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HEALTH SCIENCES

Leaf extracts of *Campomanesia xanthocarpa* positively regulates atherosclerotic-related protein expression

EDUARDO B.B. CUNHA, NATÁLIA F. DA SILVA, JEAN DE LIMA, JULIA A. SERRATO, CARLOS A.M. AITA & ROBERTO H. HERAI

Abstract: Atherosclerosis is caused by a monocyte-mediated inflammatory process that, in turn, is stimulated by cytokines and adhesion molecules. Monocytes are then differentiated into macrophages, leading to the formation of arterial atherosclerotic plaques. Recently, guavirova leaf extracts from *Campomanesia xanthocarpa* (EG) have shown potential effects on the treatment of plaque formation by reducing cholesterol, LDL levels and serum oxidative stress. We evaluated the effect of EG on the viability of human monocytic and endothelial cell lines at three time points (24, 48 and 72 hours) and whether it can modulate the migration and *in vitro* expression of CD14, PECAM-1, ICAM-1, HLA-DR and CD105. Cell viability was affected only at higher concentrations and times. We observed decreased ICAM-1 expression in cells treated with 50 µg/ml EG and CD14 expression with IFN-γ and without IFN-γ. CD14 also decreased endothelial cell expression in the presence of IFN-γ and GE. We also found decreased expression of PECAM-1 when treated with EG and IFN-γ. In addition, EG-treated endothelial cells showed higher migration than the control group. Reduced expression of these markers and increased migration may lead to decreased cytokines, which may be contributing to decreased chronic inflammatory response during atherosclerosis and protecting endothelial integrity.

Key words: *Campomanesia xanthocarpa*, plant extract, atherosclerosis, *in vitro* cell treatment, protein expression.

INTRODUCTION

Atherosclerosis is a chronic inflammatory disease, and it is characterized by the formation of atherosclerotic plaques. When ruptured and depending on where it is lodged, plaques can cause acute myocardial infarction (Zhu et al. 2017).

At cellular level, the atherogenesis is caused by the oxidized low-density lipoprotein (ox-LDL), and along with minimally modified low-density lipoprotein (mmLDL), they stimulate the appearance of leukocyte adhesion molecules, such as the vascular cell adhesion protein 1 (VCAM-1), the intercellular adhesion molecule 1 (ICAM-1) and the platelet endothelial cell adhesion molecule (PECAM-1), and together are responsible for attracting monocytes and lymphocytes within the intima (Eisenhardt et al. 2012). In this process, when a monocyte penetrates the endothelium, there is an increase in expression of PECAM-1 and a decrease of the LV-cadherin expression, thus causing a weakening of the endothelial integrity (Hashimoto et al. 2011). In addition, overexpression of PECAM-1 also directly induces ICAM-1 overexpression. This protein, through the leukocyte trans-endothelial migration pathway,

plays an essential role in the process of adhesion and transmigration of monocytes into the intima (Privratsky & Newman 2014), and thus causing the formation of atherosclerotic plaques inside blood vessels. During the cell migration and plaque formation process, the mmLDL lipoprotein stimulates the onset of leukocyte adhesion molecules, and also stimulates the expression of tool-like receptor 4 (TLR4) (Estruch et al. 2013). This molecule precisely with CD14 (co-receptor required for the activation of TLR4) (Estruch et al. 2013) is responsible for the production of pro-inflammatory cytokines (interleukin 1 beta (IL-1β), interleukin 6 (IL6), tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ)) and VCAM-1. The production of IFN-γ also modulates an increase in the HLA-DR expression by the cells (Hansson & Jonasson 2009). Another important molecule in the context of the atherosclerotic process is the CD105. It is a transmembrane protein, being one of the main glycoproteins expressed on the surface of the endothelial cells, playing a fundamental role in the cardiovascular system (López-Novoa & Bernabeu 2010). Genetic alterations in the gene that codes for this protein can cause hereditary hemorrhagic (telangiectasia), besides being involved in pre-eclampsia and in several types of cancer (Di Paolo et al. 2018, Metcalfe et al. 2018). Recent evidence shows that CD105 also plays an important role in platelet adhesion within the endothelium by interacting with integrins (Rossi et al. 2018). Together, these molecules directly contribute to the increase of the inflammatory process that culminates in atherosclerosis (de Vries et al. 2014, Rocha et al. 2016).

It was previously demonstrated that PECAM-1 is directly involved in the development of atherosclerotic lesions (Hashimoto et al. 2007). In another work in the offspring of PECAM-1 deficient mice, it was also observed a decrease in atherosclerotic lesion and leukocyte migration and reduced expression of the proteins VCAM-1, ICAM-1, P-selectin, and decreased activation of NF-kB (Tzima et al. 2005).

Recently, it was demonstrated that the use of plant leaves of the *Myrtaceae* group, such as guavirova (*Campomanesia xanthocarpa Berg*), have the potential to be used as a natural treatment for atherosclerosis (Dickel et al. 2007, Klafke et al. 2016a). In hypercholesterolemic patients, the administration of guavirova leaves have been shown to reduce plasma oxidative stress (Viecili et al. 2014, Klafke et al. 2010) and decreased total cholesterol and LDL levels by inhibiting the concentration-dependent activity of the precursor cholesterol synthesis enzyme HMGR (Klafke et al. 2010, Islam et al. 2015). In animal models, guavirova leaves showed antithrombotic and fibrinolytic effects (Klafke et al. 2012), and also decreased levels of nitric oxide (Viecili et al. 2014) and proinflammatory cytokines such as IL-1, IL6, TNF-α and IFN-γ (Klafke et al. 2016b). In an *in vitro* study, it was also demonstrated that the guavirova leaves can act as an antiplatelet formation (Klafke et al. 2012).

Although the previous studies have correlated positive effects of *C. xanthocarpa* in patients with hypercholesterolemia, it is still missing studies describing, at cellular and molecular levels, the role of EG in regulating the molecules involved in hypercholesterolemia and plaque formation.

In this work, we investigated whether EG is able to modulate *in vitro* atherosclerotic-related cellular phenotypes. We show that leaf extracts of *C. xanthocarpa* significantly affect cellular viability under different concentrations and at different time intervals. We also show that EG significantly induces endothelial cell migration. At molecular level, we demonstrate that EG also modulates atherosclerotic-related proteins (such as CD14, ICAM-1, VCAM-1 and CD105,

HLA-DR) that are involved in the inflammatory process correlated with the plaque formation in atherogenesis. Together, our findings suggest that EG can modulate distinct cellular phenotypes that protects cells against atherosclerosis (Figure 1).

MATERIALS AND METHODS

Extract preparation

The leaves of *Campomanesia xanthocarpa* were collected during the year 2010 in the city of Cruz Alta (RS, Brazil) and an exsicata number 1088 was

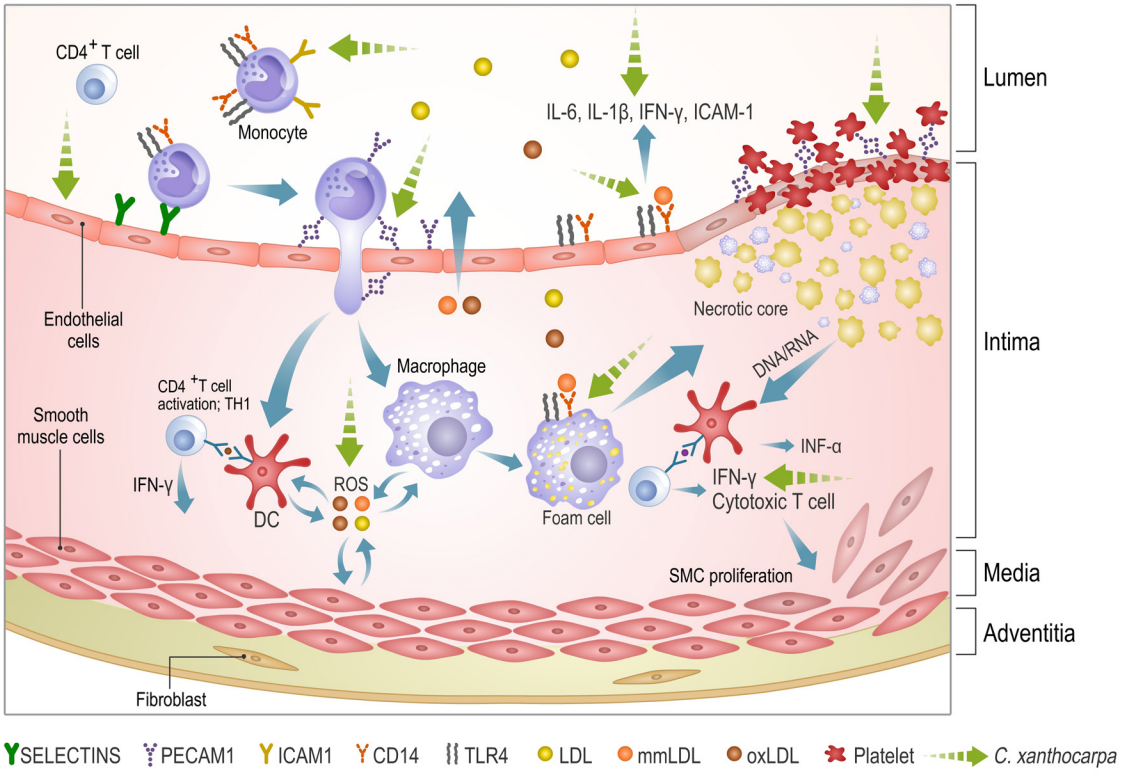

Figure 1. Guavirova plant extract associated with the regulation of molecules involved in atherosclerosis. Schematic summary of the main mechanisms of atherosclerosis and molecules modulated by aqueous extract of *C. xanthocarpa* (EG) leaves. Continuous exposure to risk factors leads to endothelial dysfunction, making the endothelium more permeable for the entrance of LDL within intimal region, also inducing increased expression of selectin on the surface of monocytes and endothelial cells. This process culminates with increased monocyte migration (dependent on binding with PECAM-1, which is EG-modulated) to the intimal region, that differentiates into dendritic (DC) cells (highly specialized in the activation of TH1-secreting TH lymphocytes). IFN-γ: EG decreases the amount of IFN-γ) and / or macrophages that begin to phagocyte LDL. The interactions between dendritic cells, macrophages and smooth muscle cells (SMC) with LDL produce reactive oxygen species (ROS), which is decreased by EG. LDL molecules can be transformed into oxLDL (EG-decreased molecules) and mmLDL. These mmLDL are capable of binding to the CD14-TLR4 complex (EG decreases CD14), increasing the production of IL-6, IL-1β, IFN-γ and ICAM-1 (both negatively modulated molecules by EG), that together potentiates the inflammatory process. This process favors the transformation of macrophages into foam cells and leads to the proliferation of MCS from the middle layer to the intima of the vessel, which secretes extracellular matrix, forming the fibrous capsule. This imbalance within the intima leads to apoptosis of these cells, leading to the formation of the necrotic nucleus plaque, where DNA / RNA fragments are present and stimulates the activation of TH1 lymphocytes by dendritic cells, leading to increased IFN-γ production. Exposure of this plaque content triggers platelet activation and aggregation, leading to thrombus formation, with the participation of PECAM-1, by binding to platelets / endothelium and platelets / platelets.

deposited in the Herbarium of the University of Cruz Alta (RS, Brazil). The collected material was subjected to a cleaning process using sodium hypochlorite at 0.4%, immediately followed by washing in running potable water for 15 min. Then, the material was dried at 40-45 °C and ground to a fine powder (Klafke et al. 2010).

For the tests, an aqueous extract of leaves of *Campomanesia xanthocarpa* (EG) was prepared. Initially, 50 g of dried leaves were added to 500 ml of distilled water, 37 °C under constant stirring for 30 min. Then the solution was filtered, frozen and lyophilized to determine the total dry matter content. The final powder was diluted in Milli-Q water, filtered on a 20 µm filter and adjusted to the desired concentration (25, 50, 100 and 500 µg/ml) to perform the tests (Klafke et al. 2012).

Cellular lines

Cells from the THP-1 human monocytic strain were cultured and expanded in RPMI medium containing 10% fetal bovine serum (FBS), 1% antibiotic (penicillin and streptomycin) at 37° C, in 5% CO_2 atmosphere until used in experiments.

EA.hy926 is a permanent cell line derived by fusing human umbilical vein endothelial cells (HUVECs) with cell line A549 (Edgell et al. 1983) lineage were obtained from the Rio de Janeiro Cell Bank (BCRJ, RJ, Brazil) and ATCC (American Type Culture Collection) CRL 2922. Cells were cultured and expanded in Dulbecco's modified eagle medium (DMEM) supplemented with 4000 mg/L glucose, 4 mM glutamine, 1 mM sodium pyruvate, 1500 mg/L sodium bicarbonate and 10% FBS, 1% antibiotic at 37 °C, in 5% atmosphere of CO_2 until used in the experiments.

Cellular viability

Viability measured using MTT (3-4,5-dimetiltiazol-2yl-2,5-difenil brometo de tetrazolina) assay ($1x10^4$ cells/well) in 96-well plates, incubated for 24, 48 and 72 hours at 37 °C with different concentrations of EG (25, 50, 100 and 500 µg/ml in 2% RPMI). Then 10 µl of the MTT solution (5 mg / ml) was added for a further 3 h of incubation and the reaction was stopped by the addition of dimethylsulfoxide (DMSO). The optical density (OD) reading will be performed by spectrophotometry at 570 nm.

Analysis of the expression of cell markers

For flow cytometry experiments, cells were initially maintained for at least 24 hours in culture medium containing 2% FBS. Next, the cells were distributed in 24-well plates at a concentration of approximately $1x10^6$ cells / well. To induce activation of the cells incubated with 1000 U/ml IFN-γ for 30 minutes. The cells were then incubated with the EG at 50 µg/ml for 24 hours. At the end of the incubation the cells were washed 2x with phosphate-buffered saline (PBS) and transferred to citometry tubes.

Immunophenotyping of THP-1 cells was performed with the use of fluorochrome-conjugated monoclonal antibodies (FITC, PE or APC) directed against human CD14 (cluster of differentiation 14), ICAM-1, human HLA-DR molecules. Immunophenotyping of EA-hy926 cells was performed using fluorochromes-conjugated monoclonal antibodies (FITC, PE or APC) directed against human PECAM-1, CD105, ICAM-1 and HLA-DR molecules. As controls was used isotypic monoclonal antibodies conjugated to FITC, PE and APC markers. Cells will be incubated with the specific antibodies for 30 min at 40°C and then washed with PBS, centrifuged for 5 min at 400xg, resuspended and fixed in 500 µl PBS with 2% formaldehyde (Merck) for flow cytometry.

Cell analysis was performed on FACSCalibur flow cytometer (BD Biosciences, USA). The equipment was adjusted to the conditions of analysis of cell size and complexity, and the

fluorescence adjustment was performed using the FITC, PE and APC isotype controls. Data acquisition and analysis will be performed using the software CellQuest Pro (BD Biosciences, USA).

Cellular migration

For cellular migration the *scratch healing-wound* method was used, which is based on the creation of a wound in the cellular monolayer. In this work, the cells were analyzed by observation in an inverted phase microscope (Liang et al. 2007).

For the migration analysis, EA-hy926 cells were cultured and expanded in DMEM supplemented with 4,000 mg/L glucose, 4 mM glutamine, 1 mM sodium pyruvate, 1500 mg/L sodium bicarbonate and 2% FBS at 37 °C, in an atmosphere of 5% CO_2. These cells were plated at $5x10^5$ cells, in triplicates for each experimental group. After checking the confluence of the monolayer, the wound was performed with the aid of a p200-type pipette tip, after which a complete medium wash (2% FBS, 1% antibiotic) was done to remove the debris. Then the plates received 2 ml of complete medium (control) and EG at the concentrations of 50 µg/ml and 100 µg/ml of the extract. In all, 8 trials were performed for each condition evaluated. *Scratch wound* area was measured using *TScratch software version 1.0* (CSE Lab, SWITZERLAND).

Statistical analysis

For the normality analysis the *Shapiro-Wilk* test was used, for the parametric data the student T test and for the nonparametric data of *Wilcoxon*. All tests were two-tailed and the p value was considered significant when less than 0.05. For simplicity of presentation, outputs the results on average of percentages. To analyze the data, the IBM SPSS 20 Statistics for *Windows* software was used.

RESULTS

C. xanthocarpa alters cellular viability under different extract concentrations and time intervals

To assess whether EG interferes with cell viability, the MTT method was applied to two cell lines: monocytic THP-1 (Figure 2) and endothelial EA-hy926 (Figure 3).

In monocytes, we did not detect significant alterations in cell viability in 24 hours of incubation with EG, at concentrations of 25, 50, 100 and 500 µg/ml of EG (Figure 2). Only after 48 hours of incubation of the cells with 500 µg/ml of EG, a significant decrease (p-value = 0.012) of 19% of cell viability was observed for monocytic cells (Figure 2). This same observation was made after 72 hours of cell incubation at concentrations of 100 and 500 µg/ml, in which a significant decrease of 28% (p-value = 0.000) and 41% (p-value = 0.000) were observed, respectively (Figure 2).

Additionally, we also found that at 48 hours of incubation, there was a significant decrease (p-value = 0.019) in cell viability at the concentration of 500 µg/ml for both investigated cell lines (Figures 2, 3). After 72 hours of incubation, it was also observed a significant decrease (p-value = 0.000) in cell viability at 100 and 500 µg/ml, the highest used concentrations of EG (Figures 2, 3).

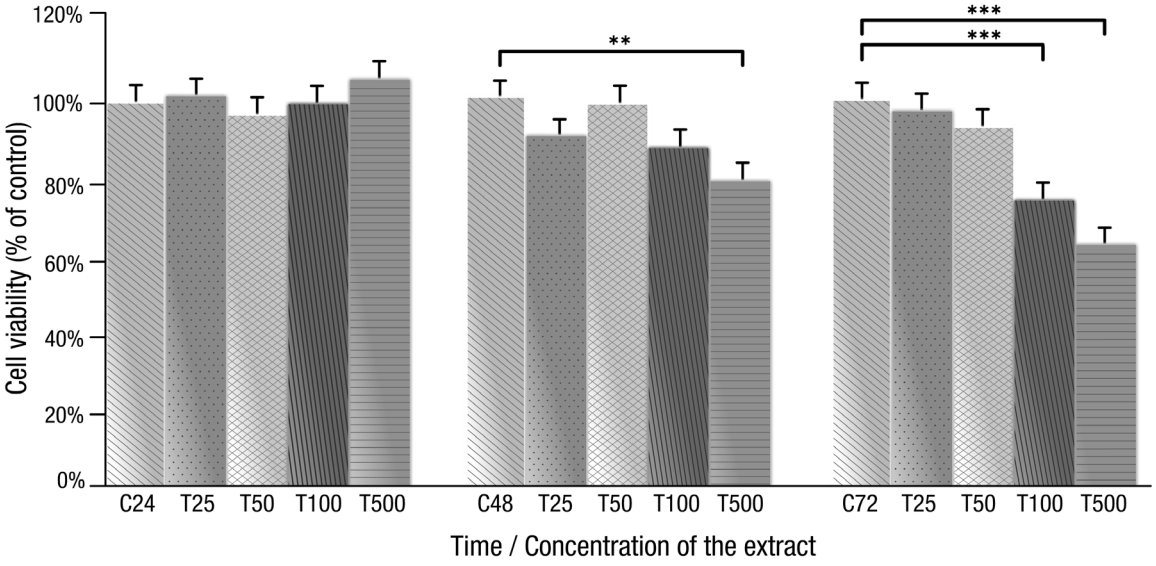

Figure 2. Cell viability analysis of GE-treated THP-1 lineage cells. Cells were treated with different concentrations of *C. xanthocarpa* (25, 50, 100 and 500 µg/ml) and subsequently submitted to MTT cell viability analysis at 24, 48 and 72 hours. The treated groups were compared to the control group C (without extract), and the variations were expressed as percentage and mean (µ) and standard deviation (σ). **p-value = 0.012 compared group C48 (48 hours) with group T500 (µ = 81, σ = 0.02) (500 µg/ml), ***p-value < 0.000 compared group C72 (72 hours) with group T100 (µ = 72, σ = 0.09) (100 µg/ml) and C T500 (µ = 59, σ = 0.08) (500 µg/ml). Six triplicates were performed.

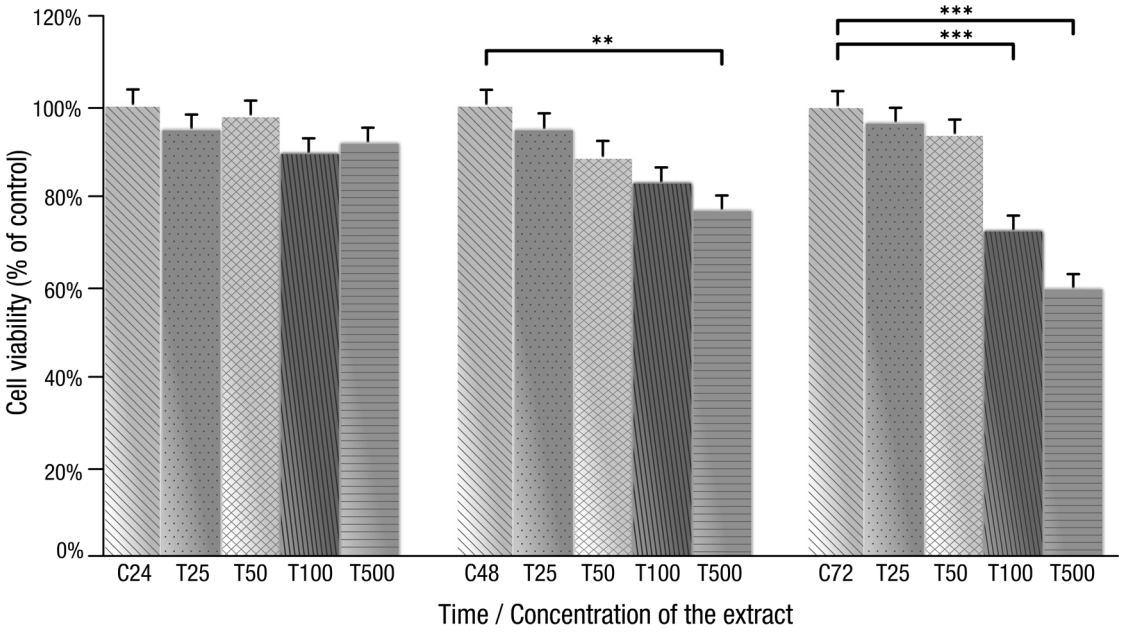

Figure 3. Cell viability analysis of EA-HY926 line cells treated with GE. Cells were treated with different concentrations of *C. xanthocarpa* (25, 50, 100 and 500 µg/ml) and subsequently submitted to MTT cell viability analysis at 24, 48 and 72 hours. The treated groups were compared to the control group C (without extract), and the variations were expressed as percentage and mean (µ) and standard deviation (σ). **p-value = 0.019 compared group C48 (48 hours) with group T500 (µ = 77, σ = 0.04) (500 µg/ml), ***p-value < 0.000 compared group C72 (72 hours) with group T100 (µ = 72, σ = 0.09) (100 µg/ml) and C T500 (µ = 58, σ = 0.10) (500 µg/ml). Six triplicates were performed.

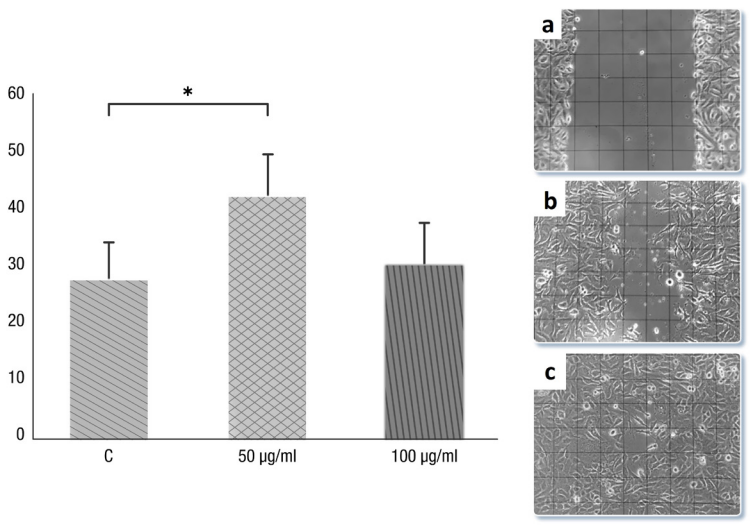

Figure 4. Analysis of cell migration of EA-HY926 strain treated with GE. Cells were treated with different concentrations of *C. xanthocarpa* (50 and 100 µg/ml), and then quantified the number of cells after 24 hours. The treated groups were compared to the control group c (without extract), and the variations were represented as mean of cells (µ) and standard deviation (σ). a: represents the moment of injury; b: 24 hours after injury in the control group (µ = 31.55, σ = 3.71); c: 24 hours after injury in the group treated with 50 µg/ml (µ = 43.3, σ = 3.58) of C. xanthocarpa. *p-value = 0.042 compared to group c. Eight assays were performed.

C. xanthocarpa reduces expression of proteins involved with inflammatory process of atherosclerosis

To assess whether EG alters the expression of proteins involved with inflammatory process of atherosclerosis, we performed flow cytometry experiments over the same cells lines we used in the previous experiments: monocytic THP-1 and endothelial EA-hy926 (Figure 5).

In monocytic cells, EG at the concentration of 50 µg/ml significantly decreased (p-value = 0.02771) the percentage expression of CD14 protein (mean = 46.38%) (Figure 5c) when compared to the control group (mean = 56.51%) (Figure 5b). In these same cells, the percentage of ICAM-1 expression did not show significant alteration (p-value > 0.05) in the treated cells (Figure 6c) when compared to control cells (Figure 6b).

We then stimulated the monocytic cells by IFN-γ using the same concentration of 50 µg/ml of EG. We also observed significant decrease (p-value = 0.04484) in the percentage expression of CD14 protein (mean = 54.98%) (Figure 5e) when compared to the control group (mean = 65.13%) (Figure 5f). We also observed a significant decrease (p-value < 0.05) in the percentage expression of ICAM-1 protein (mean = 58.57%) (Figure 6f) when compared to relative controls (Figure 6e) (mean = 66.90%).

Next, we investigated similar experiments in endothelial cells incubated by EG at 50 µg/ml. The expression of the protein PECAM-1 did not show significant change (p-value > 0.05) in the treated cells (Figure 7c) when compared to control cells (Figure 7b). The percentage of the proteins CD14 (Figures 8b, 8c), HLA-DR and CD105 also did not show significant change in treated cells when compared to relative controls.

We then stimulated the endothelial cells by IFN-γ using the same concentration of 50 µg/ml of EG. We observed a significant decrease (p-value = 0.032) in the percentage of the protein PECAM-1 expression in treated cells (mean = 49.93%) (Figures 7e, 7f) when compared to relative controls (mean = 57.45%). Similarly, we also found a significant decrease (p-value = 0.036) in the percentage of expression of the protein CD14 in treated cells (mean = 11.92%) (Figure 8f) when compared to relative controls (Figure 8e) (mean = 20.84%). However, the percentage of the CD105 HLA-DR protein expression did not show significant change (p-value > 0.05).

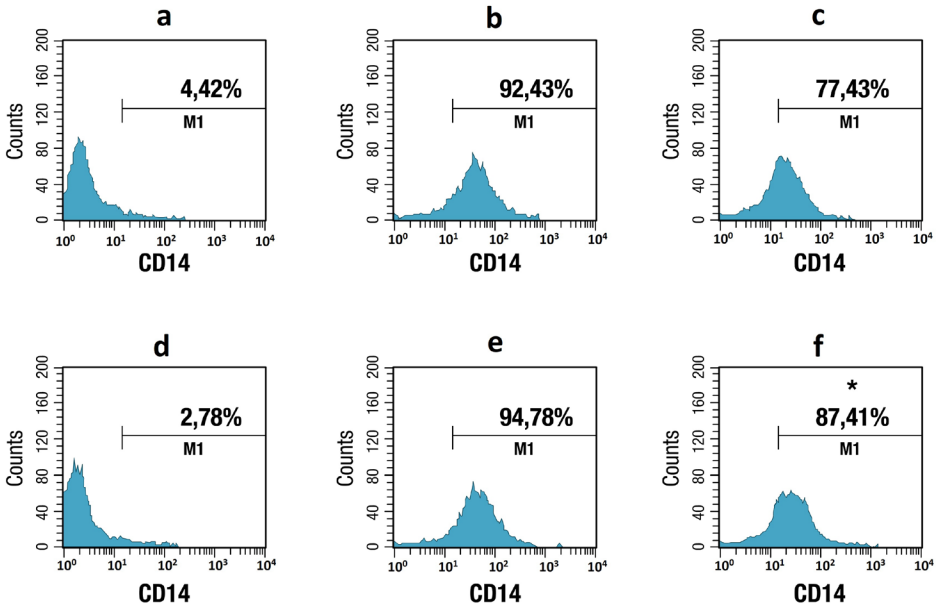

Figure 5. Immunophenotypic analysis of CD14 expression in cell lines (THP-1) treated with EG. Results are expressed as mean fluorescence (μ) and standard error ($\sigma_{\bar{x}}$). **a**: Isotype control; **b**: Expression of CD14 by THP-1 cells (μ = 56.51, $\sigma_{\bar{x}}$ = 6.73); **c**: Expression of CD14 by THP-1 cells in the presence of the extract of *C. xanthocarpa* at 50 μg/ml (μ = 46.38, $\sigma_{\bar{x}}$ = 7.64), *p-value = 0.018 compared to group **b**; **d**: Isotypic control stimulated with IFNγ; **e**: Expression of CD14 by IFNγ-stimulated THP-1 cells (μ = 65.15, $\sigma_{\bar{x}}$ = 8.03); **f**: CD14 expression by IFNγ-stimulated THP-1 cells and in the presence of the extract *of C. xanthocarpa* at 50 μg/ml (μ = 54.98, $\sigma_{\bar{x}}$ = 9.04), *p-value = 0.045 compared to group **e**. Six assays were performed, and 10,000 events were collected in each trial.

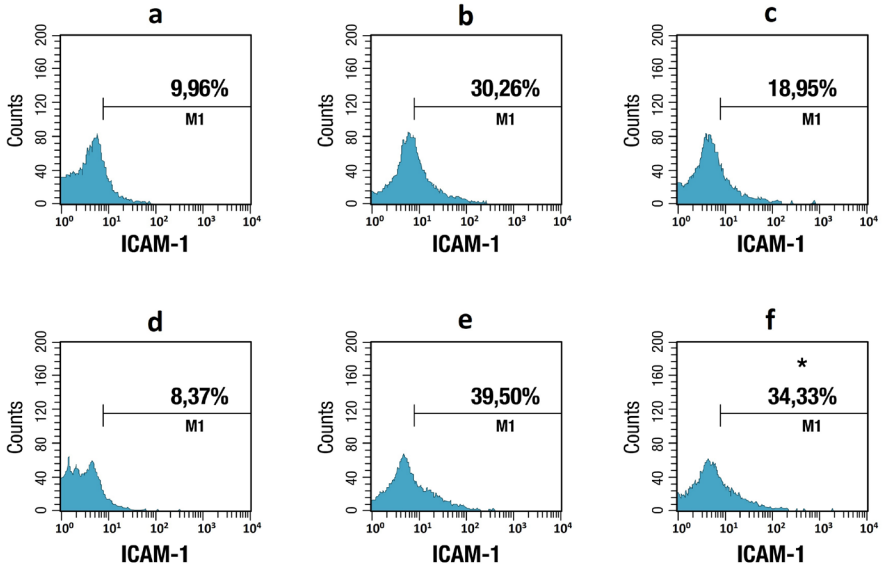

Figure 6. Immunophenotypic analysis of ICAM-1 expression in cell lines treated with EG. Results are expressed as mean fluorescence (μ) and standard error ($\sigma_{\bar{x}}$). **a**: Isotype control; **b**: Expression of ICAM-1 by THP-1 cells (μ = 30.26, $\sigma_{\bar{x}}$ = 12.95); **c**: Expression of ICAM-1 by THP-1 cells in the presence of the extract of *C. xanthocarpa* at 50 μg/ml (μ = 18.95, $\sigma_{\bar{x}}$ = 12.82); **d**: Isotypic control stimulated with IFNγ; **e**: Expression of CD54 by IFNγ-stimulated THP-1 cells (μ = 66.90, $\sigma_{\bar{x}}$ = 8.01); **f**: Expression of ICAM-1 by IFNγ-stimulated THP-1 cells and in the presence of the extract of *C. xanthocarpa* at 50 μg/ml (μ = 58.57, $\sigma_{\bar{x}}$ = 7.08), *p-value = 0.005 compared to group **e**. Six assays were performed, and 10,000 events were collected in each trial.

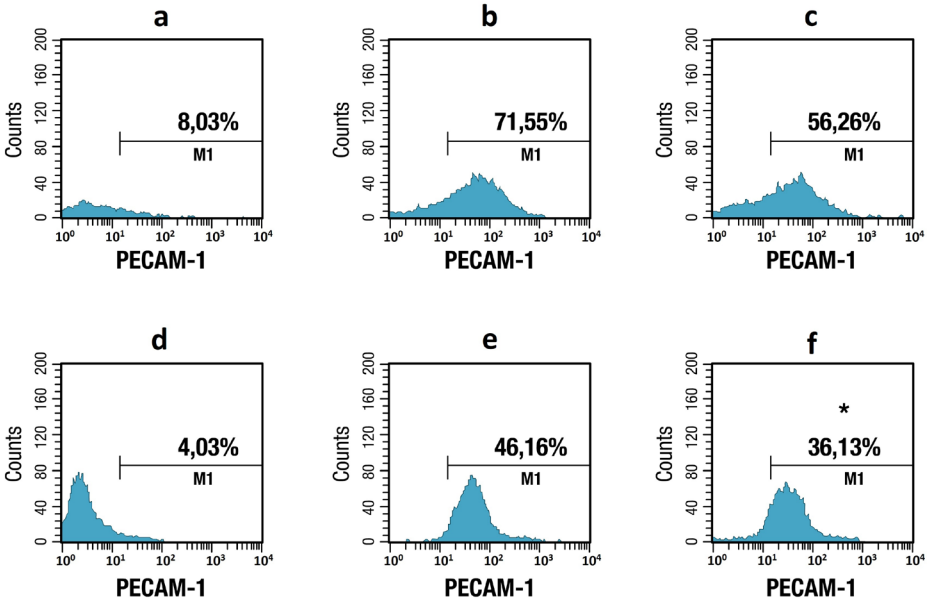

Figure 7. Immunophenotypic analysis of PECAM-1 expression in EG cell lines EA.HY926 treated with EG. Results are expressed as mean fluorescence (μ) and standard error ($\sigma_{\bar{x}}$). **a**: Isotypic control; **b**: Expression of PECAM-1 by EA.HY926 cells (μ = 46.12, $\sigma_{\bar{x}}$ = 9.79); **c**: Expression of CD31 by EA.HY926 cells in the presence of the extract of *C. xanthocarpa* at 50 μg/ml (μ = 40.32, $\sigma_{\bar{x}}$ = 8.34); **d**: Isotypic control stimulated with IFNγ; **e**: Expression of PECAM-1 by IFNγ-stimulated EA.HY926 cells (μ = 57.45, $\sigma_{\bar{x}}$ = 7.91); **f**: Expression of PECAM-1 by IFNγ stimulated cells EA.HY926 and in the presence of the extract of *C. xanthocarpa* at 50 μg/ml (μ = 49.93, $\sigma_{\bar{x}}$ = 6.10), *p-value = 0.032 compared to group **e**. Six assays were performed, and 10,000 events were collected in each trial.

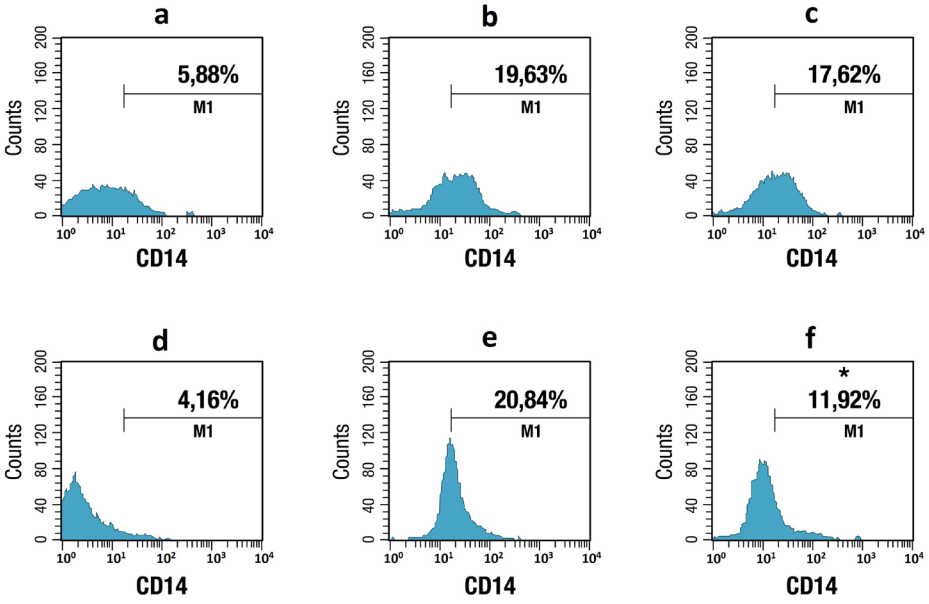

Figure 8. Immunophenotypic analysis of CD14 expression in EG-treated cell lines EA.HY926. Results are expressed as mean fluorescence (μ) and standard error ($\sigma_{\bar{x}}$). **a**: Isotype control; **b**: CD14 expression by EA.HY926 cells (μ = 19.63, $\sigma_{\bar{x}}$ = 7.97); **c**: Expression of CD14 by EA.HY926 cells in the presence of the extract of *C. xanthocarpa* at 50 μg/ml (μ = 17.62, $\sigma_{\bar{x}}$ = 6.69); **d**: Isotypic control stimulated with IFNγ; **e**: CD14 expression by IFN-γ stimulated cells EA.HY926 (μ = 20.84, $\sigma_{\bar{x}}$ = 6.49); **f**: CD14 expression by the IFNγ stimulated cells EA.HY926 and in the presence of the extract of C. xanthocarpa at 50 μg/ml (μ = 11.92, $\sigma_{\bar{x}}$ = 3.58), *p-value = 0.036 compared to group **e**. Six assays were performed, and 10,000 events were collect

Migration of endothelial cells are induced by *C. xanthocarpa*

The use of EG at the concentration of 50 µg/ml with cultured endothelial cells induced a significant increase (p-value = 0.042) in the cell migration (mean = 43.30%) when compared to the control experiment without use of the extract (mean = 31.55%), corresponding to an average of 1.5 fold increase. However, when EG concentration was increased to 100 µg/ml, no change in endothelial cell migration was observed compared to controls (Figure 4), 7 independent trials were performed.

DISCUSSION

In this work (Figure 1), we investigated how extracts of guavirova leaves (EG) can modulate cellular phenotypes that are correlated with the onset of the atherosclerotic process. We have demonstrated that EG significantly reduced ICAM-1 protein expression in monocytic cell lineages, and the reduction of PECAM-1 protein in endothelial cells when stimulated with IFN-γ. It has been previously reported that the decreased expression of ICAM-1 (Liu et al. 2017) and the unstable expression of PECAM-1 can increase the permeability of the endothelium as they act as key molecules for this phenotype (Liu et al. 2011, Woodfin et al. 2007). This observation was corroborated by a recent study that has revealed that PECAM-1 may have anti-inflammatory and proinflammatory roles in the cells (Malik et al. 2019). In addition to this process, the increase in vascular permeability is dependent on the binding of monocytes to endothelial cells, causing activation of the ICAM-1-mediated Src Kinase signaling protein (Liu et al. 2012).

The expression modulation of the PECAM-1 molecule may be one of the ways in which EG acts to promote the antithrombotic and fibrinolytic capacity that has previously been observed by *in vivo* experiments with mice (Klafke et al. 2012), and its antiplatelet property supported by *in vitro* experiments (Klafke et al. 2012, Otero et al. 2017). By modulating these molecules, we hypothesize that EG can act reducing leukocyte transmigration to the intima layer of a blood vessel.

In addition, we also detected that EG significantly reduced CD14 expression in the two cell lines tested (endothelial and monocytic), especially after stimulation with IFN-γ. Chavez-Sanchez and colleagues demonstrated that increases the activation of CD14 by mmLDL increases the secretion of IL-1 and IL-6, and after blocking CD14 these same molecules reduced their secretion (Chavez-Sanchez et al. 2010). Corroborated with these findings, Klafke et al. (2016b) observed that guavirova decreases serum levels of IL-1, IL-6 (Klafke et al. 2016b). Together, our data suggest that EG can also control the inflammatory process that is associated with the development of atherosclerosis and plaque formation.

Another important molecule in the context of the atherosclerotic process is CD105. In our experiments with endothelial cell lines, EG did not significantly interfere in the expression of CD105. Such transmembrane protein is one of the major glycoproteins expressed on the surface of endothelial cells and plays a key role by ensuring homeostasis of the cardiovascular system (López-Novoa & Bernabeu 2010). Recent evidences also show that CD105 plays an important role in platelet adhesion to the endothelium by interacting with integrins (Rossi et al. 2018). When this function is impaired, it may be related to abnormal bleeding and thrombosis (Rossi et al. 2018), and also could affect the vascular integrity, which is crucial to avoid the progress of atherosclerosis (Torres et al. 2015). In our data, the fact that EG did not

significantly affects the expression of CD105 (which is required by the molecules of the atherosclerotic-related inflammation process (de Vries et al. 2014, Rocha et al. 2016) in EG treated cells versus controls, might suggest that the EG is acting as a protective treatment to atherosclerosis.

Due to the importance of vascular endothelial integrity in atherosclerosis, we also investigated whether EG altered cellular migration. We demonstrated that EG promoted increased endothelial cell migration, and at a higher concentration did not cause significant changes. As previously described, endothelium integrity is extremely important to avoid atherosclerosis (Torres et al. 2015), and migration of endothelial cells is one of the key processes for maintaining vascular integrity (Vitorino et al. 2011). As previously demonstrated, in the same cell line used by this study (EA.hy926), atorvastatin promoted increased endothelial migration at concentrations up to 0.1 μM, but inhibited cell migration at higher doses (1 μM) (Korybalska et al. 2012). At low doses, statin-derived drugs, such as atorvastatin, can inhibit cholesterol synthesis but do not impair the synthesis of farnesyl and geranylgeranyl pyrophosphates, key intermediates in cell growth pathways. However, at high doses they inhibit synthesis of *prenyl* radicals, in addition to cholesterol synthesis, resulting in inhibition of cell migration and proliferation (Korybalska et al. 2012).

CONCLUSION

Together, our findings suggest that EG can directly modulate atherosclerotic-related molecules, protecting the cells against markers that are altered during the early onset of the disease. The presented results are also supported by the literature, and although additional experiments are required, they suggest that EG could be used as an alternative for hypercholesterolemia treatment (Figure 1).

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EDUARDO B.B. CUNHA[1,2]
https://orcid.org/0000-0002-0677-4061

NATÁLIA F. DA SILVA[3]
https://orcid.org/0000-0001-8122-5911

JEAN DE LIMA[4]
https://orcid.org/0000-0001-7223-914X

JULIA A. SERRATO[1]
https://orcid.org/0000-0002-7049-9498

CARLOS A.M. AITA[5]
https://orcid.org/0000-0003-4871-7740

ROBERTO H. HERAI[1,6]
https://orcid.org/0000-0001-9885-2735

[1]Programa de Pós-Graduação em Ciências da Saúde/PPGCS, Pontifícia Universidade Católica do Paraná/PUCPR, Escola de Medicina, Laboratório Experimental Multiusuário/LEM, Rua Imaculada Conceição, 1155, 80215-901 Curitiba, PR, Brazil

[2]Centro Universitário - UNIFACEAR, Av. das Araucárias, 3803, 83707-067 Araucária, PR, Brazil

[3]Divisão de Pneumologia do Instituto do Coração do Hospital das Clínicas/ HCFMUSP, Av. Dr. Enéas Carvalho de Aguiar, 44, 05403-900 São Paulo, SP, Brazil

[4]Universidade de São Paulo/USP, Instituto de Ciências Biomédicas/ICB-IV, Laboratório de Imunobiologia de Transplantes/LIT, Av. Prof. Lineu Prestes, 137, 05508-000 São Paulo, SP, Brazil

[5]Laboratório Diagnósticos Brasil/DB, Rua Manoel Ribas, 245, 83010-030 São José dos Pinhais, PR, Brazil

[6]Instituto Lico Kaesemodel/ILK, Av. Sete de Setembro, 5402, 80240-000 Curitiba, PR, Brazil

Correspondence to: **Roberto Hirochi Herai, Carlos Alberto Mayora Aita**
E-mail: roberto.herai@pucpr.br
E-mail: mayoracam@gmail.com

Author contributions

Eduardo B.B. Cunha and Carlos A.M. Aita conceived and planned the experiments. Eduardo B.B. Cunha, Natália F. da Silva, Jean de Lima and Julia A. Serrato carried out the experiments. Eduardo B.B. Cunha and Natália F. da Silva contributed to the preparation of the extract. Eduardo B.B. Cunha, Carlos A.M. Aita and Roberto H. Herai contributed to the interpretation of the results. Eduardo B.B. Cunha and Roberto H. Herai took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.