Anti-Angiogenic Properties of Cafestol and Kahweol Palmitate Diterpene Esters

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ABSTRACT

Epidemiological studies support the association of coffee-specific diterpenes, with various beneficial health effects. Although anti-antiangiogenic properties of free cafestol and kahweol have been recently described, available data regarding their esterified form, in particular palmitate esters as the main diterpene esters present in coffee, are still rare. Given that angiogenesis plays an important role in many pathological conditions, including cancer growth and metastasis, this study aimed to assess and compare the potential anti-angiogenic effects of cafestol palmitate (CP) and kahweol palmitate (KP) in an in vitro angiogenesis model. According to our findings, both compounds inhibited angiogenesis steps on human microvascular endothelial cells (HMVECs), although a more significant effect was observed for KP. Compared to control, HMVECs viability decreased in a dose-dependent manner upon incubation either with CP or KP. Concentrations of 75 and 100 μM o f each compound were cytotoxic. Cell proliferation was also dramatically reduced by both diterpene esters at 50 μM, although KP had a stronger inhibitory effect. However, CP and KP did not induce apoptosis on HMVECs. Both compounds reduced cell migration, but this effect was only statistically significant after KP incubation. Inhibition of VEGFR2 expression and its downstream effector Akt, but not Erk, was also observed in CP- and KP-treated HMVECs. These findings were confirmed using ELISA assay for phosphorylated (active) VEGFR-2. Taken together, these data indicate that both CP and KP can be considered potent compounds against angiogenesis-dependent disorders. Our findings further indicate that KP exerts more potent anti-angiogenic effects than CP, in most of assays.

KEY WORDS: ANGIOGENESIS; ESTERIFIED DITERPENES; COFFEE FAT-SOLUBLE COMPOUNDS; HMVEC

Angiogenesis, the generation of new capillaries from pre-existing ones, is an essential step involved in physiologic situations, such as embryo development, tissue growth, and wound repair [Costa et al., 2009; Wang et al., 2012]. It is a complex multistep process involving endothelial cell proliferation, extracellular matrix invasion, migration, anastomosis, and ending up by the recruitment of supporting cells [Wang et al., 2012]. On the other hand, angiogenesis may be regarded as a detrimental process as it plays an important role in many pathological conditions as well, particularly in tumor proliferation, expansion, and metastasis [Yang et al., 2006]. Providing nutrients and oxygen, as well as removing waste products through new vasculature, is necessary for further tumor growth [Gong et al., 2013]. The normal adult vasculature is quiescent as endothelial cells (ECs) division occurs

approximately every 10 years [Oronsky et al., 2012]. However, endothelial cells division in normal vessels may be induced by angiogenic signals, which allows degradative enzymes synthesis and release, followed by migration, proliferation, and differentiation of ECs [Rodriguez-Nieto et al., 2002]. In fact, tumor angiogenesis occurs due to the imbalance between stimulatory and inhibitory factors [Rodriguez-Nieto et al., 2002; Costa et al., 2009]. Therefore, preventing the expansion of new blood vessel networks is a way of reducing tumor size and metastases development [Lopes et al., 2009].

Although there is a huge list of proangiogenic factors described, vascular endothelial growth factor (VEGF) family members are considered surrogate markers of neovascularization. Accordingly, VEGFs are growth factors with angiogenic activities involved in the whole angiogenic process. They stimulate the proliferative, invasive and migratory capacities of ECs and vascular permeability by acting through Akt and Erk intracellular signaling pathways. Moreover, they are well-established ECs survival factors [Carneiro et al., 2009]. Due to the crucial role of VEGF on angiogenesis, several molecules were tested to develop anti-angiogenic therapy targeting these factors. Thus, compounds targeting VEGF ligands or receptors are used clinically in anti-angiogenic therapies [Tarallo and De Falco, 2015].

Several modulators of angiogenesis derived from natural plants have been reported in the literatures [Rodriguez-Nieto et al., 2002; Lopes et al., 2009; Cárdenas et al., 2011]. Among several compounds playing anti-angiogenic roles, diterpenes and diterpene esters are currently under development due to their potential impact on human health [Cavin et al., 2002] and their high intake through coffee consumption, mainly Turkish and Scandinavian boiled coffee [Gross et al., 1997]. Cafestol, kahweol, and their related esters are fat-soluble compounds that belong to the class of diterpenes. They are exclusively found in Arabica coffee, comprising up to 20% of coffee lipids [Kurzrock and Speer, 2007]. These compounds are known not only for their contribution to human health, but also for their application to discriminate between coffee species [Schievano et al., 2014]. Principally, diterpenes exist in coffee oil as monoesters on C_{17} as esterified diterpenes comprise almost 98% of total diterpenes content. Nevertheless, in quantitative point of view, palmitate esters are the predominant ones in Arabica coffees [Kurzrock and Speer, 2007; Erny et al., 2015], while free diterpenes occur as minor components (less than 3.5%) [Speer and Kölling-Speer, 2006].

Several studies, either in vitro or in vivo, support the inverse correlation between diterpenes consumption and risk of colon or liver cancer [Huber et al., 1997; Lee et al., 2007; Cavin et al., 2008]. Protection against carcinogens such as 7,12-dimethylbenz[a] anthracene (DMBA), 2-amino-l-methyl-6-phenylimidazo[4,5-b]-pyridine (PhIP), and aflatoxin B1 (AFB1) have been also documented [Lee and Jeong, 2007]. It has been also reported that cafestol and kahweol protect against induced genotoxicity through reduction of the expression of the activating enzymes as well as induction of the expression of the glutathione-S-transferase (GST) as detoxifying enzymes [Cavin et al., 1998]. Another approach is induction of phase II detoxifying enzymes which results in inhibition of formation or detoxification of electrophilic or oxidant metabolites. This process interfere the initiation of tumor formation through reduction of DNA damage [Muriel and Arauz, 2010]. Along with diterpenes and diterpene esters, coffee contains a variety of chemicals including caffeine and chlorogenic acids. It suggests that chemical component in coffee may play an important role in protecting against the occurrence and development of several disease. A study by Kim et al. [2010] showed that chlorogenic acids, known as an antioxidant, has anti-angiogenic effects on choroidal neovascularization. Caffeine also has antioxidant properties along with quenching effect on the production of hydroxyl radicals, as well as on oxidative DNA breakage [Azam et al., 2003].

According to the literature review, moderate coffee consumption or in another hand daily intake of ~2–3 cups of coffee appears to be safe and is associated with neutral to beneficial effects for most of the studied health outcomes [O'Keefe et al., 2013]. Furthermore, low or moderate coffee consumption were contribute to less risk for hepatic cirrhosis when compared with no coffee consumption [Liu et al., 2015]. Nevertheless, the possibility of coffee diterpenes for an elevation in plasma lipid profiles has been also reported which has been indicated to substantially depend on the method of brewing. Each extra 2 mg of cafestol consumed per day producing a 1 mg/dL increase in cholesterol [Weusten-Van der Wouw et al., 1994]. Several meta analyses revealed significant positive association between caffeinated or boiled coffee consumption and raise in serum cholesterol [Jee et al., 2001; Cai et al., 2012]. While paper-filtered coffee has been less frequently reported to raise serum cholesterol [Jee et al., 2001; Cai et al., 2012; Corrêa et al., 2013]. Therefore, during these last decades, the global view on the impact of coffee on health has been displaced from a mostly harmful balance toward a more beneficial profile [Cano-Marquina et al., 2013].

Although anti-carcinogenic properties of diterpenes have been extensively studied from a pharmacological point of view [Huber et al., 1997; Cavin et al., 2002; Cavin et al., 2003], less attention has been paid to their anti-angiogenic properties. Previous contributions have reported the antiangiogenic activity of free cafestol (in vitro) [Wang et al., 2012] and free kahweol in two in vivo and one ex vivo models of angiogenesis [Cárdenas et al., 2011]. Nevertheless, as previously mentioned, most of these compounds exist as esterified form (98%), not free moieties.

To the best of our knowledge, the anti-angiogenic properties of the main two diterpene esters present in coffee, namely cafestol and kahweol palmitate (Fig. 1) have not been reported elsewhere. Considering the lack of information about the angiogenesis properties of diterpene esters as promising candidates for the development of anti-angiogenic agents, we studied the ability of these two diterpene esters to inhibit angiogenesis response of endothelial cells.

Therefore, in the present study, we examined whether cafestol and kahweol palmitate could inhibit angiogenesis in an in vitro model. Cell viability, cell migration, proliferation, and apoptosis were assessed. VEGF signaling was then evaluated, confirming the VEGF-dependent anti-angiogenic properties of these two diterpene esters.

MATERIALS AND METHODS

CELL CULTURE

HMVECs were cultured in RPMI 1640 medium (Invitrogen Life Technologies, Paisley, UK) supplemented with 10% fetal bovine serum

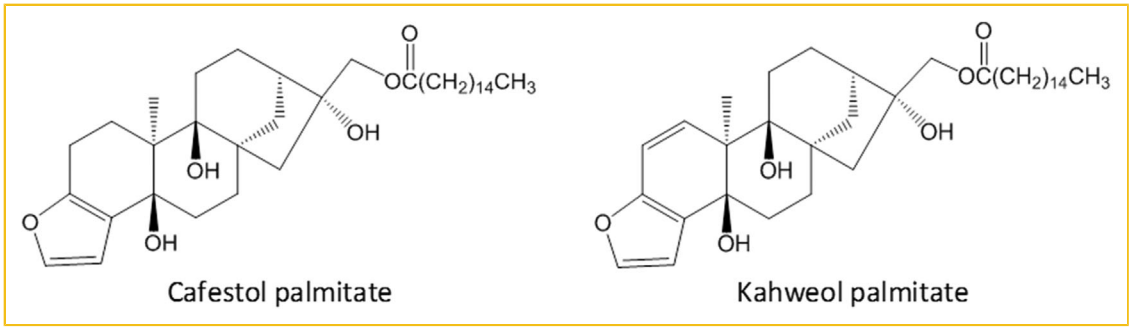

Fig. 1. Structural formula of the cafestol and kahweol palmitate [Erny et al., 2015].

(FBS) (Invitrogen Life Technologies), 1% penicillin/streptomycin (Invitrogen Life Technologies, Scotland, UK), 0.1% endothelial cell growth factor (EGF) (Sigma–Aldrich, Lisbon, Portugal), 1.176 g/L sodium bicarbonate (Merck, Darmstadt, Germany), 4.76 g/L HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Sigma–Aldrich), 0.001 g/L hydrocortisone (purity $\geq$ 98%—Sigma, Portugal).

Cafestol palmitate (CP) and kahweol palmitate (KP) were obtained from LKT Laboratories (St. Paul, MN). Stock solution of each compound (10 mM) was prepared in dimethyl sulfoxide (DMSO) and kept at −20°C. Stock solutions were diluted in 2% FBS cell culture medium to prepare distinct treatments, as the vehicle concentration (DMSO) never exceeded 1% (v/v) and had no significant effect on the assays. Control procedures were performed using medium containing 2% FBS. Except for cell viability assay (MTS) which was performed to identify the IC_{50}. The distinct treatment at 50 μM, both for CP and KP, was used in rest of assays.

MTS TOXICITY ASSAY

Cell viability was examined using the MTS assay according to Costa et al. [2009]. Briefly, HMVECs (1×10^5 cells/mL) are let to grow to 70–90% confluence. Then, cells were incubated with various concentrations of CP and KP (50, 75, 100 μM) as treatments and were incubated for another 24 h. Cell viability was determined using Cell Titer 96® Aqueous ONE Solution Reagent (MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] through MTS colorimetric assay at 492 nm according to the instructions provided by the manufacturer. Half maximal inhibitory concentration (IC_{50}) values were calculated and define as concentration of the drug that resulted in 50% inhibition in absorbance [Yang et al., 2006]. Experimental conditions were examined in triplicate. All experiments were also performed in duplicates. Results are expressed as percentage of control, which was considered to be 100%.

BrdU PROLIFERATION ASSAY

The HMVECs (1×10^5 cells/mL) were treated with CP or KP (50 μM) along with BrdU labeling solution (5-bromo-2′-deoxyuridine) at final concentration of 10 μM and incubated for 24 h. Detection was performed by ELISA Kit using anti-BrdU specific antibodies (Roche, Germany), according to the manufacturer's instructions [Machado et al., 2015]. The absorbance of the samples was measured against a background control (blank). Results were expressed as percentage of control (100%). Experimental conditions were tested in triplicate followed by duplicate repetitions of the whole experiment.

TUNEL ASSAY

HMVECs (6×10^4 cells/mL) were grown on glass coverslips and were treated with CP or KP at 50 μM concentration for 24 h. TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling) assay was performed as reported previously by Carneiro et al. [2009] using the In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Glass coverslips were visualized and photographed under a fluorescence microscope (Nikon, Tokyo, Japan) at a magnification of 200×. The percentages of TUNEL-stained nuclei were evaluated in relation to every DAPI-stained nuclei (4′,6-diamidino-2-phenylindole, DAPI) under fluorescence microscopy. Experimental conditions were performed in triplicate in two independent experiments.

MIGRATION ANALYSIS

Injury assay was performed as previously described [Costa et al., 2009]. The HMVECs (1×10^5 cells/mL) are let to grow until 90% confluence. After complete adherence, a single wound was scrapped in the center of the cell monolayers of each well. Subsequently, cells were incubated with respective treatment of CP or KP (50 μM) for another 24 h. To measure the migration of ECs to the damaged area, cells were photographed on a phase contrast microscope (Nikon, UK) at a magnification of 100×, immediately after wounding and after a 24 h incubation period (37°C, 5% CO_2). Inhibition percentage was expressed as percentage of the control as 100. Experimental conditions were repeated twice and tested in duplicates.

WESTERN BLOTTING ASSAY

Western blotting assay was performed as previously described [Machado et al., 2015]. Cells were grown to 90% confluence and were incubated with 50 μM CP or KP. Brifely, the HMVECs lysis was performed using RIPA buffer (Radio-Immunoprecipitation Assay) (Chemicon International, Temecula, CA) and proteins were separated by 8% SDS–PAGE and were transferred to Hybond nitrocellulose membrane (Amersham, Arlington, VA). Afterward, the membrane was incubated overnight at 4°C with primary antibodies including

total VEGFR-2 (Cell Signalling, Danvers, MA), total AKT, total Erk, and β-actin (Abcam, Cambridge, UK). The following day, the membrane was rinsed with TBST and incubation with secondary antibodies was accomplished at room temperature. Immunoreactive proteins were detected using the enhanced chemiluminescence detection system (ThermoFisher Scientific, Lisbon, Portugal).

ELISA ASSAY

The lysis of HMVECs treated with 50 μM CP or KP, was performed using RIPA buffer (Radio-Immunoprecipitation Assay) (Chemicon International) and proteins were separated by 8% SDS–PAGE. Subsequently, the levels of tyrosine-phosphorylated VEGFR-2 (pVEGFR-2) in HMVECs lysates were measured using a Duoset human phospho-VEGFR2 (Tyr1175) ELISA kit (R&D Systems, Abingdon, UK). The quantifications were performed in accordance to the manufacturer's instructions at 450 and 550 nm using a plate reader (Thermo Electron Corporation, Lviv, Ukraine). Results were expressed as percentage of control which was considered as 100%.

STATISTICAL ANALYSIS

All experiments were performed in duplicates. Quantifications are expressed as mean ± standard deviation (SD) of three independent experiments and expressed as percentage of control, which was considered to be 100%. A one-way ANOVA analysis was performed to evaluate differences among the groups followed by the Tukey multiple comparisons test. Differences were considered significant when $P \leq 0.05$. All statistical analysis was performed by Minitab 16 Software (Paris, France).

RESULTS

EFFECT OF CAFESTOL AND KAHWEOL PALMITATE ON HMVECs VIABILITY

We confirmed the HMVECs growth inhibitory effect of CP and KP using the MTS assay. The results indicated that HMVECs viability decreased after 24 h incubation with CP and KP in dose-dependent manner (Fig. 2). Incubation with 50 μM CP did not significantly affect the number of viable cells relative to control whereas 75 and 100 μM CP, significantly inhibited cell viability ($P \leq 0.05$ vs. control). Cell survival reduction was more pronounced after incubation with KP as HMVECs in all treatments were significantly less viable than those obtained for control (Fig. 2) as 75 and 100 μM treatments with KP were cytotoxic and dramatically inhibited the viability of HMVECs ($P \leq 0.05$ vs. control). Upon incubation with 100 μM KP, only 5% of cells remained viable, suggesting that KP exerted stronger inhibitory effects on HMVECs viability. In general, around 57% and 82% cell viability in relative to control was found upon treatment with 50 μM KP or CP, respectively. These findings prompted use a BrdU incorporation assay at 50 μM of CP and KP.

CAFESTOL AND KAHWEOL PALMITATE INHIBIT HMVECs PROLIFERATION

ECs proliferation is one of the main steps of angiogenesis [Wang et al., 2012]. As shown in Figure 3A, both compounds revealed significant anti-proliferative effects on HMVECs at concentration of 50 μM (Fig. 3A) as CP and KP treatments of HMVECs for 24 h, led to a decrease in the percentage of proliferating cells when compared to control group ($P \leq 0.05$). Although KP revealed higher inhibitory effects on proliferation of ECs, no statistical difference was found between CP and KP ($P \geq 0.05$). This result is compatible with MTS assay which showed higher inhibitory effect of KP on HMVECs viability.

CAFESTOL AND KAHWEOL PALMITATE DO NOT INDUCE APOPTOSIS ON HMVECs

We next examined whether 50 μM CP or KP enhanced HMVECs apoptosis by TUNEL assay. Both compounds exhibited apoptosis-inducing activity by 24 h, as compared with control, although the differences were not statistically significant ($P \geq 0.05$). As illustrated in Figure 3B and C, control cells revealed the lowest percentage of apoptotic cell number (6.0%), while the percentage of apoptotic cells upon CP incubation was less than those obtained upon KP treatment (mean values of 6.6% and 14.4%, respectively). These latter findings

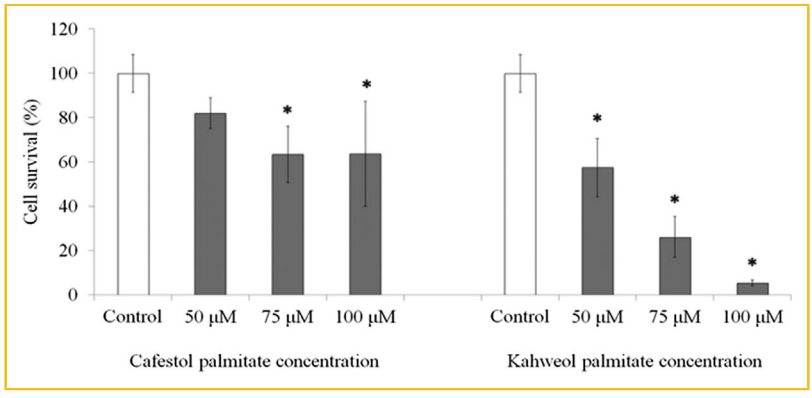

Fig. 2. Effect of CP and KP on confluent HMVECs cultures viability evaluated by MTS assay. HMVECs were seeded in 96-well plates and cultured in the presence or absence of various concentrations of CP and KP (50, 75, and 100 μM) for 24 h and viability of cells was determined by MTS assay. Incubation of cells upon treatment with CP and KP at concentration of 50 μM led to 82 ± 7% and 57 ± 13% cell viabilities, respectively. Results are expressed as percentage versus control as 100% viability. Depicted data are means of values ± SD. Assays were repeated three times and performed in duplicates, $^{*}P < 0.05$ versus control. CP, cafestol palmitate; KP, kahweol palmitate.

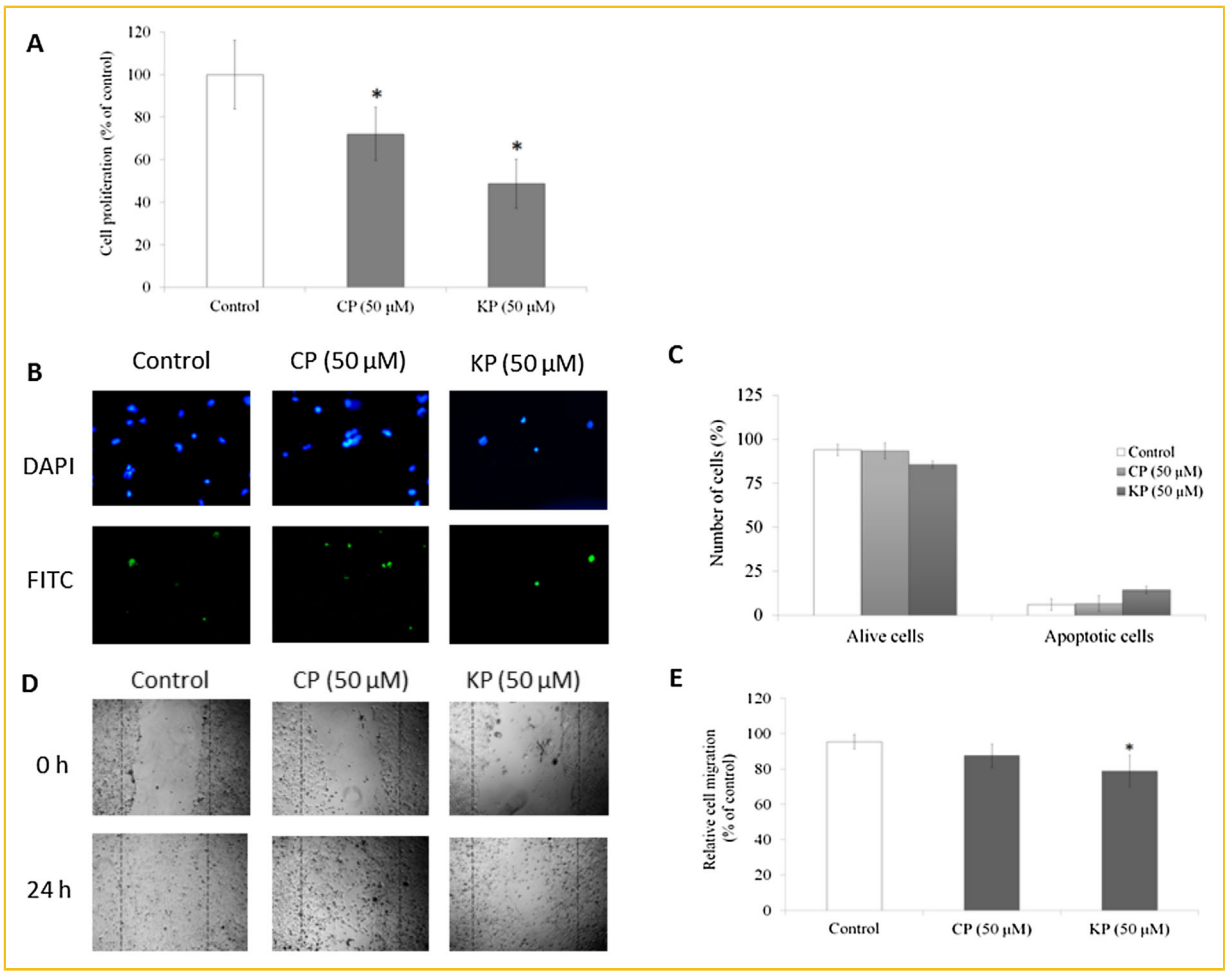

Fig. 3. Study of cell proliferation, migration, and apoptosis in HMVECs with the indicated concentration of CP and KP. (A) Cell proliferation was performed using BrdU assay at 50 μM of CP and KP for 24 h. Anti-proliferative effect was found for CP or KP at the concentration of 50 μM ($P \leq 0.05$ vs. control). There was no significant difference between the CP- and KP-treated groups ($P \geq 0.05$). (B) Effect of CP and KP on HMVECs apoptosis assessed by TUNEL assay at 50 μM of CP and KP. Fluorescent images (Magnification 200×) of cells treated with CP and KP versus control after 24 h incubation with treatments at 37°C under 5% CO_2. Green, apoptotic cells; Blue, Dapi-stained nuclei. (C) HMVECs incubation with CP and KP resulted in increased apoptosis after 24 h but in non-significant manner ($P \geq 0.05$ vs. control). (D) Effect of CP and KP on HMVECs migration at 50 μM of CP and KP. These compounds inhibited HMVECs migration in injury assay after 24 h incubation at 37°C under 5% CO_2, being significant in KP-treated HMVECs. (E) The inhibitory effect on HMVECs migration was found significant ($P \leq 0.05$ vs. control) only when ECs incubated with KP. Graphs are the mean ± SD values from at least two independent experiments, repeated three times, $^*P \leq 0.05$ versus control; CP, cafestol palmitate; KP, kahweol palmitate.

were compatible with the previous ones from BrdU proliferation assay (Fig. 3A). These findings suggest that CP and KP do not induce apoptosis on HMVECs, compare to control ($P \geq 0.05$).

KAHWEOL PALMITATE INHIBITS HMVECs MIGRATION

As shown in Figure 3D and E, 24 h treatment of HMVECs with CP and KP led to a decrease in migratory capacity at the concentration of 50 μM compared to control. However, this inhibitory effect was not significant for CP ($P \geq 0.05$) and cells were still able to migrate toward the damaged area (Fig. 3D and E). This decrease was more pronounced when HMVECs were incubated with KP ($P \leq 0.05$ vs. control). Comparison between the inhibitory effects of these two compounds revealed the higher effect for KP. Nevertheless, no significant difference of migration inhibition was observed between KP and CP ($P \geq 0.05$) (Fig. 3E).

CAFESTOL AND KAHWEOL PALMITATE SUPPRESS VEGFR-2 SIGNALING PATHWAY

According to our results, VEGFR-2 signaling pathway was significantly affected ($P \leq 0.05$ vs. control) in the presence of CP and KP at the concentration of 50 μM. As illustrated in Figure 4A and B, both compounds decreased VEGFR-2 expression, being CP the one with stronger effect. Treatment with CP and KP significantly inhibited the expression of Akt, a downstream effector of VEGFR-2 signaling pathway compare to control group ($P \leq 0.05$). However, both compounds were unable to affect Erk expression in HMVECs ($P \geq 0.05$) (Fig. 4A and B). To confirm these findings, an ELISA assay for phosphorylated (active) VEGFR-2 was performed. CP- and KP-treated HMVECs at the concentration of 50 μM displayed less phosphorylated VEGFR2, compare to control (Fig. 4C), confirming the less expression of VEGFR-2 in both treatments.

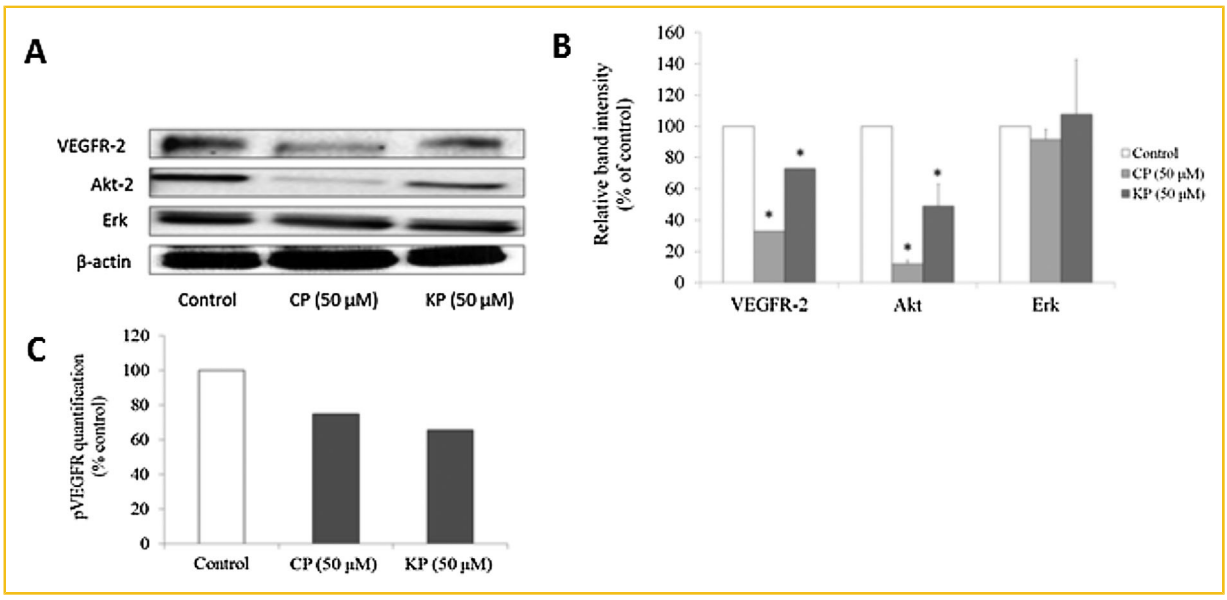

Fig. 4. Effect of cafestol and kahweol palmitate on VEGFR-2 signaling pathway. (A and B) Effect of incubation of HMVECs with CP and KP at concentrations of 50 μM on total VEGFR-2, Akt, and Erk expression was tested by Western blotting analysis. (A) Representative bands obtained after immunostaining are shown. (B) Quantification carried out through densitometry and the relative band intensity ratio after normalization with β-actin are shown compared to control as 100%. Total VEGFR-2 expression decreased in CP- and KP-treated cells. A significant reduction for Akt expression was also presented upon treatment with CP and KP. Erk immunostaining was not affected after incubation with compounds analyzed ($P \geq 0.05$ vs. control) (C) Phosphorylated VEGFR-2 (pVEGFR-2) in HMVECs after incubation with 50 μM CP and KP was measured by ELISA assay. Comparisons of the relative intensity of activated VEGFR-2 compare to control (as 100%) are shown. Both compounds inhibited the expression of activated VEGFR-2 in treated ECs. $^{*}P < 0.05$ versus control. CP, cafestol palmitate; KP, kahweol palmitate.

DISCUSSION

Availability of ECs and to some extent awareness about the angiogenesis process led to the development of in vitro assays to investigate natural biological active compounds. So, the present paper aimed to characterize and compare the effect of two bioactive compounds present in coffee on angiogenesis in an in vitro model.

Studying the biological effects of coffee diterpenes has gained substantial attention in the last decades. Several studies demonstrated that coffee-specific diterpenes, cafestol and kahweol, may diminish the incidence of certain types of cancer [Cavin et al., 2002; Cárdenas et al., 2014]. According to the literature, free cafestol and kahweol revealed anti-angiogenic properties in vitro and ex vivo models [Cárdenas et al., 2011; Kim et al., 2012; Wang et al., 2012]. Nevertheless, free diterpenes are found in small quantities (0.7–3.5%) in green and roasted beans [Kölling-Speer et al., 1999] and the anti-angiogenic properties of diterpene esters which compose up to 98% of diterpenes of coffee are not understood so far. Therefore, in the present work, HMVECs were treated with the main two diterpene esters namely CP and KP in a number of assays to test the potential anti-angiogenic properties of these compounds.

Angiogenesis plays a crucial role in a wide variety of diseases, including tumor growth and metastasis [Kim et al., 2012; Wang et al., 2012]. Accordingly, multi-target compounds are of clinical interest due to their ability to prevent different steps involved in angiogenesis [Cárdenas et al., 2011]. Our results showed that CP and KP are multitasking molecules that play a role in the regulation of angiogenesis processes via inhibition of several steps involved in angiogenesis, in particular the secretion of pro-angiogenic factor VEGF in HMVECs. Furthermore, we observed stronger inhibitory effects for KP than CP, in most assays.

We started the study of the anti-angiogenic properties of diterpenes by assessing the non-toxic concentration of these compounds on HMVECs. The study of cytotoxicity by MTS assay showed that CP has less toxicity on ECs than KP. Although cell viability decreased in comparison to control, at 50 μM CP it did not reach statistical significance. KP revealed an enhanced toxicity compared to control. Nevertheless, more than 50% of cells were alive after incubation for 24 h. These results indicate that upon treatment with 50 μM CP and KP, the HMVECs maintain their metabolic activity. Therefore, this concentration was used in the following experiments. When treated HUVECs with various concentration of pure kahweol, estimated IC_{50} values through MTT assay were 50 ± 1 and 147 ± 7 μM for proliferative and non-proliferative HUVECs, respectively [Cárdenas et al., 2011]. In another study, the IC_{50} value of the cafestol and kahweol after 48 h of incubation was estimated as 82.07 and 56 μM in human mesothelioma cells (MSTO-211H), respectively [Lee et al., 2012]. Regarding pure cafestol, treatment HUVECs up to 10 μM did not inhibited cell survival, whereas treatment with 20–80 μM, inhibited cell survival significantly, as compared with control [Wang et al., 2012]. However, as far as the authors know, there is no published work comparing the cytotoxic effect of diterpene esters, namely cafestol and kahweol palmitate on HMVECs.

Concerning cell proliferation, a stronger anti-proliferative effect of KP was observed in comparison with CP. It was clearly obvious that both compounds considerably inhibit HMVECs proliferation

versus the control group. The findings obtained for CP and KP are in agreement with the results of other authors who reported inhibition of ECs proliferation in the presence of free cafestol and kahweol [Cárdenas et al., 2011; Wang et al., 2012]. In another study, cafestol induced anti-proliferation in Caki cells during 24 h incubation at 30 and 40 M concentrations [Choi et al., 2011].

ECs, which constitute the inner layer of blood vessels, compose the blood vessels along with perivascular cells which surround them. To achieve new blood vessels, ECs must leave the vessel basement membrane through secretion of several proteases. This allows proliferative ECs to invade and migrate toward the source of the angiogenic stimulus [Bergers and Song, 2005]. ECs migration is a mechanically integrated molecular process that involves dynamic, coordinate changes in cell adhesion, signal transduction, and cytoskeletal dynamics and organization [Lamalice et al., 2007]. To investigate whether kahweol inhibits migration of HMVECs, migration assay was performed on CP- and KP-treated HMVECs. We demonstrated that the number of cells migrating toward the damaged area reduced significantly in KP-treated cells ($P \leq 0.05$). Previous study conducted by Wang et al. [2012], indicated that HUVECs migration was significantly inhibited by 6 h treatment with 10 and 20 μM cafestol. However, such a conclusion is not reached after incubation of HMVECs by CP. According to Cárdenas et al. [2011], treatment of HUVECs with 75 μM kahweol led to 30% and 60% inhibition in cell migration after 8 and 24 h of treatment, respectively. Investigation proposed by Kim et al. [2010] showed that in all kahweol-treated cancer cell lines with 1, 5, and 10 μM pure kahweol, the number of cells that was migrated to the lower chamber was reduced in a concentration-dependent manner, being statistically significant for 5 and 10 μM.

Although in our study, apoptosis did not play a major role in inhibition of angiogenesis, its role in reducing this process could not be ignored. Also, the anti-angiogenic effect of free kahweol without apoptosis among non-tumoral cells has been previously reported as 25 μM kahweol was not able to induce apoptosis in HUVECs [Cárdenas et al., 2011]. However, in another study conducted by Lee et al. [2012], the apoptotic cell death was increased in MSTO-211H as a result of incubation with cafestol (40 and 60 μM) and kahweol (60 and 90 μM) for 48 h.

Among pro-angiogenic agents, VEGF is the most potent and has a critical role in angiogenesis and can be accounted as the promoter for ECs growth, migration, and invasion [Costa et al., 2009]. VEGFR-2, which is expressed in most adult vascular ECs upon angiogenic stimuli, is considered the main transducer of VEGFA effects on endothelial cell differentiation, proliferation, migration, and formation of the vascular tube [Tugues et al., 2011]. VEGFR-2 undergoes autophosphorylation and the activated form (pVEGFR-2) triggering signaling pathways which is critical for pathological angiogenesis [Machado et al., 2015]. Accordingly, anti-angiogenic therapy is searching for molecules able to target VEGF ligands or receptors [Tarallo and De Falco, 2015]. We verified that CP and KP strongly suppressed the total VEGFR-2 and Akt expression compared to control. In contrast, exposure of HMVECs to CP and KP has no effects on total Erk expression during 24 h incubation. Our findings were corroborated by another study [Wang et al., 2012], which reported that Erk phosphorylation was not affected by free cafestol in HUVECs. Besides that, due to the crucial role of VEGF signaling pathway through the VEGFR-2 tyrosine kinase phosphorylation, inhibition of this pathway could be an effective approach against angiogenesis. Therefore, the presence of less activated VEGFR-2 (pVEGFR-2) in CP- and KP-treated HMVECs, suggests these compounds as VEGFR-2 inhibitors. The inhibitory effect of free cafestol on phosphorylation of VEGFR-2 in HUVECs at concentration of 20 μM was previously reported [Wang et al., 2012]. They concluded that free cafestol mainly influences signaling pathways downstream of VEGF subsequently leading to anti-angiogenesis [Wang et al., 2012]. Kim et al. [2012] reported that free kahweol inhibited VEGF secretion and VEGF promoter activity in treated human cancer cells. However, no inhibitory effect was observed on normal human epithelial cells. They suggested that kahweol mediates VEGF expression at the transcriptional level [Kim et al., 2012]. Kim et al. [2012] reported that the inhibitory effect of kahweol on cancer metastasis may contribute to inhibition of the expression and secretion of some MMPs (matrix metalloproteinases), inhibition of activity of STAT3 (signal transducer and activator of transcription), as well as inhibition of secretion of pro-angiogenic factor VEGF by cancer cells. However, such a report does not exist for cafestol. Therefore, kahweol may be a potential candidate for both preventive and therapeutic strategies [Kim et al., 2012].

In general, more recent reports have provided new insights into anti-angiogenic effects of kahweol and cafestol; however, the anti-angiogenic activities of CP and KP remain unclear. Investigation on pure cafestol showed that this compound could induce apoptosis in renal carcinoma Caki cells via reduction of mitochondrial membrane potential (MMP), activation of caspase 3, cytochrome *c* release, and down-regulation of anti-apoptotic proteins (Bcl-2, Bcl-xL, Mcl-1, and cFLIP) [Choi et al., 2011]. Likewise, Wang et al. [2012] concluded that cafestol inhibits angiogenesis of HUVECs via inhibition of proliferation, migration, and tube formation through several pathways including inhibition of phosphorylation of FAK and Akt and by a decrease in nitric oxide production. Similarly, kahweol exerted anti-angiogenic properties with inhibitory effects on ECs proliferation, migration, invasion, and tube formation on Matrigel [Wang et al., 2012]. The potential role of kahweol on remodeling of extracellular matrix by targeting MMP-2 and uPA has also been proved [Cárdenas et al., 2011]. Inhibition of metastasis by kahweol through the modulating expressions of MMPs and VEGF via STAT3 inactivation was also reported previously [Kim et al., 2012]. The higher reactivity of kahweol rather than cafestol may be attributed to the presence of one extra conjugated double bound in furan ring. Presence of this extra double bond increases the susceptibility of furan rings to electrophilic attack and subsequently oxidation [de Lucia et al., 2009]. Therefore, kahweol may be a more potent inhibitor of other molecules oxidation than cafestol. However, palmitate ester of cafestol (CP) had greater inhibitory effect on VEGFR expression than palmitate ester of kahweol (KP). This observation may suggest that chemical modification of cafestol to esterified form could improve its biological activities in some cases. According to Guo et al. [2008], incubation of endothelial progenitor cells (EPCs) with palmitic acid at concentrations of 0.2 μM or higher inhibited EPCs proliferation, migration, and tube formation in a dose-dependent manner via downregulation of Akt/eNOS signal

pathway. In another more recent study, Jiang et al. [2010] have demonstrated that palmitic acid could increase EPCs apoptosis in a dose- and time-dependent manner via p38 and JNK MAPKs pathways; however, Erk expression was not affected under treatment with palmitic acid. Since palmitic acid also has anti-angiogenic properties, esterification of cafestol and kahweol with palmitic acid tend to improve their properties. Therefore, probably different obtained results between CP and KP could be attributed to the presence of fatty acid in their molecular structures which may induce synergetic effects in some assays.

In conclusion, this study provides new insights into the anti-angiogenic properties of the main diterpene esters. This results support the potential application of these two biological active compounds against angiogenesis-dependent disorders. The present study demonstrated that CP and KP, as the main diterpene esters present in Arabica coffee are able to prevent several steps involved in angiogenesis. Prevention of VEGF release to HMVECs culture medium through suppression of Akt signaling pathway as well as their inhibitory effects on phosphorylated (active) VEGFR-2 was clearly indicated. Natural VEGFR-2 expression inhibitors such as CP and KP combined with other therapies may improve the efficiency and quality of cancer treatment or anti-angiogenic therapies. Our findings further indicate that in most cases, KP exerts more potent anti-angiogenic effects than CP. Although further studies, in particular, in vivo models are required to support these findings. Research targeting the action of these compounds on different types of cells is required, as well.

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