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MECHANISMS UNDERLYING THE ENDOTHELIUM-DEPENDENT VASODILATORY EFFECT OF AN AQUEOUS EXTRACT OF *ELAEIS GUINEENSIS* JACQ. (ARECACEAE) IN PORCINE CORONARY ARTERY RINGS

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**Key words:** *Elaeis guineensis*, endothelium, eNOS, coronary artery

**Abbreviations:** EGE, *Elaeis guineensis* extract; NO, nitric oxide; eNOS, endothelial NO synthase; EDHF, endothelium-derived hyperpolarizing factor.

## Abstract

This study was undertaken to investigate the vasodilatory effect of an aqueous extract of *Elaeis guineensis* Jacq (EGE) in the porcine coronary artery and elicit its possible mechanism(s) of action. Vascular effects of crude extract of dried and powdered leaves of *Elaeis guineensis* were evaluated on isolated coronary arteries on organ chambers. Determination of eNOS expression and the phosphorylation level of eNOS were determined by Western blot analysis. In the presence of indomethacin, EGE caused pronounced relaxations in endothelium-intact but not in endothelium-denuded coronary artery rings. Relaxations to EGE were significantly reduced by N<sup>o</sup>-nitro-L-arginine (L-NA, a competitive inhibitor of NO synthase), slightly but not significantly by charybdotoxin plus apamin (two potent inhibitors of EDHF-mediated responses) and abolished by the combination of L-NA and charybdotoxin plus apamin. Relaxations to EGE were abolished by the membrane permeant, SOD mimetic, MnTMPyP, and significantly reduced by wortmannin, an inhibitor of PI3-kinase. Exposure of endothelial cells to EGE increased the phosphorylation level of eNOS at Ser1177 in a time and concentration-dependent manner. MnTMPyP abolished the EGE-induced phosphorylation of eNOS. In conclusion, the obtained data indicate that EGE induces pronounced endothelium-dependent relaxations of the porcine coronary artery, which involve predominantly NO. The stimulatory effect of EGE on eNOS involves the redox-sensitive phosphorylation of eNOS at Ser1177 most likely via the PI3-kinase pathway.

## Introduction

Endothelium-derived relaxing factors, nitric oxide (NO) and endothelium-derived relaxing factor (EDHF), play an important role in the control of vascular homeostasis. NO inhibits vascular tone, and the proliferation and migration of vascular smooth muscle cells, and it also decreases platelet adhesion and aggregation (Ignarro, 1989; Radomski et al., 1987). NO also decreases the adherence of other blood cells such as leukocytes (Kubes et al., 1991). Endothelial dysfunction characterized by a blunted formation and/or bioavailability of NO is an early hallmark of most types of cardiovascular diseases including atherosclerosis and hypertension (Cines et al., 1998; Luscher and Barton, 1997). Therefore, the development of new therapeutic agents capable of restoring the formation and/or improving the bioavailability of NO is warranted to retard/ameliorate the development of major cardiovascular diseases.

Numerous experimental and clinical studies suggest that polyphenols present in fruit, vegetables and plants, might be of interest to restore the protective function of endothelial cells (Curin and Andriantsitohaina, 2005). Indeed, natural dietary polyphenolic compounds and natural products from plant induced endothelium-dependent vasorelaxation via enhanced formation of NO (Nishioka *et al.*, 2007; MnNeill and Jurgens, 2006). They act on many signaling cascades such as the activation of the Src/PI3-kinase/Akt signaling pathway through a redox-sensitive mechanism, by increasing the influx of extracellular  $Ca^{2+}$  and the mobilization of intracellular in endothelial cells (Dell'Agli *et al.*, 2004; Anselm *et al.*, 2007; Edirisinghe *et al.*, 2008). *Elaeis guineensis* is a big tree of about 15 to 30 m high, growing across the world mainly in Southeast Asia (Indonesia and Malaysia), South America (Brasilia) and Africa (Nigeria, Cameroon, Senegal etc). The fresh palm oil produced from the fruits of *Elaeis guineensis* is consumed in these countries in the diet (Ebong *et al.*, 1999). In addition, an anti-inflammatory activity has been observed with a water extract of *Elaeis guineensis* leaves (Kweifio-Okai, 1991). Also, 1.5 ml/kg of supernatant of fresh palm oil has equally been reported to protect the liver against acetaminophen-induced hepatotoxicity, which was postulated to be mediated via antioxidant and/or free radical scavenging activities (Adeneye and Benebo, 2007). Moreover, the methanolic palm leaves extract of *Elaeis guineensis* induced marked endothelium-dependent relaxations of the rat thoracic aorta and the mesenteric vascular bed (Abeywardena *et al.*, 2002). The aim of the present study was to investigate the endothelium-dependent vasodilatory activity of an *Elaeis guineensis* leaves in the porcine coronary artery and to determine the role of both NO and EDHF. In addition, the signaling pathway leading to the endothelial formation of NO was also investigated.

## Materials and Methods

### Chemicals

Apamin, charybdotoxin, indomethacin, bradykinin, and  $N^{\circ}$ -nitro-L-arginine (L-NA) were from Sigma. Wortmannin, and MnTMPyP were obtained from Alexis Chemicals. U46619 (9,11-dideoxy-11 $\alpha$ , 9 $\alpha$ -epoxymethano-prostaglandin F<sub>2 $\alpha$</sub> ) was purchased from Cayman Chemical Company (USA). When used the plant extract was dissolved in water.

### Preparation of *Elaeis guineensis* extract

Leaves of *Elaeis guineensis* voucher No. 1650) were collected in the Botanical Garden in July 2006 in Senegal, West Africa. The authenticity of *Elaeis guineensis* was confirmed by Doctor Diatta Williams a botanist at the Laboratoire de Pharmacognosie, Faculté de Médecine, Pharmacie et Odonto-Stomatologie, Université Cheikh Anta Diop, Dakar, Senegal. Leaves were dried during a week at room temperature, to avoid the risks of mold formation. Dried and powdered leaves (100g) were boiled for 30 min with 1000 ml of water. After filtration, the solvent of the filtrate was evaporated in a rotavapor to give a *Elaeis guineensis* extract (yield 14.78 %; w/w). The residue obtained was brown.

### Phytochemical study

Qualitative phytochemical groups of polyphenols and alkaloids were detected by colorimetric assay (Bruneton, 1993). Tannins and flavonoids were identified by 5% aqueous ferric chloride and alkaloids using the Dragendorff's reagent. The concentration of polyphenols in the aqueous extract of *Elaeis guineensis* was evaluated by measuring for total phenol content using the Folin-Ciocalteu procedure (Bruneton, 1993).

### Vascular Reactivity Studies

Left anterior descending coronary arteries (obtained from the local slaughterhouse) were cleaned of connective tissue and cut into rings (4-5 mm in length). To ensure tissue integrity, the coronary arteries were transported by a cooler containing Krebs. Rings were suspended in organ baths containing oxygenated (95% O<sub>2</sub>; 5% CO<sub>2</sub>) Krebs bicarbonate solution (composition in mmol/liter: NaCl 119, KCl 4.7, KH<sub>2</sub>PO<sub>4</sub> 1.18, MgSO<sub>4</sub> 1.18, CaCl<sub>2</sub> 1.25, NaHCO<sub>3</sub> 25 and D-glucose 11, pH 7.4, 37°C) and the cyclooxygenase inhibitor indomethacin (10  $\mu$ mol/liter), for the determination of changes in isometric tension. Following equilibration for 90 mins under a resting tension of 5g, rings were twice contracted with KCl (80 mmol/liter). Thereafter, the rings were contracted with the thromboxane mimetic U46619 (1 to 60 nmol/liter) to about 80% of the maximal contraction and the integrity of the endothelium was checked with bradykinin (0.3  $\mu$ mol/liter). After washout and a 30-min equilibration period, rings were again contracted with U46619 before a concentration-relaxation curve to either EGE or bradykinin was constructed. In some experiments, rings were exposed to an inhibitor for 30 mins before the addition of U46619.

### Culture of porcine coronary endothelial cells

Porcine coronary artery segments were flushed with phosphate buffered saline solution (pH 7.4) without calcium to remove remaining blood. Thereafter, endothelial cells were isolated by collagenase treatment (type I, Worthington, 1 mg/ml for 12 mins at 37°C) and cultured in culture dishes coated with collagen (type I prepared from rat tail; 60 ng/ml) containing medium RPMI1640/M199 (vol/vol) and 15% fetal calf serum supplemented with penicillin (100 U/ml), streptomycin (100 U/ml), fungizone (250 µg/ml), L-glutamine (2 mmol/liter) and grown for 48-72 hr. All experiments were performed with confluent cultures of cells used at first passage. They were exposed to serum-free culture medium in the presence of 0.1% bovine serum albumin for 6 h prior to treatment.

### Determination of eNOS expression and the phosphorylation level of eNOS

After treatment, cells were washed twice with phosphate buffered saline solution and then lysed in extraction buffer (composition in mmol/liter: Tris/HCl 20 (pH 7.5), NaCl 150, Na<sub>3</sub>VO<sub>4</sub> 1, sodium pyrophosphate 10, NaF 20, okadaic acid 0.01, a tablet of protease inhibitor and 1% Triton X-100). Total proteins (20 µg) were separated on 20% SDS-polyacrylamide gels at 70 V for 2.5 h. Separated proteins were transferred electrophoretically onto polyvinylidene difluoride membranes (Amersham) at 100 V for 120 mins. Membranes were blocked with blocking buffer containing 3% bovine serum albumin, Tris-buffered saline solution and 0.1 % Tween 20 (TBS-T) for 1 hr. For detection of phosphorylated eNOS and eNOS, membranes were incubated with the respective primary antibody (p-eNOS Ser1177 and eNOS; dilution of 1:1,000; Cell Signaling Technology, Beverly, MA) overnight at 4°C. After washing, membranes were incubated with the secondary antibody (peroxidase-labeled anti-rabbit IgG, 1:5000 for p-eNOS and peroxidase-labeled anti-mouse IgG; 1:20000 for eNOS; Cell Signaling Technology, Beverly, MA) at room temperature for 60 mins. Prestained markers (Invitrogen, Life Technologies, Carlsbad, CA) were used for molecular mass determinations. The immunoreactive bands were detected by enhanced chemiluminescence (Amersham).

### Statistical analysis

Data are expressed as means ± SEM. Statistical evaluation was performed with Student's *t* test for paired data or ANOVA followed by Fischer's protected least significant difference test where appropriate. Values of *P* < 0.05 were considered statistically significant.

## Results

### Characterization of the *Elaeis guineensis* extract

The phytochemical screening of the aqueous leaves extract indicated that the main chemical substances included flavonoids, tannins and alkaloids (Data not shown). In addition, using the Folin-Ciocalteu procedure, our data show that the total polyphenolic content of the extract was 107 mg gallic acid equivalent per g of extract.

### EGE induces endothelium-dependent relaxations of coronary arteries

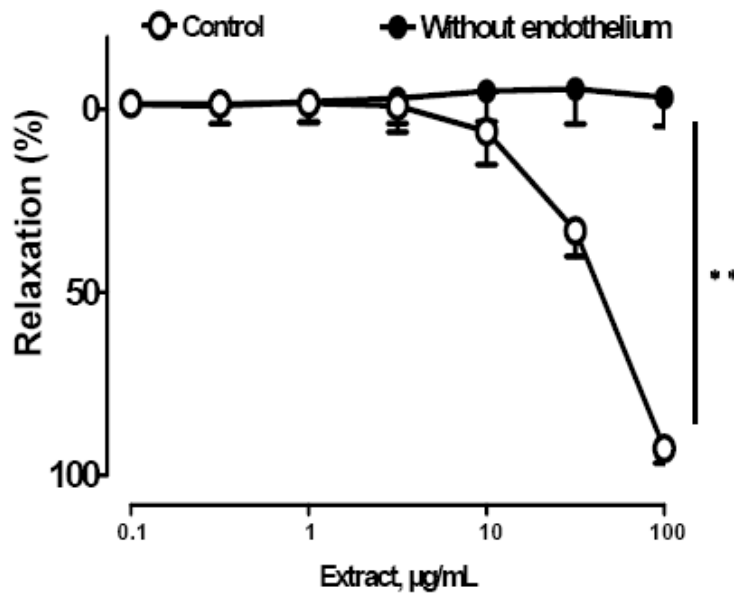
In the presence of indomethacin, EGE induced pronounced concentration-dependent relaxations in porcine coronary artery rings with endothelium but not in those following mechanical removal of endothelial cells (Figure 1). Relaxations to EGE were markedly reduced by L-NA (a competitive inhibitor of NO synthase) (*P*<0.01) and slightly but not significantly by the combination charybdotoxin plus apamin (two potent inhibitors of EDHF-mediated responses, Figure 2). In addition, they were also abolished by the combination of L-NA and charybdotoxin plus apamin (Figure 2) (*P*<0.01).

### Role of ROS and the PI3-kinase in the NO-mediated relaxation to EGE

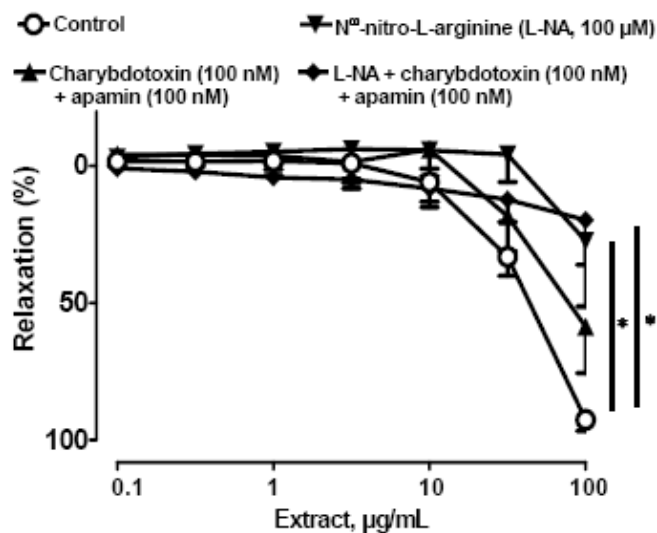
The role of superoxide anions and the PI3-kinase in the EGE-induced endothelium-dependent NO-mediated relaxation was assessed. Relaxations to EGE were abolished by the membrane permeant SOD mimetic MnTMPyP and by wortmannin, an inhibitor of PI3-kinase (Figure 3) (*P*<0.01). In contrast to EGE, bradykinin-induced endothelium-dependent relaxations were not affected by wortmannin (data not shown). Thus, these findings indicate that the EGE-induced NO-mediated endothelium-dependent relaxations is dependent on a redox-sensitive event and the PI3-kinase pathway.

**EGE induces the phosphorylation of endothelial NO synthase**

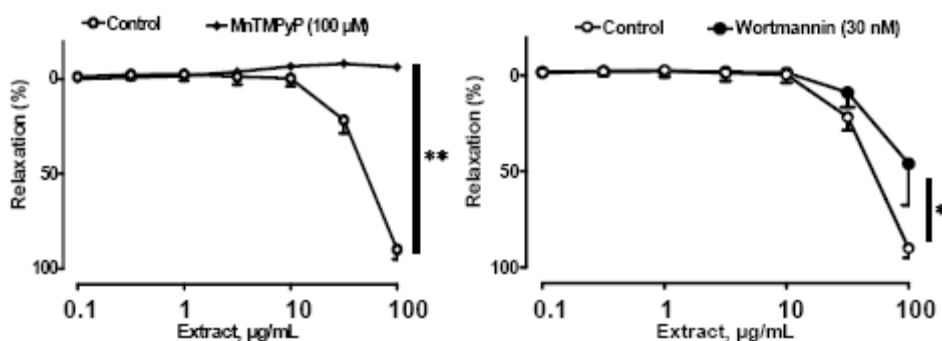
Unstimulated endothelial cells had a low level of phosphorylated *eNOS* at Ser1177 (Figure 4). Exposure of cells to EGE increased within 10 mins the phosphorylation level of *eNOS* at Ser1177, which was observed at concentrations of or greater than 100 µg/ml (Fig. 4). The EGE-induced phosphorylation of *eNOS* at Ser1177 was time dependent starting at 3 mins and this effect persisted at least for 30 mins (Figure 4). The EGE-induced phosphorylation of *eNOS* at Ser1177 was abolished by pretreatment of cells with the SOD mimetic MnTMPyP (Figure 4). Altogether, these results suggest that EGE caused the phosphorylation of *eNOS* at Ser1177 through the redox-sensitive event.



**Figure 1:** Concentration-dependent relaxations of porcine coronary artery rings to EGE in the presence or absence of endothelium. All experiments were performed in the presence of indomethacin (10 µmol/liter). Results are shown as means ± SEM of 6 different experiments. \**P* < 0.05 versus control; EGE produced a significant effect from 50 µg/mL. **EGE produced a significant effect from 50 µg/mL**

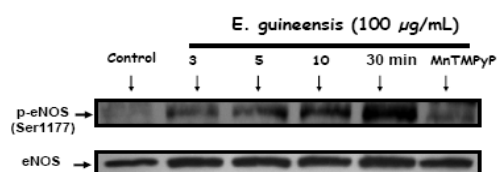


**Figure 2:** Endothelium-dependent relaxations of porcine coronary artery rings to EGE involve mainly NO. Coronary artery rings with endothelium were untreated or exposed to *N*<sup>o</sup>-nitro-L-arginine (L-NA, 100 µmol/liter) or the combination of charybdotoxin (100 nmol/liter) plus apamin (100 nmol/liter, two inhibitors of EDHF-mediated responses) or L-NA, charybdotoxin plus apamin for 30 min before addition of increasing concentrations of EGE. All experiments were performed in the presence of indomethacin (10 µmol/liter). Results are shown as means ± SEM of 6 different experiments. \**P* < 0.05 versus control; EGE produced a significant effect from 50 µg/mL. **EGE produced a significant effect from 50 µg/mL**

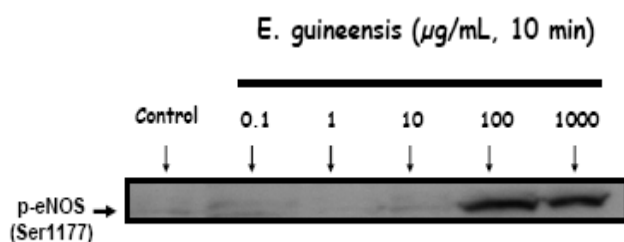


**Figure 3:** Role of superoxide anions and the PI3-kinase in the EGE-induced endothelium-dependent relaxations of coronary artery rings. Rings were exposed to either solvent, MnTMPyP (a membrane permeant superoxide dismutase mimetic, 100 μmol/liter, A) or wortmannin (an inhibitor of PI3-kinase, 30 nmol/liter, B) for 30 min before the contraction to U46619. All experiments were performed in the presence of indomethacin (10 μmol/liter) and charybdotoxin (100 nmol/liter) plus apamin (100 nmol/liter). Results are shown as means ± SEM of 6 different experiments. \**P* < 0.05 versus control; EGE produced a significant effect from 50 μg/mL (Figure A) and from 100 μg/mL (Figure B). EGE produced a significant effect from 50 μg/mL (Figure A) and from 100 μg/mL (Figure B)

(A)



(B)



**Figure 4:** EGE caused time-dependent (A) and concentration-dependent (B) phosphorylation of eNOS at Ser1177. The level of p-eNOS and eNOS expression was determined by Western blot analysis. All experiments were performed in the presence of indomethacin (10 μmol/liter) and charybdotoxin (100 nmol/liter) plus apamin (100 nmol/liter). Similar findings were observed with four different cultures of endothelial cells.

**Discussion**

The present findings indicate that the aqueous polyphenol rich extract of *Elaeis guineensis* induced pronounced endothelium-dependent relaxations of coronary artery rings, which involved predominantly NO. A

previous study has also shown that a polyphenolic rich extract of oil palm fronds of *Elaeis guineensis* caused endothelium-dependent relaxations in the rat aorta and the isolated perfused mesenteric arterial bed (Abeywardena et al., 2002). In addition, endothelium-dependent relaxations have been observed to a variety of plant extracts such as those from *Euterpe oleracea* (Aracaceae), *Epimedii herba* (Berberidaceae), *Mitragyna inermis* (Rubiaceae) and *Paulinia pinnata* (Sapindaceae) (Rocha et al., 2007; Zamble et al., 2006; Ouedraogo et al., 2004; Ajay et al., 2007; El-Hilaly et al., 2004; Ghayur et al., 2005).

Several studies have indicated that ROS and in particular superoxide anions have a determinant role in the signal transduction pathway leading to eNOS activation in response to red wine extract, Concord grape juice and epigallocatechin-3-o-gallate (Anselm et al., 2007; Ndiaye et al., 2005; Kim et al., 2007a). The moderate pro-oxidant signal has been shown to trigger activation of the redox-sensitive signal transduction pathway involving Src/PI3-kinase/Akt, which ultimately increases the endothelial formation of NO by changing the phosphorylation level of eNOS (Anselm et al., 2007; Ndiaye et al., 2005). Consistent with those previous findings, the present ones indicate that EGE induced endothelium-dependent NO-mediated relaxations in coronary artery rings and the phosphorylation eNOS at Ser1177 in cultured endothelial cells, both of which are abolished by the SOD mimetic MnTMPyP indicating the involvement of superoxide anions. They are also consistent with the fact that inhibition of the PI3-kinase by wortmannin significantly reduced endothelium-dependent relaxations to EGE.

The present findings indicate also that exposure of endothelial cells to EGE for 30 mins did not affect the expression level of eNOS. Similarly, eNOS expression was not affected by short-term treatment of endothelial cells with red wine polyphenols, Concord grape juice or icariin, a flavonoid from *Epimedii herba* (Anselm et al., 2007; Ndiaye et al., 2005; Xu and Huang, 2007). In contrast, the Chinese herb Danshen significantly increased total eNOS and p-eNOS in endothelial cells after an exposure period of 5 mins and 1hr (Kim et al., 2007b). Moreover, long-term exposure of endothelial cells to several sources of polyphenols caused an up-regulation of the expression level of eNOS (Leikert et al., 2002; Wallerath et al., 2005).

Altogether, the present findings indicate that EGE is a potent endothelium-dependent vasodilator and that this effect is due to the increased endothelial formation of NO via redox- and PI3-kinase-dependent mechanisms. Further studies are needed to identify the compounds responsible for the vaso-relaxant activity of EGE. The present results may contribute to explain the antihypertensive effects of palm oil, which are due, at least in part, to an increased formation of endothelium-derived relaxing factors (Bayorh et al., 2005; Ganafa et al., 2002).

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