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Vitexin Promotes Nitric Oxide Production in Human Umbilical Vein Endothelial Cells

(Viteksin Merangsang Penghasilan Nitrik Oksida dalam Sel Endotelium Vena Umbilikus Manusia)

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ABSTRACT

Endothelial nitric oxide synthase (eNOS) plays a pivotal vasculoprotective role by synthesising nitric oxide (NO), a vital anti-atherogenic agent in the vascular system. Vitexin, a flavonoid, has gained attention for its extensive vasculoprotective effects. The aim of this study was to explore the effect of vitexin on eNOS and NO synthesis in human umbilical vein endothelial cells (HUVECs). Cultured HUVECs were assigned to four distinct groups: control, oxidative stress induced by 180 µM hydrogen peroxide (H_2O_2), treatment with 300 µM vitexin, and simultaneous treatment with H_2O_2 and vitexin. After 24 h, HUVECs were harvested and the mRNA expression, protein levels and activity of eNOS were measured. Additionally, NO levels were determined. The study findings showed that the HUVECs treated with vitexin demonstrated an enhanced NO production compared to the control group. Vitexin facilitated production of NO in HUVECs by upregulating the expression of the eNOS gene, promoting eNOS protein synthesis, and increasing eNOS activity. In conclusion, vitexin enhances endothelial function by stimulating endothelial NO production by eNOS, suggesting its potential as a therapeutic agent for vascular health.

Keywords: Endothelial nitric oxide synthase; human umbilical vein endothelial cells; nitric oxide; oxidative stress; vitexin

ABSTRAK

Endotelium nitrik oksida sintase (eNOS) memainkan peranan penting dalam perlindungan vaskular dengan menghasilkan nitrat oksida (NO) yang merupakan agen anti-aterogenik penting dalam sistem vaskular. Viteksin, sejenis flavonoid, telah mendapat perhatian kerana kesan perlindungan vaskularnya yang menyeluruh. Tujuan penyelidikan ini dijalankan adalah untuk mengkaji kesan viteksin terhadap sintesis eNOS dan NO dalam sel endotelium vena umbilikal manusia (HUVECs). Kultur HUVECs dibahagikan kepada empat kumpulan yang berbeza: kumpulan kawalan, tekanan oksidatif yang diaruh oleh 180 µM hidrogen peroksida (H_2O_2), rawatan dengan 300 µM viteksin dan rawatan serentak dengan H_2O_2 dan viteksin. Selepas 24 jam, HUVECs dituai dan ekspresi mRNA, paras protein dan aktiviti eNOS diukur. Selain itu paras NO juga ditentukan. Penemuan kajian menunjukkan bahawa HUVECs yang dirawat dengan viteksin menunjukkan peningkatan dalam penghasilan NO berbanding kumpulan kawalan. Viteksin membantu penghasilan NO dalam HUVECs dengan meningkatkan ekspresi gen eNOS, merangsang penghasilan protein eNOS dan meningkatkan aktiviti eNOS. Kesimpulannya, viteksin meningkatkan fungsi endotelium dengan merangsang penghasilan NO oleh eNOS, seterusnya mencadangkan potensinya sebagai agen terapeutik untuk kesihatan vaskular.

Kata kunci: Endotelium nitrik oksida sintase; nitrik oksida; sel endotelium vena umbilikus manusia; tekanan oksidatif; viteksin

INTRODUCTION

Endothelial nitric oxide (NO) is a crucial element in preserving cardiovascular homeostasis through its anti-atherogenic properties (Janaszak-Jasiecka et al. 2023). Identified in 1986 by Furchgott and Ignarro as a vasorelaxant, it plays a pivotal role in counteracting various processes that contribute to atherosclerosis, such as the oxidation of low-density lipoproteins, aggregation of platelets, production of reactive oxygen species, infiltration of inflammatory cells, and aberrant proliferation of vascular myocytes (Batty, Bennett & Yu 2022). Decreased NO bioavailability in the endothelium is frequently observed as a hallmark of endothelial dysfunction, highlighting its clinical significance (Ambrosino et al. 2022). Therefore,

increasing NO production to leverage its vasculoprotective effects could be a promising therapeutic approach for the prevention and treatment of cardiovascular diseases (Daiber et al. 2019).

Endothelial nitric oxide synthase (eNOS) is the primary enzyme that is responsible for the production of vascular NO (Nguyen et al. 2023). Increased expression and enzymatic activity of eNOS significantly elevate the NO output (Tran et al. 2022). Furthermore, genetic modulation of eNOS through key regulatory pathways, including growth factors such as vascular endothelial growth factor (VEGF), transforming growth factor beta-1 (TGF-β1), and basic fibroblast growth factor (bFGF), further underscores the intricate nature of endothelial NO production (Ray, Gulati & Rehman 2023; Ugusman et al. 2014).

Oxidative stress, characterized by an imbalance between pro-oxidant and antioxidant defense systems, is primarily generated through hydrogen peroxide (H_2O_2) redox reactions (Konno et al. 2021; Olufunmilayo, Gerke-Duncan & Holsinger 2023). This method has also been widely utilised to trigger oxidative stress *in vitro* studies (Ransy et al. 2020). Oxidative stress is instrumental in the pathogenesis of cardiovascular diseases and atherosclerosis by aggravating endothelial dysfunction, promoting inflammatory responses, and inducing lipid peroxidation, which ultimately reduce NO bioavailability (Panda et al. 2022).

Dietary flavonoids, a class of phenolic plant compounds, have been associated with a reduced cardiovascular risk, as evidenced by numerous epidemiological studies (Bondonno et al. 2023; Zhou, Gu & Zhou 2023). The cardiovascular protective effects of flavonoids are complex and multifaceted. It may involve the upregulation of eNOS expression and subsequent NO synthesis, thereby inhibiting the pathophysiological cascades associated with endothelial dysfunction and atherosclerosis (Chen & Zhang 2021).

Apigenin-8-C-β-D-glucopyranoside, also known as vitexin, is a flavonoid derivative found in several botanical sources, including hawthorns, mung beans, beetroots, passion flowers, millet, bamboo leaves, and barley (Ranjan et al. 2023). Numerous studies demonstrated that vitexin exhibits diverse metabolic activities, including antioxidant, anti-arteriosclerotic, antihypertensive, anti-inflammatory, antiviral, anticancer, and neuroprotective effects (Chen & Zhang 2021; Chen et al. 2022).

Notably, vitexin is one of the active compounds found in the aqueous extract of *Piper sarmentosum* Roxb. leaves (Azmi et al. 2021). *P. sarmentosum* has been demonstrated to enhance NO synthesis in HUVECs, though the particular bioactive compound responsible for this effect has yet to be identified (Ugusman et al. 2014). Therefore, the purpose of this study was to elucidate the specific effects of vitexin on eNOS activity and NO synthesis in HUVECs. Our findings could provide insights into the management and prevention of endothelial dysfunction, which is connected to cardiovascular diseases. In addition, the positive outcomes of this study could support the scientific rationale for using *P. sarmentosum* as a supplement to promote cardiovascular well-being.

MATERIALS AND METHODS

CELL CULTURE AND TREATMENT PROTOCOL

Human umbilical cords were collected under sterile conditions. HUVECs were isolated from the umbilical cord veins through enzymatic digestion with 0.1% collagenase type I (Gibco-Invitrogen Corp, Grand Island, NY, USA). The cells were cultured in Medium 200 (Cascade Biologics, Grand Island, NY, USA), and maintained in an incubator with 5% CO_2 at 37 °C. The authenticity of HUVECs was established by their cobblestone morphology and positive expression of CD31 and von Willebrand factor in immunocytochemistry. The culture medium was replaced every two days, and HUVECs from passage 3, with 80% confluency, were utilized for the experiments. Vitexin (95% purity) was obtained from Sigma (St. Louis, USA). HUVECs were categorised into four groups: untreated control, 180 μM H_2O_2 to induce oxidative stress, treatment with 300 μM vitexin, and concomitant treatment with 180 μM H_2O_2 and with 300 μM vitexin. Each treatment was administered for 24 h. The doses of H_2O_2 and vitexin used were based on previous studies (Ugusman et al. 2014).

REAL-TIME QUANTITATIVE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (QPCR) FOR THE MEASUREMENT OF eNOS MRNA EXPRESSION IN HUVECs

Following a 24-h treatment, total ribonucleic acid (RNA) was extracted from HUVECs using TRI Reagent (Molecular Research Center, Cincinnati, USA), as previously described (Ugusman et al. 2014). The purity and concentration of the extracted RNA were evaluated with Nanodrop ND-100 spectrophotometer (Wilmington DE, USA). Complimentary DNA (cDNA) was synthesized using the SuperScript III First-Strand Synthesis SuperMix (Invitrogen, Carlsbad, USA). Then, qPCR was conducted to evaluate the eNOS mRNA expression, with glycerylaldehyde-3-phosphate dehydrogenase (GAPDH) serving as the housekeeping gene. Primer3 software was utilized to design the primers of eNOS and GAPDH based on sequences available in the NIH GenBank database (Table 1). The qPCR was conducted by incorporating 1 μL cDNA, 12.5 μL of IQ SYBR Green Supermix (Bio-Rad, CA, USA), and 5 μM of each forward and reverse primer and in a Bio-Rad iCycler (Bio-Rad, CA, USA). The reaction specificity was confirmed based on agarose gel electrophoresis and the melting curve profile. The values of the threshold cycle (CT) were recorded, and eNOS mRNA expression was calculated using the 2-ΔΔCT method.

TABLE 1. List of primers for qPCR analysis

Gene	Genbank accession no.	Primer sequence	PCR product size (bp)
GAPDH[a]	NM_002046	F: tcc ctg agc tga acg gga ag R: gga gga gtg ggt gtc gct gt	217
eNOS [b]	NM_000603	F: ttt gcc ctt atg gat gtg aag R: cgc atc aaa gaa agc tca gtc	139

eNOS: endothelial nitric oxide synthase; GAPDH: glycerylaldehyde-3-phosphate dehydrogenase

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE MEASUREMENT OF eNOS PROTEIN IN HUVECs

The determination of eNOS protein concentration in HUVECs was accomplished using the Quantikine® human eNOS ELISA kit (R&D Systems Inc., Minneapolis, USA). HUVECs were initially lysed with lysis buffer. The lysed cells were added into 96-well plate, allowing any eNOS in the sample to bind to the antibody inside the plate. Then, the conjugate of eNOS was pipetted to the plate prior to the addition of substrate and stop solutions. The optical density was measured at a wavelength of 450 nm with a microplate reader.

MEASUREMENT OF eNOS ACTIVITY IN HUVECs

The Calbiochem® nitric oxide synthase assay kit (EMD Chemicals, Darmstadt, Germany) was utilised to determine eNOS activity, as previously described (Ugusman et al. 2014). The assay measured the rate of nitrite production by eNOS over a specified time period. HUVECs were scraped from the flasks, and centrifuged at 10,000 g for 20 min. Next, the samples were added to a mixture of cofactor preparation solution, nitrate reductase, NADPH, and lactate dehydrogenase. The absorbance was recorded at 540 nm and eNOS activity was calculated as nmol of nitrite/min/mL of the sample.

MEASUREMENT OF NITRIC OXIDE LEVEL IN HUVECs

The Bioxytech® nitric oxide assay kit (Oxis Research, Portland, USA) was used to measure NO produced by HUVECs based on the level of nitrite, which is NO's stable oxidation product. A 50 µL aliquot of the HUVECs culture medium was diluted with assay buffer, followed by the addition of nitrate reductase and NADH. The mixture was then incubated for 20 min to transform nitrate to nitrite. Subsequently, Griess reagents (sulfanilamide and naphthalene–ethylenediamine dihydrochloride) were added and the absorbance was measured at 540 nm wavelength.

ETHICAL STATEMENT

This study was approved by the Ethical Research Committee of Universiti Kebangsaan Malaysia (Approval number: UKM PPI/111/8/JEP-2019-671). Informed consent was obtained from all subjects.

STATISTICAL ANALYSIS

The Kolmogorov-Smirnov test was used to test the normality of the data, confirming that all variables followed a normal distribution. Data was presented as mean ± standard error of the mean (SEM). Statistical comparison between the two groups was conducted using the Student's t-test in SPSS version 29.0. Statistical significance was defined as values with $p < 0.05$.

RESULTS

EFFECT OF VITEXIN ON eNOS mRNA EXPRESSION IN HUVECs

Vitexin treatment resulted in a 1.8-fold upregulation of eNOS mRNA expression in HUVECs compared to the control group ($p < 0.05$) (Figure 1). In the group subjected to oxidative stress, HUVECs treated with H_2O_2 showed a 1.7-fold increase in eNOS mRNA expression than the control group ($p < 0.05$). However, simultaneous treatment with both vitexin and H_2O_2 did not lead to a significant upregulation in eNOS mRNA expression compared to the control.

EFFECT OF VITEXIN ON eNOS PROTEIN LEVEL IN HUVECs

Treatment with vitexin significantly increased eNOS protein level in HUVECs (1.55 ± 0.06 × 103 pg/mL) compared to the control group (1.30 ± 0.06 × 103 pg/mL) ($p < 0.05$) (Figure 2). Furthermore, the group exposed to H_2O_2 (1.43 ± 0.04 × 103 pg/mL) also exhibited a notable eNOS protein level elevation ($p < 0.05$) compared to the control group. This increase in eNOS protein levels was concurrent with the increment in eNOS mRNA expression (Figure 1). However, HUVECs treated with both vitexin and H_2O_2 (1.35 ± 0.06 × 103 pg/mL) did not show a significant increment in eNOS protein level relative to the control.

EFFECT OF VITEXIN ON eNOS ACTIVITY IN HUVECs

Vitexin treatment resulted in an increase in the eNOS activity in HUVECs (4.88 ± 0.07 × 10-2 nmoles/mL/min) compared to the control group (4.46 ± 0.04 × 10-2 nmoles/mL/min) ($p < 0.05$) (Figure 3). Similarly, the H_2O_2 group demonstrated a rise in eNOS activity (4.77 ± 0.67 × 10-2 nmoles/mL/min) compared to the control

($p < 0.05$). However, HUVECs treated with both vitexin and H_2O_2 (4.56 ± 0.07 × 10-2 nmoles/mL/min) did not exhibit a statistically significant difference in their eNOS activity compared to the control cells.

EFFECT OF VITEXIN ON NO PRODUCTION IN HUVECs

HUVECs treated with vitexin demonstrated a significant increase in NO level (3.82 ± 0.27 μM) compared to the control group (2.39 ± 0.10 μM) ($p < 0.01$) (Figure 4). Furthermore, HUVECs exposed to H_2O_2 produced a greater amount of NO (2.75 ± 0.10 μM) than the control group ($p < 0.05$). The highest NO level was recorded in HUVECs treated with both vitexin and H_2O_2 (4.13 ± 0.34 μM), which was significantly greater than the control group ($p < 0.01$) and the H_2O_2 group ($p < 0.01$).

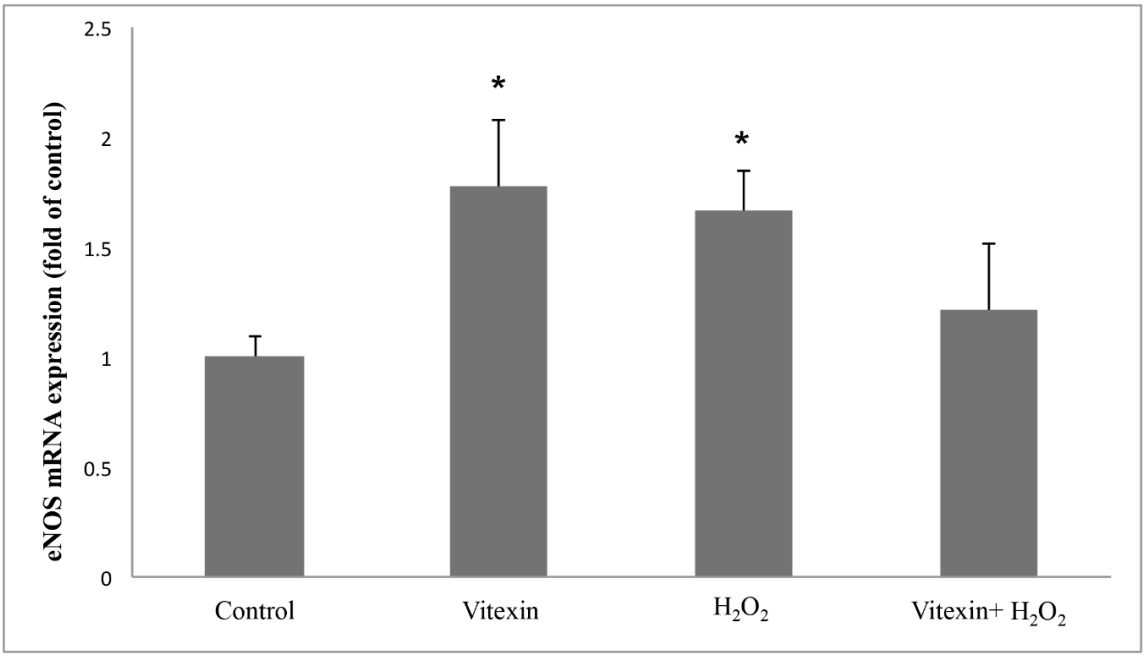
DISCUSSION

The findings of this study showed that vitexin significantly enhanced the production of NO in HUVECs, primarily attributed to the upregulation of eNOS mRNA expression, which increased the synthesis of eNOS protein and stimulated the activity of eNOS. These results corroborate the hypothesis that vitexin exerts beneficial effects on vascular health by modulating the eNOS signaling pathway.

Oxidative stress is a critical contributor to the pathogenesis of atherosclerosis, causing endothelial dysfunction, inflammatory responses, lipid peroxidation, and diminished bioavailability of NO (Higashi 2022). In this study, inducing oxidative stress in HUVECs through the administration of 180 μM H_2O_2 resulted in increased eNOS mRNA expression, protein levels, and activity, which subsequently enhanced NO production. The responses to H_2O_2 observed in this study were consistent with previous findings reported in similar experimental settings (Aini et al. 2023).

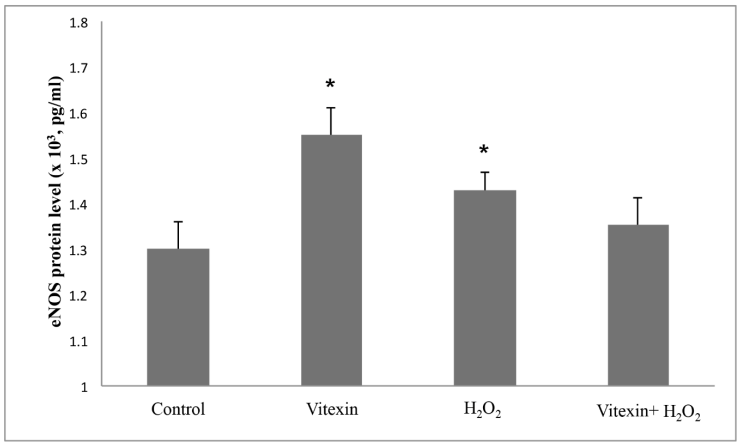
We found that the level of NO was greater in the H_2O_2 group compared to the control. This increase can be attributed to the stimulation of NO synthesis by H_2O_2, which acts as a component of the cellular defense mechanism (Zhou et al. 2019). Moreover, the concentration of H_2O_2 applied was non-lethal to HUVECs, allowing these cells to enhance their endogenous production of NO in response to the H_2O_2 challenge. Nevertheless, the presence of H_2O_2 resulted in the oxidative degradation of the synthesised NO, elucidating why the augmentation of NO in the H_2O_2-treated group was not as pronounced as that observed in other experimental groups, such as the vitexin alone and the combined vitexin and H_2O_2 groups. Furthermore, the upregulation of eNOS expression by H_2O_2 indicates a self-protective response by endothelial cells to preserve NO bioavailability during oxidative stress (Liao et al. 2021).

Similarly, it was observed that NO levels were elevated in the group treated with vitexin compared to the control. We postulate that this could be attributed to the antioxidant and cytoprotective effects of vitexin against oxidative cell



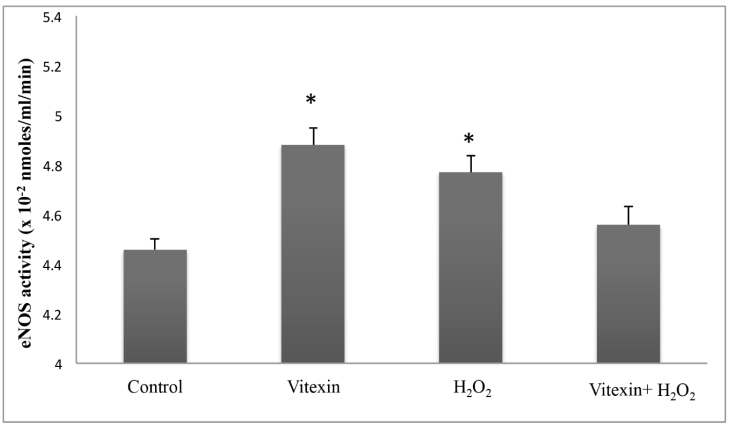
Values are expressed as mean ± SEM, n =8, *$p < 0.05$ vs. control. SEM: Standard error of the mean

FIGURE 1. Endothelial nitric oxide synthase (eNOS) mRNA expression in the control, vitexin, hydrogen peroxide (H_2O_2) and vitexin + H_2O_2 groups



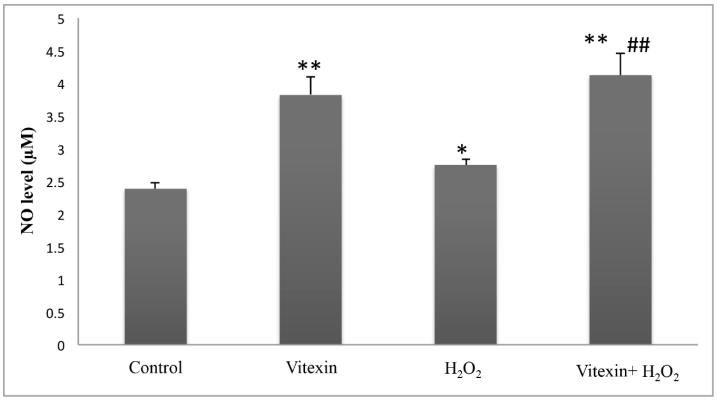
Values are expressed as mean ± SEM, n = 8, *p < 0.05 vs. control. SEM: Standard error of the mean

FIGURE 2. Endothelial nitric oxide synthase (eNOS) protein levels in the control, vitexin, hydrogen peroxide (H_2O_2), and vitexin + H_2O_2 groups



Values are expressed as mean ± SEM, n = 8, *p < 0.05 vs. control. SEM: Standard error of the mean

FIGURE 3. Endothelial nitric oxide synthase (eNOS) activity in the control, vitexin, hydrogen peroxide (H_2O_2), and vitexin + H_2O_2 groups



Values are expressed as mean ± SEM, n = 8, *p < 0.05 vs. control; **p < 0.01 vs. control; ##p < 0.01 vs. H_2O_2. SEM: Standard error of the mean

FIGURE 4. Nitric oxide (NO) levels in the control, vitexin, hydrogen peroxide (H_2O_2), and vitexin + H_2O_2 groups

damage induced by H_2O_2 (Zhang et al. 2022). Antioxidants are recognised for their ability to augment the biological effects of NO by preserving NO against oxidative damage caused by ROS (Kunter, Zabib & Kosar 2019). This indicates that vitexin directly protects NO from oxidative destruction by H_2O_2. Thus, all of these mechanisms contribute significantly to elevated levels of endothelial NO following vitexin treatment.

Furthermore, we observed that cells subjected to vitexin and H_2O_2 treatment demonstrated elevated levels of NO. However, there was no significant elevation in eNOS mRNA expression, protein level, or activity. This phenomenon can be explained by a classic example of negative feedback mechanism, whereby an increase in NO bioavailability results in the downregulation of eNOS mRNA expression (Janaszak-Jasiecka et al. 2021). This regulatory effect is likely facilitated by the antioxidative property of vitexin, which protects NO from degradation by H_2O_2, thereby maintaining high NO levels within endothelial cells, as previously discussed. Consequently, increased NO concentration initiates a feedback loop that leads to the suppression of eNOS mRNA and protein expression.

P. sarmentosum has been reported to enhance endothelial NO synthesis (Sundar et al. 2019). Given that vitexin is one of the major flavonoids present in this plant, it is plausible that vitexin significantly contributes to the ability of *P. sarmentosum* to stimulate NO production (Azmi et al. 2021). This relationship suggests a pivotal role of vitexin in modulating endothelial function through its impact on NO levels, underscoring the therapeutic potential of natural flavonoids in vascular health.

This study offers valuable insights on the mechanisms by which vitexin enhances NO production in HUVECs, providing evidence of its potential to improve vascular health. These findings not only deepen our understanding of vitexin's pharmacological actions but also underscore its relevance in the broader context of cardiovascular disease prevention. Additionally, the identification of vitexin as an active component with potential for use in dietary supplements carries substantial implications for its integration into clinical practice, particularly for improving cardiovascular health.

Nevertheless, the lack of *in vivo* data remains a major barrier to the translation of these *in vitro* findings into clinical applications, where the physiological response may affect the effectiveness of the compound. Future research should focus on conducting *in vivo* studies using appropriate animal models to evaluate the pharmacokinetics, bioavailability, and systemic effects of vitexin. These studies would provide crucial insights into how vitexin acts under physiological conditions, including its metabolism, distribution, and potential side effects. Ultimately, clinical trials will be essential to determine its safety, efficacy, and optimal dosage in human populations. Addressing these gaps will be crucial for fully uncovering vitexin's therapeutic potential for endothelial dysfunction.

CONCLUSIONS

This study elucidates the significant role of vitexin in promoting NO synthesis in HUVECs by stimulating eNOS mRNA expression, protein synthesis and activity. In addition, vitexin exhibits protective antioxidant properties that mitigate oxidative stress and preserve NO bioactivity, thereby emphasizing its dual beneficial effects on endothelial function. Future research should focus on conducting *in vivo* studies to assess the efficacy of vitexin and to facilitate the translation of these findings into human clinical trials.

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