

Myricetin inhibits angiotensin converting enzyme and induces nitric oxide production in HUVEC cell line

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Abstract. Nitric oxide is known as relaxing factor because it acts as a vasodilator, increases blood flow, and inhibits platelet aggregation and adhesion, on the other hand nitric oxide can modulate cellular and physiological processes to limit oxidative injury, limiting processes such as leukocyte adhesion. As the complete mechanism of myricetin and its cardiovascular benefits is not completely understood, the aim of this study was to investigate the antihypertensive activity of myricetin in human umbilical vein endothelial cell (HUVEC). Angiotensin converting enzyme (ACE) activity, nitric oxide production, reactive oxygen species (ROS) scavenger activity, cellular calcium concentration, and endothelial nitric oxide synthase (eNOS) activity and protein expression was investigated in HUVEC treated with different concentration of myricetin (1–60 μ M). Myricetin increased nitric oxide production in HUVEC through decreased ROS levels and increased nitric oxide production and eNOS activation. Activation of eNOS enzyme was achieved by an increase of cellular calcium concentration. At the same examined concentration of myricetin, the activity of ACE was significantly inhibited. These findings indicate that myricetin may be helpful for lowering blood pressure; this could be achieved through dietary intervention or by the production of new antihypertensive treatments from a natural product.

Key words: Angiotensin converting enzyme — Antihypertensive activity — Endothelial nitric oxide synthase — HUVEC cell line — Myricetin

Abbreviations: ACE, angiotensin converting enzyme; eNOS, endothelial nitric oxide synthase; HUVEC, human umbilical vein endothelial cell; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species.

Introduction

High blood pressure is a major risk factor for some chronic diseases such as stroke, renal disease, and cardiovascular disease (Mittal and Singh 2010). In patients with hypertension, angiotensin II is produced from angiotensin I by the catalytic effect of angiotensin converting enzyme (ACE) in higher amounts than normal (Fuchs et al. 2008; Gowrisankar and

Clark 2016). Angiotensin II acts as vasoconstrictor, increasing blood pressure. For this reason, inhibition of ACE activity is a pharmacological target for the treatment of hypertension (Li et al. 2017). The ACE enzyme has a Zn^{2+} ion in its two active sites (Wang et al. 2011). Substrate binding and catalysis of ACE indicate the mechanism of Zn^{2+} ion (Bernstein et al. 2018). One of the mechanisms of ACE inhibitors is the ability to bind the Zn^{2+} ion (Braun and Rosenfeldt 2013). High and unregulated production of reactive oxygen species (ROS) is another risk factor for heart disease (Dong et al. 2019; Nanayakkara et al. 2019). In endothelial cells, enzyme systems involving NADPH oxidase, xanthine oxidase, and the mitochondrial respiratory chain are responsible for ROS

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production (Burtenshaw et al. 2017). ROS production should be regulated or adverse effects like oxidation will be cause to cell macronutrients such as proteins, lipids, and nucleic acids. Balance in ROS concentration can be achieved by the antioxidant system that exists in the human body and by antioxidant supplement (Bolisetty and Jaimes 2013). Moreover, ROS have the ability to oxidize nitric oxide produced from endothelial cells, leading to endothelial dysfunction and the initiation and development of cardiac disease (Cervantes-Gracia et al. 2017; Khaddaj et al. 2017).

Nitric oxide is known as relaxing factor because it acts as a vasodilator, increases blood flow, and inhibits platelet aggregation and adhesion. On the other hand, nitric oxide can also abate the oxidation chemistry mediated by reactive oxygen species such as H_2O_2 and O^{2-} that occurs at physiological levels of nitric oxide. In addition to the antioxidant chemistry, nitric oxide protects against cell death mediated by hydrogen peroxide, alkylhydroperoxides, and xanthine oxidase. The attenuation of metal/peroxide oxidative chemistry, as well as lipid peroxidation, appears to be the major chemical mechanisms by which nitric oxide may limit oxidative injury to mammalian cells. In addition to these chemical and biochemical properties, nitric oxide can modulate cellular and physiological processes to limit oxidative injury, limiting processes such as leukocyte adhesion (Maiuolo et al. 2019). Calcium-dependent endothelial nitric oxide synthase (eNOS) is one of the important factors responsible for the production of nitric oxide in endothelial cells. Increased levels of nitric oxide in the endothelial cells are often due to the increased protein expression of the eNOS enzyme or by scavenging the ROS produced within the cells (Shu et al. 2015).

Several research groups have reported a significant interaction between biological systems and dietary polyphenols from vegetables and fruits. Six flavonoids namely apigenin, luteolin, kaempferol-3-O- α -arabinopyranoside, kaempferol-3-O- β -galactopyranoside, quercetin-3-O- α -arabinopyranoside and luteolin-7-O- β -glucopyranoside were isolated from *Ailanthus excelsa* (Roxb) in a study conducted by Loizzo et al. (2007). The ACE inhibition activities

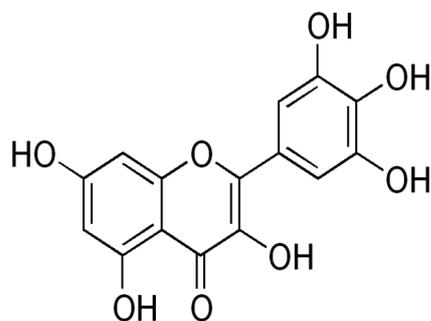


Figure 1. Chemical formula of myricetin.

of those flavonoids were tested. According to the results, all flavonoids displayed ACE inhibitory activity but the most active flavonoid was kaempferol-3-O- β -galactopyranoside with an IC_{50} value of 260 μ M. Furthermore, Balasuriya and Rupasinghe (2011) reported that quercetin, quercetin-3-glucoside, quercetin-3-galactoside, cyanidin-3-galactoside showed ACE inhibitor activity with the IC_{50} values of 151 μ M, 71 μ M, 180 μ M, 206 μ M, respectively.

Myricetin (3,3',4',5',7-hexahydroxyflavone) is a common natural flavonoid found in many fruits, vegetables, and herbs (Fig. 1). Myricetin plays a substantial role in the treatment and prevention of some diseases due to its potent iron-chelating capability, antioxidant and free-radical scavenging activities (Yao et al. 2014). It has been also used to treat inflammatory diseases and hypotensive with a variety of pharmacological functions such as anti-inflammatory, analgesic, anti-tumor activities and protecting liver, showing a high potential value for therapeutic application. In addition, recent researches indicated that myricetin can significantly depress the level of pro-inflammatory cytokines such as interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) by down regulating the activation of the Akt, mTOR and NF- κ B pathways, alleviating inflammation reaction to offer protective effect on various organs (Lee da and Lee 2016).

As the complete mechanism of myricetin and its cardiovascular benefits is not completely understood, the aim of this study was to investigate the effect of myricetin on ACE activity, nitric oxide production, ROS scavenger activity, cellular calcium concentration, and eNOS protein expression in human umbilical vein endothelial cell (HUVEC).

Materials and Methods

Materials

HUVEC cell line was purchased from American Type Culture Collection (ATCC[®] PCS-100-010). Myricetin was commercially obtained from Sigma-Aldrich, USA (Catalog No: M6760). All cell culture chemicals were bought either from Sigma-Aldrich or Merck. Antibody eNOS and β -actin mouse mAb were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All the other chemicals were bought either from Sigma-Aldrich, Merck or other standard suppliers.

Cell culture

The cells were seeded in DMEM with high glucose content (4.5 g/l), and supplemented with 10% FBS and 5% penicillin. Different myricetin concentrations (1–100 μ M) were tested on HUVEC cells to measure the cell viability and nitric oxide

production. Briefly, a stock solution of myricetin (10 mM) was prepared in DMSO. HUVEC cells were seeded into 96 well tissue culture plates at a density of 1×10^4 cells/200 μ l DMEM serum media. Once the cells reached 60–70% confluence, they were treated with myricetin for 12, 24, or 48 h (Do et al. 2015).

Nitric oxide assay

Nitric oxide levels in the cell supernatant were measured by Griess kit, at each experimental time (12, 24, or 48 h) in all groups. According to the Griess kit instructions, 100 μ l of medium supernatant was mixed with 100 μ l Griess reagent (0.1% N-naphthylethylenediamine dihydrochloride in 5% phosphoric acid and 1% sulfanilamide, mixed in a 1:1 ratio). The formation of a yellow colour was measured at 550 nm using a VersaMax Microplate Reader; the colour intensity was proportional to nitrite concentration. A sodium nitrite standard curve was obtained using a sodium nitrite standard solution supplied with the kit (Bredt et al. 1994).

Cell viability assay

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, at each experimental time (12, 24, or 48 hours). Cells pretreated with different concentrations of myricetin were incubated with 20 μ l of MTT dye (5 mg/ml PBS) at 37°C and 5% CO₂ to measure cell viability after different incubation times. At the end of each experimental time, the culture medium was aspirated and 100 μ l of DMSO was added to each well. The formazan crystals produced were dissolved completely by shaking the plate for 30 s at room temperature. The purple colour produced was measured at 492 nm using a VersaMax Microplate Reader (Mosmann 1983).

ACE activity assay

As a result of a meaningful reduction in cell viability after incubation for 48 h, 24 h incubation was performed in the following stages of the current study. Angiotensin converting enzyme activity was measured in the HUVEC cell line after 24 h of exposure to different concentrations of myricetin (1, 20, 40 and 60 μ M) using a modified fluorometric method (Kapiloff et al. 1984). Briefly, HUVEC cells were seeded to confluence in 25 cm² flasks (1×10^6 cells/ml) and then incubated with myricetin (1, 20, 40 and 60 μ M) for 24 h. Treated cells were washed three times with 3 ml Hank's Balanced Salt Solution (HBSS) buffer. The cells were scraped from the flask with 1 ml HBSS and frozen at –20°C until assayed. For the assay, frozen cells were thawed and sonicated then 20 μ l samples were added to 80 μ l of H-H-L (5 mM prepared in HBSS buffer) and incubated at 37°C for 3 h. The incubated

cells were then mixed with 1.4 ml NaOH (0.5 N) to stop the reaction. The fluorescent dye, o-phthaldialdehyde (100 μ l of 10 mg/ml in methanol) was used to detect the histidyl-leucine reaction product. The reagents were incubated for 5 min at room temperature followed by the addition of 250 μ l of HCl (6 N). A spectrofluorimeter (Hitachi F-7000, Berkshire, U.K.) was used to measure the fluorescence of the samples using an excitation wavelength of 365 nm and an emission wavelength of 495 nm. Fluorescence intensity is directly proportional to ACE activity, whereas it is inversely proportional to ACE inhibition. ACE activity was expressed as pg/ml, and the percentage of ACE inhibition was calculated. Captopril (1 μ M) was used as positive control in place of myricetin (Kapiloff et al. 1984).

Measurement of ROS

Dichlorofluorescein dye (non-fluorescent CM-H₂DCFDA) has the ability to diffuse through the cell membrane. In the cytoplasm, this dye is hydrolysed enzymatically by intracellular esterases and rapidly converted to fluorescent dye (DCF) in the presence of ROS. The fluorescence intensity is proportional to the ROS content (Osseni et al. 1999). The ROS level in the HUVEC cell line was determined by flow cytometry, using CM-H₂DCFDA dye after incubation with 1, 20, 40, and 60 μ M myricetin. HUVEC cells were seeded in 25 cm² flasks at 1×10^6 cells/ml. The treatments were added when cells reached the desired cell confluence (60–70%). After 24-h incubation time, half of pre-treated cells were exposed to 10 nM angiotensin II for 1 h, while the other left untreated. The cells were trypsinised and centrifuged for 3 min at 150 \times g. The supernatant was removed, the pellets washed with 2 ml of HBSS buffer and centrifuged. The supernatant was discarded and the cells re-suspended in 1 ml HBSS. Re-suspended cells were then incubated with 5 μ M CM-H₂DCFDA (prepared in DMSO) for 30 min at 37°C and 5% CO₂. At the end of the incubation time, cells were kept on ice under low light conditions due to the high susceptibility of the dye to photo-oxidation. Dichlorofluorescein fluorescence was then measured using a flow cytometer (FACSCalibur, Becton Dickinson, USA). At least 10,000 events were acquired in the gated regions at 488 nm excitation and 532 emission (Osseni et al. 1999).

Determination of cellular calcium concentration

Intracellular calcium concentrations were measured by fluorometry after loading the cells with fura-2/AM dye. Free intracellular calcium will bind with the membrane diffusible fluorescent dye fura-2/AM (Gryniewicz et al. 1985). Briefly, HUVEC cells were cultured in 25 cm² flasks at 1×10^6 cells/ml concentration. Different concentrations of myricetin (1, 20, 40 and 60 μ M) were incubated with the cells after they

reached 60–70% confluence. Following 24-h incubation, cells were loaded with 5 μM fura-2/AM dye and incubated for 45 min at 37°C and 5% CO_2 . Consequently, the dye was removed and the cells were washed and scraped from the flask with 2 ml HBSS buffer. The calcium concentration was detected by spectrofluorimetry (Hitachi F-7000, Berkshire, U.K.) with excitation and emission wavelengths of 340 nm and 510 nm, respectively. Intracellular calcium concentration was expressed as nM. Ethyleneglycoltetraacetic acid (EGTA) at 1 mM was used as negative control instead of myricetin (Grynkiewicz et al. 1985).

eNOS activity assay

Cells treated with different concentrations of myricetin (1, 20, 40 and 60 μM) for 24 h were lysed, and frozen at -20°C until eNOS activity was measured. 1 mM EGTA was used as a negative control in place of myricetin. eNOS activity was measured using a nitric oxide synthase assay colorimetric kit (Bioassay System, Hayward, CA, USA) (Catalog No: ENOS-100) using a direct and non-radioactive method. According to the manufacturer's instructions, this kit is designed to measure eNOS activity in two steps. The first step is an oxidation reaction by an eNOS enzyme that oxidises the amino acid arginine to nitric oxide. The second step is detection of nitric oxide *via* a reduction reaction. Nitric oxide is rapidly oxidised to nitrite and nitrate after production, thus the reduction of nitrate to nitrite by Griess method was used to measure nitric oxide production. The yellow colour produced was measured at 540 nm using a VersaMax Microplate Reader. A nitric oxide synthase standard curve was obtained using a eNOS standard solution supplied with the kit.

eNOS protein expression assay by Western blot

HUVEC cells at a density of 1×10^6 cells/ml were seeded in 25 cm^2 tissue culture flasks. Once 50% confluence was achieved, cells were treated with 1, 20, 40 and 60 μM myricetin and incubated for 24 h. After this incubation, the culture media was decanted and cells were washed with 5 ml PBS. The cells were then trypsinized and the resulting cell suspension centrifuged at $1500 \times g$ for 3 min with 5 ml PBS to wash. Supernatants were removed and the cell pellets were lysed by adding 300 μl of lysis buffer. The last step for cell lysis was to keep the cells on ice for 20 min and then store them at -80°C until protein determination and Western blot experiment were carried out. Total protein concentrations of the supernatant were determined by Pierce™ BCA Protein Assay Kit (Thermo Scientific Pierce).

An equal amount of total protein (20–50 μg) was loaded into sodium dodecyl sulphate-polyacrylamide gel electrophoresis and separated by electrophoresis under 80–100 V.

After electrophoresis, the protein bands were transferred to polyvinylidene difluoride (PVDF) membrane under 100 V for 1 h in cold water bath. Then, the PVDF membranes were blocked with phosphate-buffered saline (PBS) containing 5% nonfat milk or BSA for 1 h and incubated with the primary antibodies against eNOS (1:1000) and β -actin (1:1000) overnight at 4°C. The PVDF membrane was washed 3 times with phosphate-buffered saline containing 0.05% Tween-20 (PBST) and incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody (1:10000 dilution) at 37°C for 1 h. The PVDF membranes were washed 3 times with PBST again and labeled with the enhanced Pierce™ Fast Western blot Kit (Thermo Scientific Pierce) for 1 min at room temperature in dark. Finally, the PVDF membranes were exposed to the imaging system (ImageJ software, National Institutes of Health, Bethesda, MD, USA) to capture the light signals of protein bands. The eNOS protein level was expressed as a relative value to that of β -actin.

Statistical analyses

All experiments were performed at least in triplicate. For the 96-well microtiter tissue culture plates, 4 replicate wells were used *per* category. The data were analyzed by SPSS statistics software (version 15.0, SPSS Inc, Chicago, IL, USA). For significant differences between control and experimental values, the *p* value between groups was determined by one-way analysis of variance followed by Tukey's test. The significance level was set at $p < 0.05$.

Results

Cell viability and nitric oxide production was measured over a time course to determine the optimal experimental exposure time. The data in Fig. 2A shows that cell viability increased significantly ($p < 0.05$) after treatment with 1, 20, 40, and 60 μM myricetin for 12 h, but that nitric oxide production is similar compared with the untreated control (Fig. 2B). In contrast, there was a significant decrease in cell viability at 80 and 100 μM myricetin concentrations ($p < 0.001$) with a corresponding significant decrease in nitric oxide production at 12 h ($p < 0.001$) (Fig. 2A and B). After 24-h incubation time with the same range of myricetin concentrations, cell viability and nitric oxide production were significantly increased ($p < 0.05$) compared with untreated cells as shown in Fig. 2A and B. As for the 12-h incubation, concentrations of 80 and 100 μM myricetin caused a significant decrease in cell viability and nitric oxide production compared with untreated cells ($p < 0.05$). After 48-h incubation significant decreases in cell viability ($p < 0.05$) and nitric oxide production ($p < 0.001$) were observed at

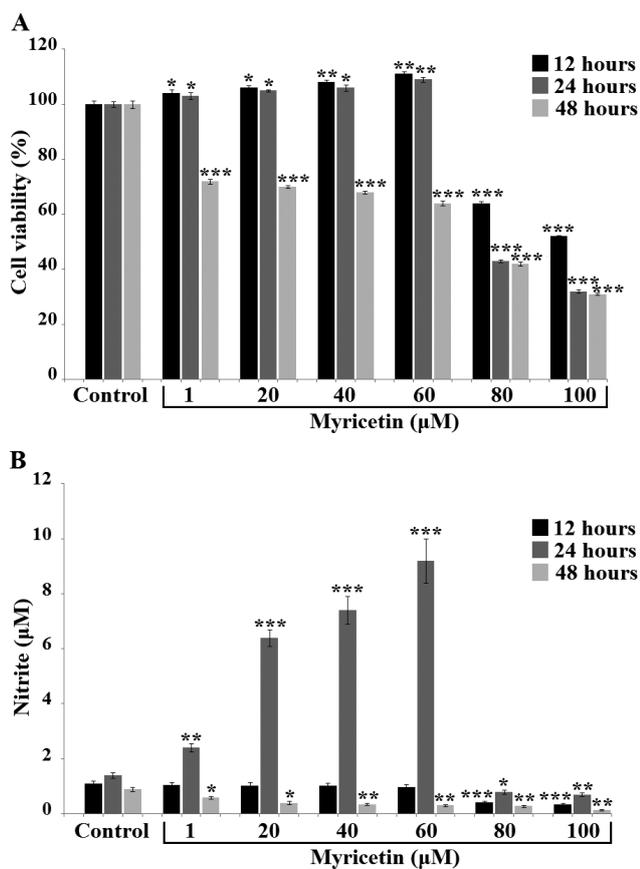


Figure 2. Effect of different concentrations of myricetin (1–100 µM) on the cell viability (A) and nitric oxide production (B) of HUVEC. Data represented as mean ± SD of three individual experiments. Comparisons of mean values were made using a one-way ANOVA followed by Tukey’s test. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$ versus Control.

all myricetin concentrations (1, 20, 40, 60, 80 and 100 µM) (Fig. 2A and B). Based on the previous cell viability and nitric oxide production results, myricetin concentrations in the range 1–60 µM were selected for exposure to cells for 24 h in future experiments because higher myricetin concentrations 80–100 µM produced a toxic effect on HUVEC cells (Fig. 2A and B).

In this study, incubation of HUVEC with different concentrations of myricetin (1, 20, 40 and 60 µM) for 24 h caused significant and dose-dependent inhibition of ACE activity compared with the control ($p < 0.001$) (Fig. 3).

ROS production in the HUVEC cell line, treated with different concentrations of myricetin for 24 h, in the absence of angiotensin II stimulation, was not significantly different to ROS production in cells not treated with myricetin ($p > 0.05$) (Fig. 4). The ability of different concentrations of myricetin added to HUVEC cells for 24 h to reduce ROS production was assessed by flow cytometer analysis after

treating cells with 10 nM angiotensin II for 1 h. The cells treated with angiotensin II showed a significant decrease in fluorescence intensity as the concentration of myricetin increased compared with cells treated with angiotensin II alone as a positive control ($p < 0.001$) (Fig. 4).

Different concentrations of myricetin (1, 20, 40 and 60 µM) exposed to HUVEC cell line for 24 h caused a significant dose-dependent increase in intracellular Ca^{2+} concentration ($p < 0.001$) (Fig. 5).

The effects of myricetin at different concentrations (1, 20, 40, and 60 µM) on activation of the eNOS enzyme were determined in HUVEC cells after a 24 h exposure time. As shown in Fig. 6, myricetin caused a significant increase in eNOS enzyme activity that was dose-dependent ($p < 0.05$).

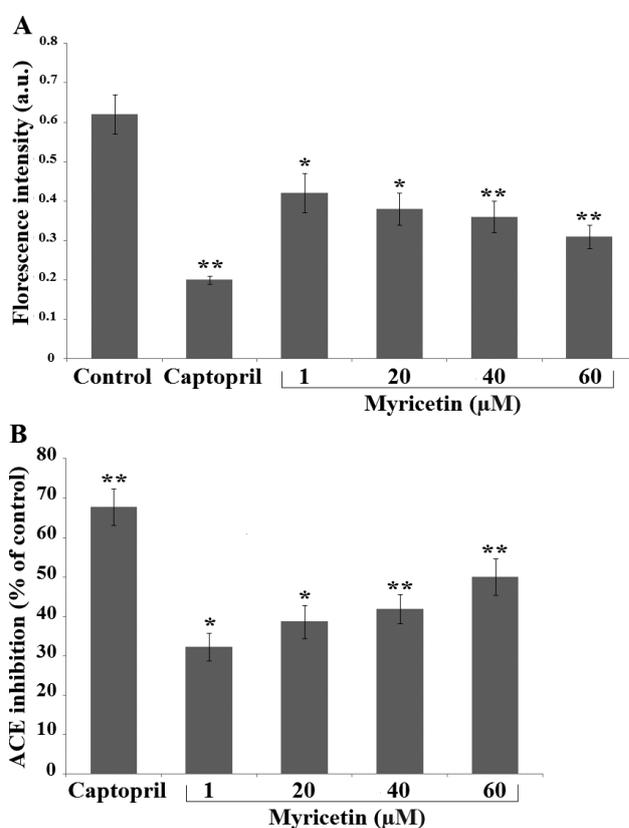


Figure 3. ACE activity in HUVEC exposed to myricetin (1–60 µM) for 24 h. **A.** Fluorescence intensity of negative control (untreated cell), positive control (1 µM captopril) and cells treated with different myricetin concentrations. The fluorescent intensity is proportional to enzyme activity. **B.** ACE inhibition activity for positive control and treated cells with different myricetin concentrations in relative to negative control (untreated cell). Data represented as mean ± SD of three individual experiments. Comparisons of mean values were made using a one-way ANOVA followed by Tukey’s test, * $p < 0.05$, ** $p < 0.001$ versus Control. ACE, angiotensin converting enzyme.

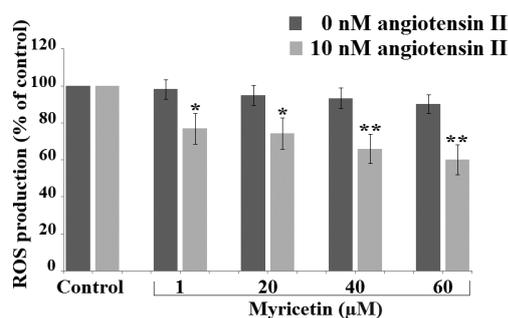


Figure 4. ROS production in HUVEC treated with different myricetin concentrations (1–60 μM) with/without 10 nM angiotensin II pre-treatment for 1 h. ROS production in the control group was considered as 100% and relative ROS production percentages of the other groups were expressed according to the control group. Data represented as mean \pm SD of three individual experiments. Comparisons of mean values were made using a one-way ANOVA followed by Tukey's test. * $p < 0.05$, ** $p < 0.001$ versus Control. ROS, reactive oxygen species.

Western blot analysis using a specific antibody against eNOS revealed no change in eNOS protein expression in HUVEC cells treated with myricetin compared with untreated control cells (Fig. 7). The calcium concentration for 1 mM EGTA was significantly decreased ($p < 0.05$); therefore, eNOS protein expression level was down regulated and eNOS enzyme activity decreased.

Discussion

Major research indicates that polyphenols have the ability to reduce the risk of cardiac disease such as atherosclerosis as well as hypertension (Persson et al. 2006). A wide concentration range of myricetin (1–100 μM) was examined using an exposure time of 24 h on HUVEC cells. At concentrations of 1–60 μM , no cytotoxicity was observed, while nitric oxide production increased dose-dependently. In contrast, at 80 and 100 μM myricetin, a reduction in cell viability and consequently reduction in nitric oxide was observed, indicating toxicity at high levels of myricetin on HUVEC cells. The results of several studies investigating the effect of polyphenol to increase the bioavailability of nitric oxide were consistent with our findings. EA.hy296 cell line incubated with different concentrations of red wine polyphenol extract (100–600 $\mu\text{g}/\text{ml}$) for 18 h produced a significant increase in nitric oxide level (Leikert et al. 2002). Uguşman et al. (2014) showed that rutin administration at concentrations of 300 μM significantly increased the level of nitric oxide after being added to HUVEC cells for 24 h. Nitric oxide level was also significantly increased in a dose-dependent manner after incubating the HUVEC cell line for 8 h with quercetin (Jones et al. 2016).

To investigate whether myricetin has the potential to reduce hypertension *via* ACE inhibition, the activity of ACE was examined. This research showed significant inhibition of ACE activity associated with increasing myricetin dose. As a consequence of enzyme inhibition, levels of angiotensin II were decreased. Angiotensin II acts as a vasoconstrictor leading to increased blood pressure (Dos Santos et al. 2016), thus reduction of angiotensin II levels should decrease blood pressure. The mechanism of inhibition has been suggested by Persson et al. (2006) to involve polyphenol binding to Zn^{2+} ions in the active site of ACE. As reported by Selvaraj et al. (2013) myricetin has the ability to chelate-metal ions like Fe^{2+} and Zn^{2+} . Numerous studies have been conducted on endothelial cell lines to examine the effect of polyphenols as ACE inhibitors (Parichatikanond et al. 2012). Hussain et al. (2018) evaluated the ACE inhibition potential of flavonoid fraction obtained from *Coriandrum*

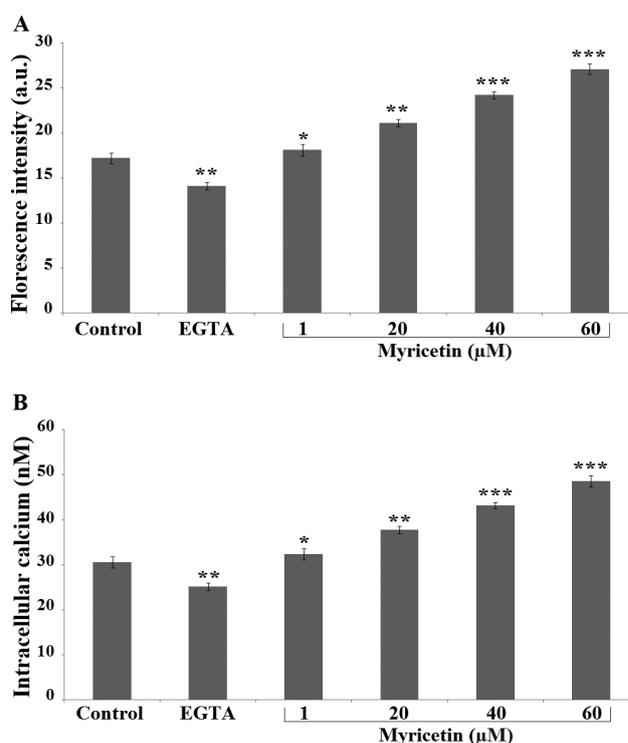


Figure 5. Intracellular calcium concentration in HUVEC exposed to myricetin (1–60 μM) for 24 h. **A.** Fluorescence intensity for control (untreated cell), negative control (1 mM EGTA) and cells treated with different myricetin concentrations. **B.** The intracellular calcium concentrations for control (untreated cell), negative control (1 mM EGTA) and cells treated with different myricetin concentrations. The fluorescent intensity is proportional to intracellular calcium concentrations. EGTA (1 mM) incubated for 3 h was used as a negative control. Data represented as mean \pm SD of three individual experiments. Comparisons of mean values were made using a one-way ANOVA followed by Tukey's test. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$ versus Control.

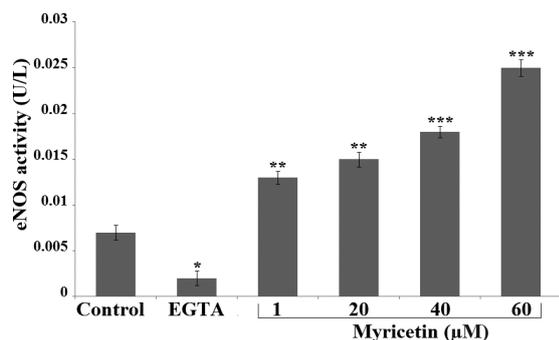


Figure 6. eNOS activity in HUVEC exposed to myricetin (1–60 μM) for 24 h. EGTA (1 mM) incubated for 3 h was used as a negative control. Data represented as mean ± SD of three individual experiments. Comparisons of mean values were made using a one-way ANOVA followed by Tukey's test. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$ versus Control. eNOS, endothelial nitric oxide synthase.

sativum. According to their results, ACE inhibition rate of the flavonoid fraction was 81.4%. Pinocembrin, apigenin, pseudobaptigenin, galangin-5-methyl ether, quercetin, baicalein trimethyl ether, kaempferol dimethyl ether, pinobanksin-5-methylether-3-O-acetate, pinobanksin-3-O-pentenoate, pinobanksin-3-O-phenylpropionate, pinobanksin-3-O-pentanoate, apigenin-7-O-glucuronide, quercetin-3-O-glucoside, apigenin-3-O-rutinoside, rutin, isorhamnetin-3-O-rutinoside, quercetin dimethyl ether-3-O-rutinoside, daidzein, luteolin, pectolinarigenin, apigenin-C-glucoside, kaempferol-3-7-dimethyl ether-3-O-glucoside, and apigenin-7-O-(6-methyl-beta-D-glucoside) were identified from the flavonoid fraction obtained from *C. sativum*. Guerrero et al. (2012) reported that the IC_{50} values of luteolin, quercetin, rutin, kaempferol, rhoifolin and apigenin were 23, 43, 64, 178, 183 and 196 μM, respectively. Epicatechin, epigallocatechin, epicatechin gallate and epigallocatechin gallate inhibited the activity of ACE in HUVEC cell line at all studied concentrations (Persson et al. 2006). In another study was performed on HUVEC cell line to investigate the inhibition of ACE enzyme after exposure to aqueous phenolic extract from *Vaccinium myrtillus*. The *Vaccinium myrtillus* extract contained several polyphenols such as quercetin, stillbene, resveratrol, ferulic acid and coumaric acid; these components significantly inhibited ACE activity in a dose dependent manner in HUVEC cell line treated for 10 min (Persson et al. 2009).

Angiotensin II has the ability to stimulate ROS production in endothelial cells through activation of a redox-sensitive signaling system. In an endothelial cell line, NADPH oxidase was considered as a source of ROS, responding to angiotensin II with the donation of an electron to reduce a molecule of oxygen to produce the superoxide anion, O_2^- (Förstermann et al. 2017). In our study, ROS production in the HUVEC

cell line, treated with different concentrations of myricetin for 24 h, in the absence of angiotensin II stimulation, was not significantly different to ROS production in cells not treated with myricetin. On the other hand, the superoxide production that was induced after incubation of cells with angiotensin II was significantly inhibited at all concentrations of myricetin. These results illustrate that myricetin has the ability to scavenge ROS and thereby protect cellular macromolecules from ROS-mediated damage. As reported previously from several *in vitro* studies, the presence of the hydroxyl group in phenolic compounds plays an important role in scavenging ROS (Bao and Lou 2006). Inhibition of superoxide levels was expected due to the presence of several hydroxyl groups within the chemical structure of myricetin (Semwal et al. 2016).

Myricetin, at studied doses, showed a significant increase in eNOS enzyme activity but there was no increase in the protein expression level of the eNOS enzyme. Myricetin may have increased the eNOS activity *via* eNOS phosphorylation which enhances activity *via* the activation of the redox-sensitive phosphatidylinositol-3 (PI3)/protein kinase B (AKT) pathways (Krga et al. 2018). Protein expression of eNOS enzyme in bovine aortic cell line was not affected after incubation for 12 h with 50 μM of punicalagin (Chen et al. 2008). In contrast, Leikert et al. (2002) found that red wine polyphenols water extract had increased eNOS protein levels after incubation in EA.hy926 cell line for 20 h. Ugusman et al. (2014) found a significant increase in protein expression and

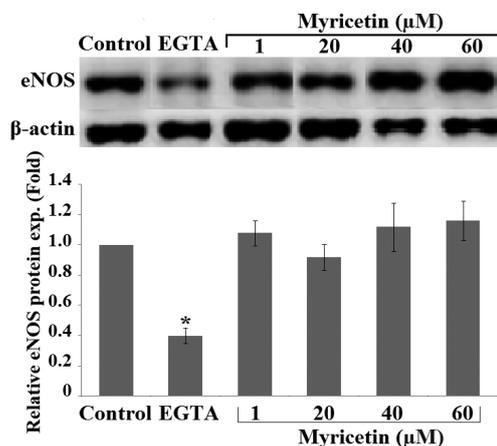


Figure 7. eNOS protein expression in HUVEC exposed to myricetin (1–60 μM) for 24 h. EGTA (1 mM) incubated for 3 h was used as a negative control. eNOS protein expression in the control group was considered as 1.00 and relative eNOS protein expressions of the other groups were expressed according to the control group. Data represented as mean ± SD of three individual experiments. Comparisons of mean values were made using a one-way ANOVA followed by Tukey's test. * $p < 0.05$ versus Control. eNOS, endothelial nitric oxide synthase.

activity of eNOS enzyme in HUVEC cell line after incubation with rutin at different concentrations for 24 h. As seen in other research on polyphenols, the increase in nitric oxide can be mediated by increased activity and protein expression of the eNOS enzyme (Chen et al. 2008) or by scavenging of ROS that would normally degrade it (Krga et al. 2018).

eNOS enzyme activity is calcium-dependent (Leikert et al. 2002), therefore, the calcium concentration in HUVEC cells treated with myricetin was measured. This study demonstrates that myricetin produced a significant increase in calcium concentration in HUVEC cell line after incubation for 24 h. Polyphenols can affect intracellular Ca^{2+} levels by releasing ions from intracellular stores or by increasing the entrance of Ca^{2+} through the cell membrane (Oak et al. 2018). One or both of these mechanisms may help to explain the significant increase of Ca^{2+} concentration caused by myricetin in the endothelial cell line. According to literature, red wine polyphenols showed a significant increase in calcium concentration in EA.hy926 cell line, which increased the eNOS protein level and showed a significant release of nitric oxide (Leikert et al. 2002). Bi et al. (2016) found a significant increase in intracellular calcium concentration in HUVEC cell line after incubation with naringin at different concentrations for 6 h. As we have seen previous research results on polyphenols as calcium inducer were similar to our findings.

In conclusion, myricetin is a type of polyphenol that could play a role in reducing the risk of cardiovascular disease. As observed previously, myricetin incubated with HUVEC cells at different concentrations for 24 h produced a significant inhibition of ACE enzyme activity and increased nitric oxide production. Increased nitric oxide production was *via* increased eNOS activity due to an increase in calcium concentration. There was no induction of eNOS enzyme expression observed. This nitric oxide level is protected from destruction by ROS through myricetin scavenging activity. Our findings indicate that myricetin may be helpful for lowering blood pressure; this could be achieved through dietary intervention or by the production of new antihypertensive treatments from a natural product.

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