover, TNF-α has been shown to induce the activation-phosphorylation and translocation of NFkB to the nucleus, promoting the VCAM-1 and ICAM-1 expression in endothelial cells (23). Thus, firstly, we examined the time course of TNF-α induced phosphorylation on Ser-536 of NFkB p65, and then we tested the impact on NFkB p65 phosphorylation of CMG neutral extract and tirucallol at the indicated concentrations. Briefly, the cells were fixed with 4% fixing buffer for 20 min, room temperature (RT). Then, plates were blocked for 1 h, RT, with blocking buffer. After the blocking, incubation with the primary antibody (either the phospho-protein or the total-protein specific antibody) for 1 h, RT, followed. Then, the plates were washed three times with 1X washing buffer and incubated with the secondary antibody for 1 h, RT. Subsequently, the plates were washed three times with 1X washing buffer, and, finally, Colorimetric Detection of Antibodies was done by measuring the absorption of each well at 450 nm. Determination of relative cell number was followed by reading the absorption of each well at 595 nm. The absorption was measured by using a microplate ELISA reader. It is important that the amount of phosphorylated NFkB is normalized to the amount of total NFkB protein, so that it is directly related to the extent of activation.

**Statistical Analysis.** Data are reported as mean ± SD of three independent experiments (each experiment was conducted in triplicate or quadruplicate). Data in figures are expressed as percentage of control, which was calculated as follows: (value for cells treated with test compound or test extract/value for control cells) × 100. Statistical analysis was performed using Student's *t*-test, two-tailed distribution, assuming two-sample unequal variance.

## Results

**CMG Extract and Isolated Phytosterol Tirucallol Inhibit Cell Adhesion Molecules Expression in HAEC.** The effect of different concentrations of TNF-α (1 ng/ml or 2 ng/ml) on VCAM-1 and ICAM-1 expression was initially determined after 6, 12, or 24 h of incubation. Incubation of confluent HAEC with TNF-α (1 ng/ml) caused a maximal surface expression of VCAM-1 and ICAM-1 after 6 h of incubation (data not shown). In subsequent experiments, we used TNF-α (1ng/ml) for 6 h to induce stimulation of cells. TNF-α increased the basal expression (control) of cell adhesion molecules VCAM-1 and ICAM-1 of confluent HAEC. α-Tocopherol significantly decreased the TNF-α–induced endothelial expression of both VCAM-1 and ICAM-1 ($P < 0.001$), as expected (24).

The effects of CMG neutral extract and tirucallol on the expression of VCAM-1 and ICAM-1 by HAEC are presented in Figure 2 and Figure 3, respectively. CMG extract (100, 200 μg/ml) caused a significant dose-dependent decrease in VCAM-1 and ICAM-1 expression ($P<0.01$, $P < 0.001$) compared to TNF-α–treated HAEC. Tirucallol (10, 100 μM) had a similar effect, as it also significantly inhibited VCAM-1 and ICAM-1 expression ($P < 0.01$, $P < 0.001$).

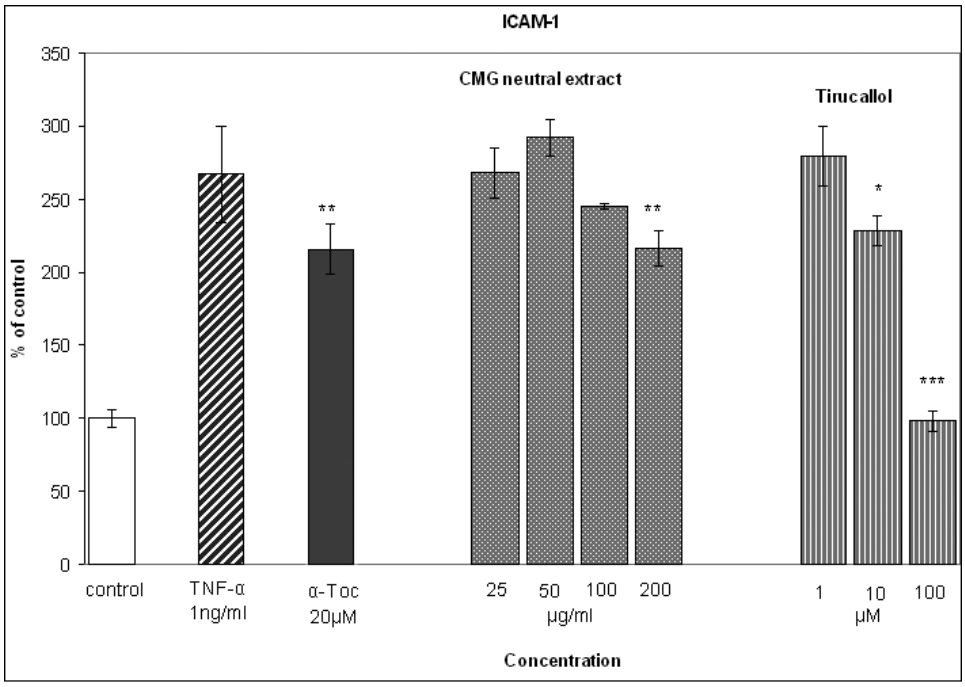


**Figure 3.** CMG neutral extract and tirucallol inhibit TNF-α–induced ICAM-1 protein expression in HAEC. HAEC were incubated as described in Methods, in absence of TNF-α or compounds (control), with α-tocopherol (α-Toc, 20 μM) or with different concentrations of CMG neutral extract (25–200 μg/ml) and tirucallol (1–100 μM) for 18 h, followed by stimulation with TNF-α (1 ng/ml) for up to 24 h. Adhesion molecules were measured by cell ELISA. Data are expressed as percentage of control and shown as means ± SD of three independent experiments (each conducted in triplicates). *$P < 0.05$ value was considered statistically significant when compared to TNF-α–treated cells (**$P < 0.01$, ***$P < 0.001$).

**Cell Viability.** The assessment of cell viability revealed that neither the morphology nor the reduction of MTT salt in HAEC cells was affected by any of the tested compounds (tirucallol, α-tocopherol or TNF-α), or by CMG neutral extract, at any concentration range or experimental conditions used. The lowering effect of the CMG extract and tirucallol on the expression of adhesion molecules without affecting cell viability supports their anti-inflammatory activity.

**CMG Extract and Isolated Phytosterol Tirucallol Inhibit Binding of U937 Cells to TNF-α–Stimulated HAEC.** The effects of the test compounds on the binding of U937 cells to TNF-α–stimulated HAEC were determined, and results are shown in Figure 4. Control confluent HAEC showed minimal binding to U937 cells, and results are expressed as percentage of control. Adhesion was significantly increased when the HAEC were treated with TNF-α (2 ng/ml). Pretreatment with α-tocopherol (20 μM), CMG neutral extract (25, 50, 100, 200 μg/ml), or tirucallol (0.1, 1, 10, 100 μM) significantly ($P < 0.001$, $P < 0.01$) reduced the adhesion of U937 cells to TNF-α–stimulated HAEC.

**CMG Extract and Isolated Phytosterol Tirucallol Attenuate Phosphorylation of NFkB p65 in TNF-α–Stimulated HAEC.** The time course experiment to monitor the NFkB phosphorylation by stimulating HAEC with TNF-α (2 ng/ml) for 5 min, 15 min, and 30 min showed that stimulation of the cells for 5 min was more significant (Figure 5). Results showed that pretreatment with either CMG neutral extract or tirucallol significantly decreased ($P < 0.01$) the phosphorylation of NFkB p65 in TNF-α–stimulated HAEC compared to control HAEC (Figure 6).

## Discussion

The cardiovascular protective effect of CMG has been related to antioxidant and hypocholesterolemic effects (9, 10, 16). In particular, CMG has been shown to exhibit *in vitro* biological protection against LDL induced oxidation (10), to inhibit oxidized LDL cytotoxic effect on PBMC (9), and to exert a beneficial action on total cholesterol (TC), LDL and TC/high-density lipoprotein (HDL) ratio in a healthy human population (16). However, data on the anti-inflammatory effects of CMG are sparse. The binding and recruitment of circulating monocytes to vascular endothelial cells are early steps in the development of inflammation and atherosclerosis, mediated through cell adhesion molecules (CAMs) that are expressed on the surface of endothelial cells. Therefore, we evaluated the potential of the neutral extract of CMG to influence the expression of VCAM-1 and ICAM-1 by HAEC. We used the Cell ELISA to measure VCAM-1 and ICAM-1, a well recognized *in vitro* assay, to evaluate the anti-inflammatory effect of test compounds or extracts (25–27). As a positive control, we used vitamin E (α-tocopherol), an antioxidant known to exert its effects through modulation of cytokines, adhesion molecules,

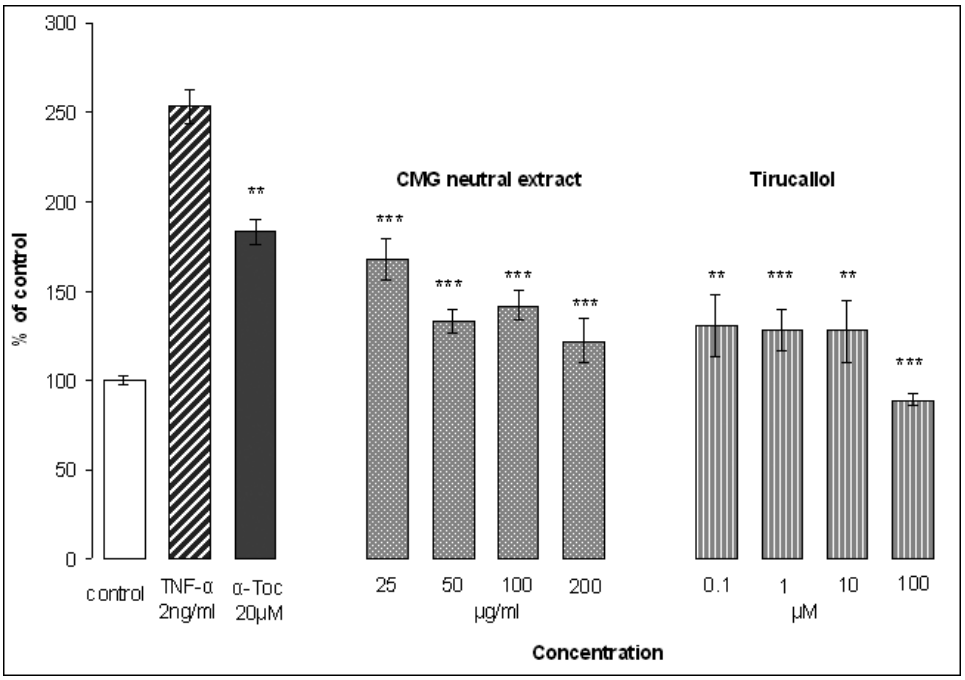


**Figure 4.** CMG neutral extract, tirucallol, and α-tocopherol (α-Toc) inhibit TNF-α–induced monocyte adhesion to HAEC. HAEC were incubated as described in Methods, in absence of TNF-α or compounds (control), with α-tocopherol (α-Toc, 20 μM) or with different concentrations of CMG neutral extract (25–200 μg/ml) and tirucallol (0.1–100 μM) for 18 h, followed by stimulation with TNF-α (2 ng/ml) for up to 24 h. Monocyte adhesion was determined by the adhesion assay. Data are expressed as percentage of control and shown as means ± SD of three independent experiments (each conducted in triplicates). $^{*}P < 0.05$ value was considered statistically significant when compared to TNF-α–treated cells ($^{**}P < 0.01$, $^{***}P < 0.001$).

mobilization of NFkB transcription factor, and interaction of immune cells with endothelial cells (21, 22). For further evaluation of the anti-inflammatory effects of CMG neutral extract, we used the Adhesion assay to measure the monocyte adhesion to TNF-α–stimulated HAEC (28). We studied CMG neutral fraction, the major components of which are five neutral triterpenic compounds: tirucallol, dammaradienone, 28-norolean-12-en-3-one, oleanonic aldehyde, and oleanolic aldehyde (11, 14). We further compared the data from the CMG neutral fraction with those from tirucallol in view of its large contribution to the total triterpenic content (10, 11, 14). In order to decide the proper dosages of CMG extract and tirucallol to be tested in our *in vitro* systems, we considered it important to take into account the following: a) 500 g of CMG total extract resulted in 135 g of neutral fraction (11), b) 17.2 g of neutral

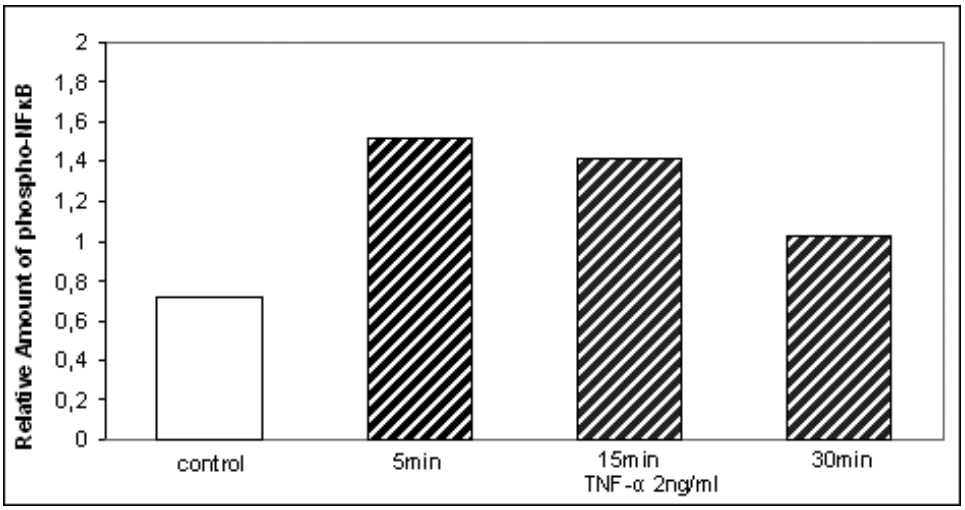


**Figure 5.** Monitoring of NFkB phosphorylation over a time course. HAEC were grown in 96-well plates and were starved before treatment. Then, cells were treated with 2 ng/ml of NFkB activator TNF-α for 5, 15, and 30 min. Phosphorylation levels at Serine-536 of NFkB p65 were measured using the CASE™ kit for NFkB S536 (Cat. No. FE-005).

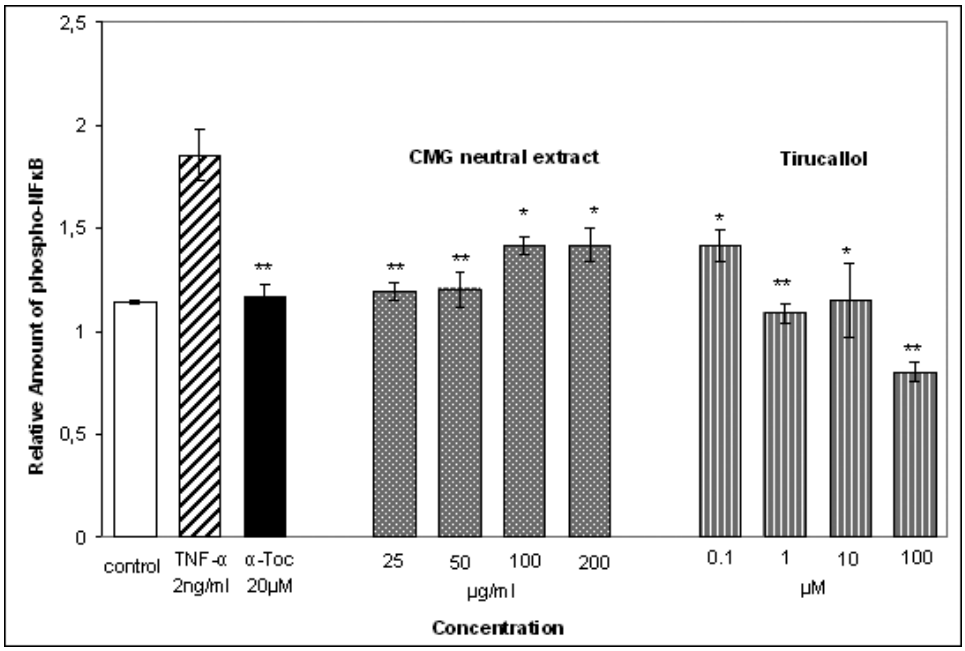


**Figure 6.** CMG neutral extract and tirucallol inhibit TNF-α–induced phosphorylation of NFkB. HAEC were grown in 96-well plates and were starved before treatment with test extract/compound. Cells were then treated with 2 ng/ml of NFkB activator TNF-α (2 ng/ml) for 5 min. Phosphorylation levels at Serine-536 of NFkB p65 were measured using the CASE$^{TM}$ kit for NFkB S536. Data are expressed as relative amount of phospho-NFkB and shown as means ± SD of three independent experiments (each conducted in triplicates). $^{*}P < 0.05$ value was considered statistically significant when compared to TNF-α–treated cells ($^{**}P < 0.01$).

fraction of CMG when submitted to column liquid chromatography and MPLC finally afforded 110 mg tirucallol (11); c) when 5 g of CMG powder was given orally to humans, it resulted in lowering the LDL levels (16). In view of the above, and considering the human blood volume and bioavailability, we decided to assess the biological effects of CMG neutral extract at a concentration range of 25–200 μg/ml and of tirucallol at a concentration range of 0.1–100 μM, which are physiologically achievable, biologically active, and comparable concentrations. Vitamin E inhibited the TNF-α–induced expression of both ICAM-1 and VCAM-1, as expected (21, 24). CMG neutral extract inhibited the TNF-α–induced endothelial activation and expression of ICAM-1 and VCAM-1 adhesion molecules, at a concentration range of 25–200 μg/ml, which is within the reported *in vitro* concentration range of CMG extract shown to inhibit LDL-oxidation (10). More importantly, the adhesion assay showed that, in the presence of CMG extract (25–200 μg/ml), the binding of human monocytic cell line U937 to TNF-α–stimulated HAEC was inhibited significantly. Tirucallol, a phytosterol that is found as a component of CMG neutral extract (11), also inhibited the TNF-α–induced endothelial activation and expression of ICAM-1 and VCAM-1 adhesion molecules in a dose-dependent manner when tested at a concentration range of 0.1–100 μM. In addition, the same treatments with tirucallol (0.1–100 μM) inhibited the binding of human monocytic cell line U937 to TNF-α–stimulated HAEC. In summary, since monocyte recruitment into the vascular wall after their adhesion to endothelial cells is a crucial step in the pathogenesis of atherosclerosis, our study shows that CMG neutral extract has a noticeable anti-inflammatory potential that can be attributed, partly, to tirucallol. However, since the CMG neutral extract contains not only tirucallol but also other triterpenic compounds (such as dammaradienone, 28-norolean-12-en-3-one, oleanonic aldehyde, and oleanolic aldehyde), which are known to exert anti-inflammatory activity (29, 30), we suggest that the combination of constituents present in CMG extract may result in synergistic/additive activity.

To further elucidate the mechanism of action of CMG neutral extract and tirucallol, we investigated their effects on the phosphorylation of NFkB p65, which is the critical process for the regulation of a variety of genes participating in inflammatory responses, including genes encoding VCAM-1 and ICAM-1 (31). NFkB is a cytoplasmatic component as an inactive complex with its inhibitor IkB. In our study we used TNF-α to stimulate endothelial cells, since it induces the phosphorylation of IkB, which results in dissociation of IkB and finally activation-phosphorylation and translocation of NFkB to the nucleus, promoting the expression of downstream genes, such as adhesion molecules VCAM-1 and ICAM-1 (23). To monitor the phosphorylation state of NFkB we used the CASE$^{TM}$ kit, since it helps verify the activation of the NFkB-dependent pathway. In this assay, it is important that the amount of phosphorylated NFkB is normalized to the amount of total

NFkB protein, so that it is directly related to the extent of activation.

Our findings showed that TNF-α treatment induced higher levels of NFkB phosphorylation in HAEC, an indication that NFkB was activated. Treatment with CMG neutral extract (25–200 μg/ml) and isolated phytosterol tirucallol (0.1–100 μM), significantly inhibited the phosphorylation of NFkB, suggesting that their anti-inflammatory activity *in vitro* is mediated, at least in part, by modulation of NFkB activation. CMG neutral extract showed a high inhibitory effect on NFkB phosphorylation at the low concentration range, whereas Tirucallol at the high concentration range. This may be due to the fact that extracts exhibit different effects than their individual components, since combination of multiple compounds present in plant extracts may result in synergism or antagonism. Of note, Singh et al (32) showed that phytosterols partly mediated their anti-inflammatory effects via modulation of NFkB activity.

In conclusion, our study does the following: a) extends existing data regarding the cardio protective effect of CMG; b) expands the spectrum of known phytosterols with potent antiatheromatic activity (33, 34); c) provides new insights into understanding the molecular mechanism underlying the beneficial effects of CMG and tirucallol on endothelial action; and finally, d) may aid in design of new therapy for intervention in atherosclerosis and other related cardiovascular diseases.


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