# Influence of Capsicum Extract and Capsaicin on Endothelial Health

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**Objective:** *To examine the effect of Capsicum spp extract (CEX) and capsaicin (CAP) on endothelial nitric oxide release and protection against lipopolysaccharide (LPS)-induced cellular apoptosis.*
**Material and Method:** *Human umbilical vein endothelial cells (HUVEC) were isolated from newborn cords. Evaluation of cytotoxicity was performed by MTT assay. Endothelial nitric oxide (NO) production was evaluated by Griess reaction. Alteration in eNOS expression was detected by westernblot analysis. To induce oxidative stress and apoptosis, lipopolysaccharide (LPS) was coincubated with HUVEC in the presence or absence of CEX or CAP, and the vanilloid receptor blocker capsazepine (CZP). Hoechst nuclear staining was used to determine percent apoptotic nuclei.*
**Results**: *The highest concentrations of CEX (1000 μg/mL) and CAP (25 μM) used in the study did not induce cytotoxicity in HUVEC. Significant increase in NO release was observed when cells were incubated with CEX (100 μg/mL) and CAP (25 μM) and this effect was inhibited by CZP only in CAP treatment group. Despite enhanced NO generation was observed, western blot analysis indicated no change in eNOS expression. Interestingly, endothelial cells incubated with L-arginine (L-ARG, 1000 μg/mL) alone significantly showed increased NO production while L-ARG co-incubation abrogated CEX or CAP effects on endothelial NO generation. CEX (10 μg/mL) and CAP (1μM) decreased apoptotic nuclei in HUVEC treated with LPS.*
**Conclusion:** *CEX and CAP improved endothelial function and protected against LPS-induced apoptosis. Regular consumption of Capsicum spp. may promote endothelial health and reduce cardiovascular disease risk.*

**Keywords**: *Capsicum spp, Capsaicin, Endothelial cells, Oxidative stress, Apoptosis, Lipopolysaccharide*

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As the rise of the incidence of cardiovascular disease (CVD) in aging population it has become serious worldwide health problem and burden in healthcare costs for the downsizing working populations. Therefore, a cost-effective preventive strategy to reduce CVD reflects a significant change in physical activity, healthy dietary practice, and reduction of the major risk factors such as hypertension, hyper-cholesterolemia, obesity, and the chronic disease of type 2 diabetes[1]. Benefit on prevention of cardiovascular events has become evidence soon after considerable research has performed to implement these strategies to the focus groups[2-4].

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The consumption of healthy diet appears to promote vascular endothelial health which slows down the process of endothelial dysfunction[5]. It is well recognized that endothelial dysfunction and endothelial damage play a crucial role in the pathogenesis of CVD which characterized by a reduction in nitric oxide (NO) production[6-8]. The reduction in NO bioavailability leads to a loss in the regulation of vascular tone, platelet aggregation and leukocyte adhesion. Thus, the normal physiological function of NO is essential for the prevention of CVD. The decreased levels of NO bioavailability are mainly due to reduction in NO synthesis or chemically attacked by free radical, especially superoxide anion, or oxidative stress condition[9-11]. Particular approaches to prevent or impede vascular endothelial dysfunction are to provide antioxidants or using nutraceuticals such as a precursor of nitric oxide synthase (NOS) L-arginine, and other bioactive com-

pounds from natural products (*e.g.*, flavonoids, polyphenols, etc) to enhance NO release[12,13].

Chili peppers (*Capsicum* spp) are native species of South America and have been cultivated widely in many tropical regions of Asia and the Caribbean Sea. Capsicum fruits and capsicum-derived ingredients demonstrate a wide array of traditional as well as clinical uses, including peptic ulcer[14], low-back pain[15], localized pain[16], and urinary incontinence[17]. Since chili pepper is essential in regular cuisines of a large group of Asian people, particularly in Thailand, it is of our interest to study the potential use of capsicum extract as neutraceuticals to promote endothelial health and subsequently minimize the CVD risk. The effect of capsicum extract was investigated on endothelial function using its active ingredient capsaicin as a functional reference for raw material quality control of the *Capsicum* spp extract. This present study was performed using human umbilical vein endothelial cells (HUVEC) in normal culture condition and in oxidative-related environment induced by lipopolysaccharide (LPS). Additionally, a potential combination of capsicum extract and a precursor of nitric oxide synthase (NOS), L-arginine (L-ARG) were also examined.

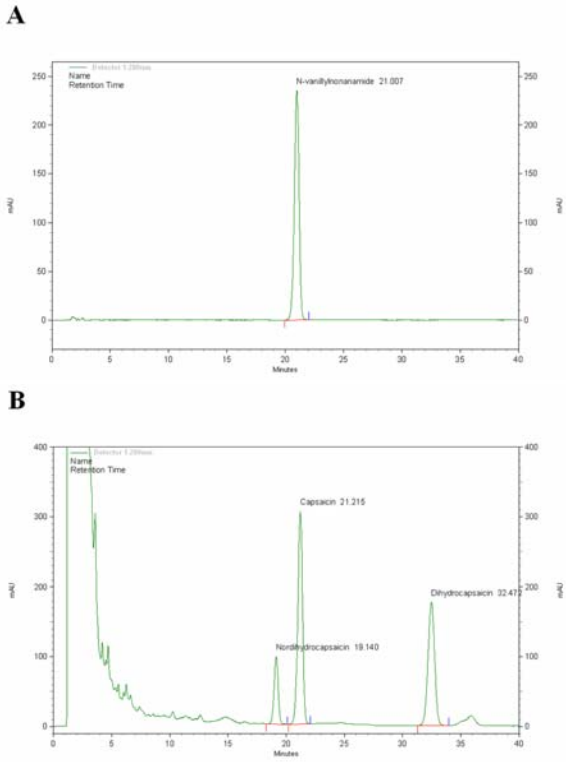


**Fig 1.** Chromatogram of HPLC analysis of capsicum extract. (A) The standard N-vanillylnonamide was peaked at the retention time of 21.007 min, (B) Chromatogram of CEX represents three major capsaicinoid peaks-nordihydrocapsaicin (13.17%), capsaicin (47.98%) and dihydrocapsaicin (38.85%).

## Material and Method

### *Chemicals and Capsicum Extract*

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or otherwise indicated. Capsicum extract (CEX) was procured from the Government Pharmaceutical Organization, Thailand (http://www.gpo.or.th). Physical appearance was described as dark red, thick viscous liquid with characteristic pungent odor of chilies. Analytical certificate reported the content of capsaicinoids as 2.37% (nordihydrocapsaicin, capsaicin, and dihydrocapsaicin) calculated from the HPLC chromatogram of the peak that had retention time corresponding to the synthetic analogue of capsaicin N-vanillynonamide (Fig. 1). Stock solutions of CEX at 10 mg/mL were prepared freshly at the time of experiment.

### *Human umbilical vein endothelial cell (HUVEC) culture*

Human umbilical cords were collected from the labor room and HUVECs were isolated within 48 h as described previously[13]. Cells were cultured in M199 medium, supplemented with 20% fetal bovine serum (FBS) with antibiotic and antimycotic agents (GIBCO), in a humidified atmosphere of 95% air and 5% CO2 at 37°C. Cells between passage 3-5 were used in the experiments and were cultured in low serum medium (1% FBS) during CEX incubation or other treatments.

### *Cytotoxicity Evaluations*

There have been reported that capsaicin (CAP) is toxic to cells[18-20]. Therefore, this experiment was aimed to find a range of concentrations that did not induce cytotoxicity. HUVECs were treated with CAP (0.001, 0.01, 0.1, 1, 10, 25 μM) or CEX (0.01, 0.1, 1, 10, 100, 1000 μg/mL) for 48 h. Cell survival was evaluated using MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay. Briefly, ten microliters of MTT stock solution (5 mg/mL) were added to the culture medium and incubated for 4 h or until dark blue-purple crystalline precipitates were visualized under an inverted microscope. Then, one hundred microliters of the detergent reagent (10% SDS in 0.01 M HCl) were added to dissolve the formazan products

which are corresponding to cell survival. The plate were then shacked at 200 rpm for 1 h and read the absorbance at 550 nm by an ELISA plate reader (Thermo, Finland).

***Nitric oxide production measurement***

The effects of CEX, CAP, and L-ARG on nitric oxide production in HUVECs were evaluated by Griess reactions as described previously[13]. Capsazepine (CZP, 10 μM), a specific vanilloid receptor antagonist, was used in the experiments to evaluate the role of vanilloid recpetors in nitric oxide production during the treatment of CEX, CAP or L-ARG. The assay began with adding 50 μL of the sample culture media in 96-well microplate in triplicate. Sulfanilamide solution (1% in 5% phosphoric acid, 50 μL) was added to all samples and standards and incubated in dark for 10 min at room temperature. The formation of azo compound was initiated by adding 0.1% N-1-napthylethylenediamine dihydrochloride (NED, 50 μL) and allowed for pseudo end point completion in 10 min. The absorbance was measured by an ELISA plate reader at 550 nm (Thermo, Finland).

***Westernblot analysis***

Following 48-h incubation, HUVECs were harvested and lysed in lysis buffer [20 mM Tris-HCl (pH 8.5), 150 mM NaCl, 1% Igepal, and 1% protease inhibitor cocktail (Sigma-Aldrich, P8340). Cell lysates were normalized for protein content using Bio-Rad protein assay kit (Biorad, USA). Twenty micrograms of protein samples were separated by 7.5% SDS-PAGE under reducing conditions and then transferred to a PVDF membrane using Biorad Mini-PROTEIN apparatus. The membrane was blocked with 3% non-fat dry milk in TBS [10 mM Tris-HCl (pH 7.5) and 0.1% Tween 20] for 1 h and then incubated at 4°C overnight with the eNOS primary antibody (Santa Cruz Biotechnology, San Francisco, CA). The blots were washed and then incubated with the peroxidase-conjugated secondary antibodies (Amersham Biosciences, GE Healthcare (Thailand) Ltd) for 1 h at room temperature. Following several washes with TBS, the membrane was developed using Opti-4CN kit (Bio-Rad Laboratories) according to the manufacturer's instructions. The relative eNOS protein expression was quantified by densitometry.

***Apoptosis assay***

HUVECs (50,000 cells) were grown on glass cover slips for 24 h. Following incubation with CEX (10 μg/mL) or CAP (1 μM), in the presence or absence of LPS (1 μg/mL), for 48 h, cells were washed with PBS and fixed with 3.7% paraformaldehyde for 15 min. The glass cover slips were washed with PBS and incubated in 1% Triton X-100 for 10 min to increase membrane permeabilization. Then, cells were incubated with Hoechst 3342 (Invitrogen) at 10 μg/mL for 5 min. After the cover slips were mounted on slides they were visualized under a UV microscope (Olympus, Japan). The relative percent of apoptotic cells were calculated using at least 500 nuclei per sample.

***Statistical analysis***

Data are expressed as mean $\pm$ SEM for at least three independent experiments. Statistical analysis was performed using one-way ANOVA with Dunnet's post hoctest or Student's t-test where appropriated. A value of $p < 0.05$ was considered significant.

**Results**

***Cytotoxicity of CEX and CAP***

The appropriate concentrations of CEX, CAP or L-ARG used in further experiments were evaluated by MTT cell survival. As shown in Fig. 2, CEX (0.01 to 1000 μg/mL) CAP (0.001 to 25 μM), and L-ARG (1 to 1000 μM) did not significantly alter HUVEC survival. The maximum CAP concentration at 25 μM was used in the experiment due to the limitation of dissolution in aqueous solution. Based on the analytical report of CEX described above and given that CAP has a MW of 305.41, it was calculated that CEX 1000 μg/mL consists of CAP 37.23 μM (capsaicinoids 77.60 μM) which is beyond the solubility limit of CAP in aqueous solution. Additionally, CEX caused cytotoxicity (< 80% survival) when incubated with HUVEC with initial cells seeded at less than 60% confluency (data not shown). Therefore, the maximum concentration of CEX at 100 μg/mL (corresponding to CAP 3.72 μM or capsaicinoids 7.76 μM) and CAP at 10 μM were used throughout the following experiments.

***Nitric oxide production was increased in HUVECs treated with CEX, CAP or L-ARG***

Shown in Fig. 3 are the effects of CEX, CAP, and L-ARG on endothelial nitric oxide production evaluated by Griess reaction. The tested compounds were co-incubated with HUVEC for 48 hr and total nitrite concentrations were determined in the cell culture media. In separate experiments, CZP (10 μM), an inhibitor of vanilloid receptors, was added to the culture media 30 min prior to the addition of test compounds and co-incubated with tested compounds throughout the ex-

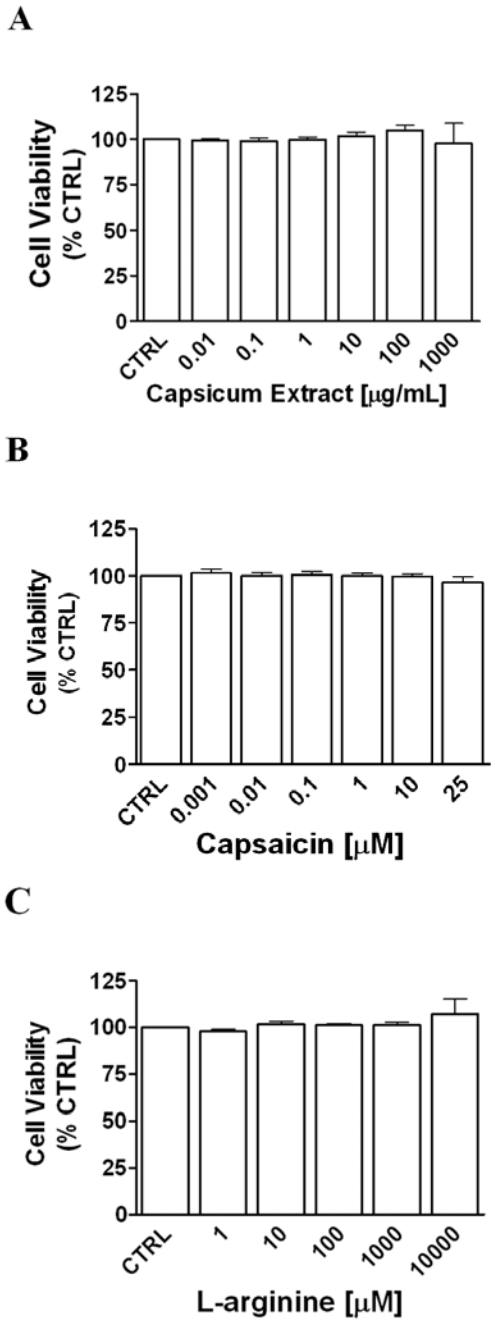


**Fig. 2** Influence of CEX, CAP, and L-arginine on cell survival. (A) cells treated with CEX; (B) cells treated with CAP; (C) cells treated with L-ARG. HUVECs were incubated for 48 h and evaluated by MTT assay as described in Material and Method.

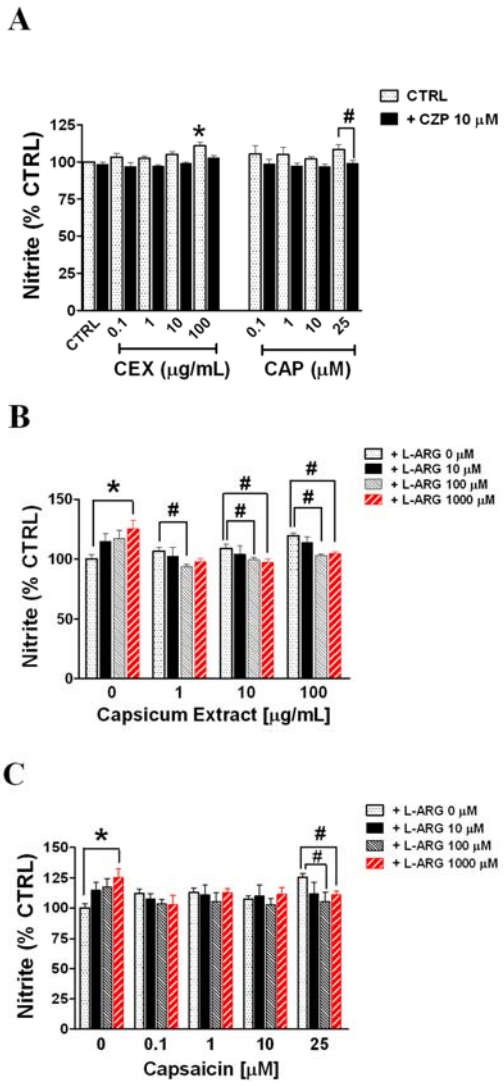


**Fig. 3** Nitrite concentrations in HUVEC culture media. (A) Nitrite contents were measured by Griess reaction in vehicle-treated cells (CTRL), CEX-treated cells at concentrations 1, 10, 100 μg/mL, and CAP-treated cells at 0.0, 1, 10 μM. Additional series of experiments, cells were co-incubated with capsazepine (CZP) 10 μM. (B,C) HUVECs were incubated with CEX or CAP in the presence or absence of L-arginine (L-ARG). Data are mean ± SEM, *p < 0.05 vs. CTRL; # p < 0.05 vs. its corresponding treatment control in the same group.

periments. CEX (100 μg/ml) significantly increased nitric oxide production and the effect was not considerably attenuated by CZP. In contrast, CAP at 25 μM did not significantly alter nitrite level but in the presence of CZP nitric oxide production was significantly attenuated (Fig. 3A). While treatment with L-ARG alone enhanced nitric oxide production at concentration of 1

mM, the combinations of L-ARG (10 or 100 μM) with CEX (10 or 100 μg/mL) significantly decreased nitric oxide production in HUVECs (Fig. 3B). However, the inhibitory effect of L-ARG only appeared in cells treated with CAP at the concentration of 25 μM (Fig. 3C).

***Effects on eNOS expression***

Relative eNOS protein expression was evaluated by westernblot analysis as shown in Fig. 4. Coincubation of CEX (1, 10, 100 μg/mL) or CAP (1, 10, 25 μM) HUVEC culture media for 48 h did not significantly alter the levels of eNOS expression.

***Apoptosis***

Hoechst staining was performed to detect the condensed or fragmented apoptotic nuclei observed under fluorescence microscope. Endothelial cells incubated with CEX (10 μg/mL), CAP (1 μM) or CZP (10 μM) alone did not change the amount of apoptotic cells while it appeared that LPS (1 μg/mL) significantly increased HUVEC apoptotic nuclei following 48 h incubation ($p < 0.05$). Interestingly, treatments with CEX (10 μg/mL), CAP (1 μM), or CZP (10 μM) abrogated LPS-induced apoptosis (Fig. 5).

**Discussion**

It is now widely accepted that dietary consumption of high antioxidant-containing food could significantly reduce CVD risk. The plants in the genus *Cappsicum* possess antioxidant properties which have been shown *in vitro, in vivo* as well as clinical trials[21-24]. The key factor in reducing CVD is to protect cells from oxidative damage (*e.g*, LDL oxidation, protein nitration, and apoptosis) and to maintain endothelial function via an increase of NO bioavailability. Our study

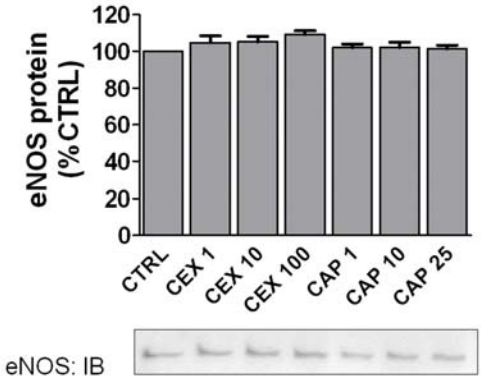


**Fig 4.** eNOS protein expression in HUVECs treated with CEX or CAP. HUVECs were treated with CEX (1, 10, 100 μg/mL) or CAP (1, 10, 25 μM) for 48 h and eNOS protein in cell lysate was separated by SDS-PAGE followed by immunoblotting (IB) as described in Materials and Methods.

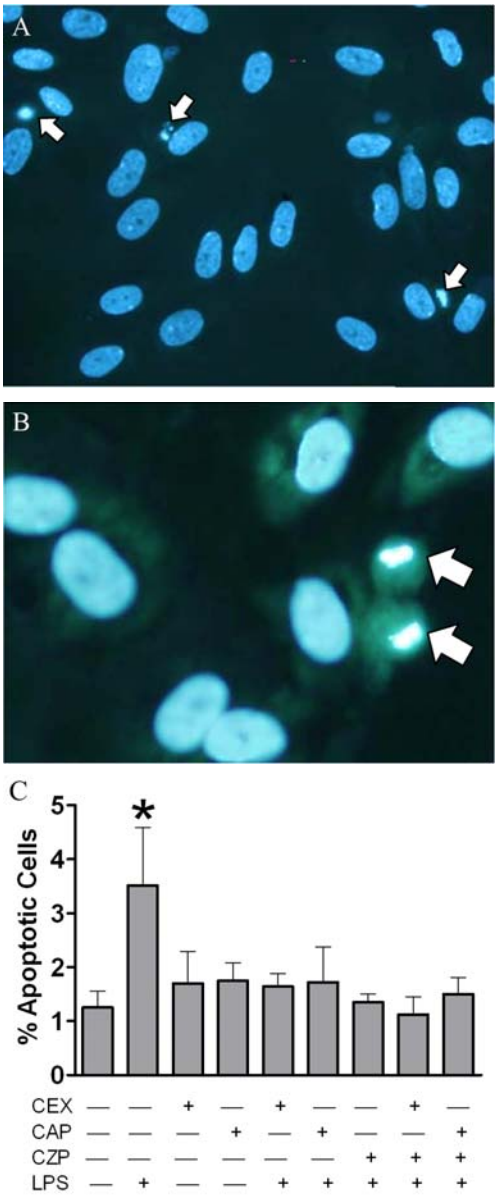


**Fig 5.** Effects of CEX (10 μg/mL), CAP (1 μM) or CZP (10 μM) on LPS-induced HUVEC apoptosis. (A, B) Representative photomicrographs of HUVECs stained with Hoechst 33342 (magnification 10X and 40X, respectively); C) Percent apoptotic cells following different treatment combinations. *$p < 0.05$

demonstrated that CEX increased NO production and protected against oxidative-induced endothelial cell apoptosis.

The maximum concentrations of CEX (1000 μg/mL) and CAP (25 μM) used in the experiments did not induce cytotoxicity in HUVEC while cytoprotective effect was observed when CEX (10 μg/mL) and CAP (1 μM) were added to the culture of LPS-induced endothelial cell apoptosis. CEX (100 μg/mL) and CAP (25 μM) significantly increased NO production but only CAP effect was inhibited by the vanilloid receptor blocker CZP. It implicated that CEX induced NO release may not rely on the action of CAP alone but also other chemical constituents containing in the extract. Despite enhanced NO generation was observed, western blot analysis indicated no change in eNOS expression when HUVECs were incubated with either CEX or CAP for 48 h. Thus, the increased NO production was not due to enhanced enzyme levels but it is likely to act via other pathways or non-specific mechanism. Interestingly, endothelial cells incubated with L-ARG (1000 μg/mL) alone significantly showed increased NO production while L-ARG co-incubation with CEX or CAP abrogated CEX or CAP effects on endothelial NO generation. This data suggest that paradoxical effect might occur when using high concentration of L-ARG in concomitant with compounds that enhance NO release.

The vasodilation effect of CEX may be due to the active ingredient CAP and other chemical constituents. Previous studies have shown that polyphenols extracted from green chili pepper (*Capsicum frutescenes*) promotes vascular relaxation via endothelium-dependent mechanisms[25]. Studies in isolated organ bath supported the association of CAP in inducing vascular relaxation in human and porcine isolated arteries[26]. In patients with ischemic heart disease, applying transdermal CAP patches increased exercise tolerance and elevated ischemic threshold potentially caused by an increased NO bioavailability[27]. Not only CAP induced vasodilation but it also protected the occurrence of endothelial dysfunction caused by the HIV protease inhibitor ritonavir[28]. The mechanisms of CAP mediated vasodilation appear to involve an activation of transient receptor potential vanilloid family, particularly TRPV1, a release of calcitonin gene-related peptide (CGRP), and an enhancement of NO production[29,30]. Similar to studies from others, our data show that TRPV1 antagonist CZP inhibited vasorelaxation induced by CAP[29].

The recently coined term "nutraceuticals" expresses the values of nutrition used as pharmaceuticals. As a consequence of a better insight into the association and significance of endothelial function and the pathophysiology of CVD, L-ARG, an amino acid precursor of NO synthesis, is highly speculated as a potential nutraceuticals. This study demonstrates that high concentration of L-ARG (1000 μg/mL) alone increased NO production in HUVEC but it mitigated effects of CEX and CAP. In the literature, conflicting results are shown as L-ARG improved endothelial function or had no impact at all. Bode-Boger et al[31] reported that giving L-ARG supplement (16 g/day) to healthy volunteers age over 70 years old for 2 weeks increased plasma L-ARG and improve endothelial function. Similar studies reveal that improved flow-mediated brachial artery dilation was observed both in volunteers with essential hypertension taken single dose L-ARG (6 g)[32], and in patients with coronary artery disease who received L-ARG supplement (21 g/day for 3 days)[33]. On the contrary, long-term L-ARG consumption (3 g/day for 6 months) elevated plasma L-ARG in patients with peripheral arterial disease but it did not increase NO availability[34]. Similarly, despite significant rise in plasma L-ARG was detected in CAD patients receiving L-ARG 9 g/day for 1 month, no evidence of enhanced NO production was observed[35]. Clinical study in children (7 to 17 years old) with chronic renal failure demonstrated no additive effect on endothelial function when L-ARG was given at the dose of 2.5 to 5 g/mm$^2$, three times a day, for 4 weeks[36]. This controversial data may derive from diff erences in duration of supplementation and pathological conditions, and possibly the level of asymmetrical dimethylarginine (ADMA), a metabolite of L-AGR, that acts as endogenous inhibitor of eNOS[37].

In addition to focusing on maintaining high level of NO bioavailability, cytoprotective effect against oxidative stress is also essential in the prevention of endothelial damage mentioned above. Our data suggest that CEX and CAP protected cellular apoptosis in oxidative stress conditions that are similar to findings reported from other laboratories about benefits of CEX or CAP in favor of cellular survival. For example, extracts from a variety of *Capsicum* spp inhibited sodium nitroprusside-induced lipid peroxidation in rat brain homogenates[22]. Ahuja et al found that regular consumption of chili (30 g/day) by healthy volunteers for 4 weeks increased the resistance of serum lipoprotein oxidation but no change in total antioxidant status was observed[23]. Chili consumption may also benefit the heart function in men as it lowered arterial stiffness and resting heart rate[38]. It is likely that anti-oxidative

stress effect may caused by many chemical constituents in CEX such as glycosides, capsaicin, carotenoids, volatiles, saponins (capsaicidin), and the derivative of CAP, 6' ',7' '-dihydro-5',5' "-dicapsaicin, and the capsaicin metabolite omega-hydroxycapsaicins[39-43].

While it is shown in this study that relatively low concentrations of CEX (10 μg/mL) and CAP (1 μM) protected against LPS-induced apoptosis, other investigators reported apoptosis induced by CAP in many cell types, including tumor cell lines and endothelial cells[44-46]. These conflicting results is likely to cause by the differences in concentrations used, a deviation in regulations of redox-sensitive pathways in particular cell types, and the dissimilarity in certain stress conditions. It appears that apoptosis was induced at relatively high concentrations of CAP in normal culture conditions while the opposite effect (antiapoptosis) occurred when tested in cells underwent oxidative stress and relatively lower concentrations of CAP were applied. For instance, antiapoptotic effect of CAP (50 μM) observed in hippocampal neurons undergone hypoxia was mediated by the PI3K/Akt signaling pathway and subsequently activation of caspase-3[47]. On the contrary, CAP (≥ 100 μM) induced endothelial inflammation and cell death in normal culture condition[44]. Similar results were observed in many cancer cell lines such as breast cancer cells[45], glioma cells[46], colon cancer cells[48] when CAP was used at relatively high concentations. Thus, the aspect of CAP and apoptosis is profoundly relied upon the experiment design and concentration of CAP used in the studies.

In summary, relatively low concentrations of CEX and CAP improved endothelial function and protected against LPS-induced apoptosis. Regular consumption of *Capsicum* spp may promote endothelial health and reduce CVD risk.

**Acknowledgments**

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*ผลของสารสกัดพริกและแคปไซซินต่อสุขภาพเซลล์เยื่อบุหลอดเลือด*

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***วัตถุประสงค์:*** *เพื่อศึกษาฤทธิ์ของสารสกัดพริกและแคปไซซิน ต่อการหลั่งไนตริกออกไซด์ในเซลล์เยื่อบุหลอดเลือด และการปกป้องเซลล์จากการตายแบบอะพอพโตซิส โดยการเหนี่ยวนำของ lipopolysaccharide (LPS)*

***วัสดุและวิธีการ:*** *เซลล์เยื่อบุหลอดเลือดสกัดได้จากสายสะดือทารกแรกเกิด และตรวจวัดความเป็นพิษต่อเซลล์โดย MTT assay ส่วนผลต่อการหลั่งไนตริกออกไซด์ตรวจวัดด้วย Griess reaction การเปลี่ยนแปลงการแสดงออกของ eNOS ตรวจวัดได้ด้วยวิธี western blot analysis การบ่ม LPS ร่วมกับเซลล์เพื่อให้เกิดภาวะ oxidative stress และ apoptosis และศึกษาผลที่เกิดขึ้นเมื่อบ่มเซลล์เยื่อบุหลอดเลือดร่วมกับสารสกัดพริกแคปไซซิน และสารยับยั้ง vanilloid receptor คือ capsazepine (CZP) โดยการนับร้อยละของจำนวนนิวเคลียสที่ย้อมด้วยสี Hoechst และมีลักษณะ apoptotic nuclei*

***ผลการศึกษา:*** *ความเข้มข้นสูงสุดของสารสกัดพริก (1000 μg/mL) และแคปไซซิน (25 μM) ที่ใช้ในการศึกษา ไม่ทำให้เกิดความเป็นพิษต่อเซลล์เยื่อบุหลอดเลือด สารสกัดพริกที่ความเข้มข้น 100 mg/mL และใช้แคปไซซินที่ความเข้มข้น 25 μM ทำให้เพิ่มการหลั่งไนตริกออกไซด์อย่างมีนัยสำคัญ และ CZP ยับยั้งผลที่เกิดขึ้นในกลุ่มของเซลล์ที่ได้รับแคปไซซินเท่านั้น ถึงแม้ว่าจะตรวจพบการเพิ่มขึ้นของการหลั่งไนตริกออกไซด์ แต่ปริมาณการแสดงออกของ eNOS โปรตีน ไม่เปลี่ยนแปลง เมื่อวิเคราะห์โดย western blot analysis เป็นที่น่าสนใจว่าการให้ L-ARG (1000 μg/mL) เดี่ยวๆ สามารถเพิ่มการหลั่งไนตริกออกไซด์ได้ แต่เมื่อให้ร่วมกับสารสกัดพริกหรือแคปไซซินกลับไปลบล้างผลการเพิ่มปริมาณไนตริกออกไซด์ที่เกิดขึ้นจากสารสกัดพริกหรือแคปไซซิน สารสกัดพริกที่ความเข้มข้น 10 μg/mL และแคปไซซินที่ความเข้มข้น 1 μM ช่วยลดการเกิด apoptotic nuclei ในเซลล์เยื่อบุหลอดเลือดที่เกิดจากการเหนี่ยวนำของ LPS*

***สรุป:*** *สารสกัดพริกและแคปไซซินช่วยเสริมการทำหน้าที่ของเซลล์เยื่อบุหลอดเลือด และปกป้องเซลล์จากการตายแบบ apoptosis ที่เกิดจากการเหนี่ยวนำของ LPS ดังนั้น การรับประทานพืชในตระกูลพริกเป็นประจำ อาจช่วยเสริมสร้างความแข็งแรงของเซลล์เยื่อบุหลอดเลือดและลดความเสี่ยงในการเกิดโรคหัวใจและหลอดเลือด*