

## Activation of Nitric Oxide Signaling Pathway Mediates Hypotensive Effect of *Muntingia calabura* L. (Tiliaceae) Leaf Extract

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**Abstract:** The cardiovascular effect of the crude methanol extract from the leaf of *Muntingia calabura* L. (Tiliaceae) was investigated in the anesthetized rats. The crude methanol extract was sequentially fractionated to obtain the water-soluble extract (WSE). Intravenous administration of the WSE (10, 25, 50, 75 or 100 mg/kg) produced an initial followed by a delayed decrease in systemic arterial pressure (SAP) in a dose-dependent manner. The *M. calabura*-induced initial hypotension lasted for 10 min and the delayed depressor effect commenced after 90 min and lasted for at least 180 min post-injection. The same treatment, on the other hand, had no appreciable effect on heart rate (HR) or the blood gas/electrolytes concentrations. Both the initial and delayed hypotensive effects of WSE (50 mg/kg, i.v.) were significantly blocked by pre-treatment with a nonselective nitric oxide (NO) synthase (NOS) inhibitor, *N*<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME, 0.325 mg/kg/min for 5 min) or a soluble guanylate cyclase (sGC) inhibitor, 1H-[1,2,4]oxadiazole[4,3- $\alpha$ ]quinoxalin-1-one (ODQ, 0.2 mg/kg/min for 5 min). Moreover, whereas the initial depressor effect of WSE was inhibited by pre-treatment with a selective endothelial NOS (eNOS) inhibitor, N5-(1-Iminoethyl)-L-ornithine (L-NIO, 1 mg/kg/min for 5 min), the delayed hypotension was attenuated by a selective inducible NOS (iNOS) inhibitor, S-methylisothiourrea (SMT, 0.5 mg/kg/min for 5 min). Administration of WSE also produced an elevation in plasma nitrate/nitrite concentration, as well as an increase in the expression of iNOS protein in the heart and thoracic aorta. These results indicate that WSE from the leaf of *M. calabura* elicited both a transient and delayed hypotensive effect via the production of NO. Furthermore, activation of NO/sGC/cGMP signaling pathway may mediate the *M. calabura*-induced hypotension.

**Keywords:** *Muntingia calabura*; Tiliaceae; Nitric Oxide; Nitric Oxide Synthase; Hypotension.

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## Introduction

*Muntingia calabura* L. (Tiliaceae), a specie of Genus *Muntingia*, is a fast-growing evergreen tree distributed in tropical American. *M. calabura* is 7–10 meter in height with branches spreading near horizontally. The flowers, borne singly, are approximately 1.5 cm in width with green sepals and white petals. Its leaves are 5–12.5 cm long in lanceolated or oblong shaped, long and pointy at the apex and covered with sticky hairs. Its red fruits are edible and the roots are used as emmenagogue and abortifacient. In some parts of Southeast Asia, the flowers of *M. calabura* are used for treatment of headaches and incipient colds. The same parts of this plant are also used as a tranquillizer, antispasmodics or antidiyspeptics (Kaneda *et al.*, 1991; Nshimo *et al.*, 1993). Recently, ethyl acetate-soluble extract of the leaves of *M. calabura*, and its major constituent, flavonoids, have been reported to have cancer chemopreventive effects (Su *et al.*, 2003).

*M. calabura* tree is widely cultivated in gardens and along roadsides for ornamental and edible purposes in southern Taiwan (Liu and Lo, 1993). Phytochemical studies of various parts of this plant have identified many bioactive flavonoids, chalcones, sesquiterpene and phenolic compounds (Seetharaman, 1990; Kaneda *et al.*, 1991; Nshimo *et al.*, 1993; Wong *et al.*, 1996; Su *et al.*, 2003; Chen *et al.*, 2005). It has been shown that flavonoid compounds present in various plants exert beneficial effects on cardiovascular diseases such as stroke, coronary artery disease, atherosclerosis and hypertension (Wang and Ng, 1999). These beneficial effects have been partly attributed to their ability to modulate nitric oxide (NO) pathways (Achike and Kwan, 2003).

NO, originally identified as an endothelium-derived messenger molecule, plays an important role in the control of cardiovascular homeostasis by eliciting a wide range of vascular functions such as vasorelaxation and anti-inflammatory activity, anti-platelet activity (Moncada and Higgs, 1993). NO is synthesized from the substrate L-arginine in the presence of NO synthase (NOS). Of the three isoforms of NOS that have been identified, endothelial and neuronal NOS (eNOS and nNOS) are expressed constitutively, whereas inducible NOS (iNOS) induced under pathological conditions (Fostermann *et al.*, 1995). At a cellular level, most of the effects of NO are mediated through its activation of soluble guanylate cyclase (sGC), resulting in the amplification of the production of cyclic guanosine 3',5'-monophosphate (cGMP) in target cells (Arnold *et al.*, 1977).

In the present study, we investigated the hypotensive effects of the water-soluble extract (WSE) from the leaf methanol extract of *M. calabura* in anesthetized normotensive rats, and delineated the involvement of the NO/sGC/cGMP signaling pathway in the cardiovascular actions. We found that the WSE from the leaf of *M. calabura* produced both a transient and delayed phases of hypotensive actions that engaged both eNOS-derived and iNOS-derived NO through cellular processes mediated by activation of NO/sGC/cGMP pathway.

## Materials and Methods

### *Plant Materials and Extraction*

The leaves of *M. calabura* L. (Tiliaceae) were collected in Kaohsiung city, Taiwan, in June 2001 and were identified by Professor Ih-Sheng Chen at Kaohsiung Medical University. A dried voucher specimen was deposited in the herbarium (No. Chen 6103) of the School of Pharmacy, Kaohsiung Medical University, Kaohsiung, Taiwan. The dried leaves (5 kg) of *M. calabura* were extracted by maceration with methanol under room temperature for three days and the extract was concentrated under reduced pressure. The crude methanol extract (290 g) was partitioned with distilled water and chloroform (1:1), yielding approximately 70 g of chloroform-soluble fraction and 211 g of aqueous fraction. The aqueous fraction was sequentially fractionated with a 1:1 mixture of distilled water and *n*-butanol, to obtain the water-soluble (112 g) and *n*-butanol-soluble extracts (90 g). The water-soluble extract (WSE) was prepared for animal study. The components in the leaf extract of *M. calabura* were analyzed by column chromatography and mass spectrometry in our laboratory (Chen *et al.*, 2005). Flavonoids were found to be the major components in the extract. This extract was subsequently used in the animal experiment to examine the cardiovascular effects of leaf extract of *M. calabura*.

### *General Animal Preparation*

Experiments were performed in adult male normotensive Sprague-Dawley rats (250–300 g, *n* = 127) purchased from the Experimental Animal Center of the National Science Council (Taipei, Taiwan). All rats were kept under conditions of constant temperature (23 ± 2°C) with a standard light/dark cycle (12-hour light/12-hour dark) and free access to food and water. All experiment procedures were conducted in compliance with the guidelines of our institutional animal care committee and were in accordance with the Guide for the Care and Use of Laboratory Animals as published by the US National Institute of Health. All efforts were made to reduce the numbers of animals used and to minimize animal suffering during the experiment.

Animals were anesthetized initially with pentobarbital sodium (50 mg/kg, i.p.) for the performance of preparatory surgery. This surgery routinely included intubation of the trachea to facilitate ventilation and cannulation of the right femoral artery and vein to measure systemic arterial pressure (SAP) and to administer test chemicals. The left femoral vein was also cannulated for continuous infusion of propofol (Zeneca Pharmaceuticals, Macclesfield, UK) at 25–30 mg/kg/h. This scheme provides satisfactory anesthetic maintenance and preserves the capacity for neural control of cardiovascular functions (Yang *et al.*, 1995).

The animal body was thereafter placed on a thermostatically controlled pad to maintain rectal temperature of  $37 \pm 0.5^\circ\text{C}$ . Pulsatile and mean systemic arterial pressure (MSAP) and heart rate (HR) were recorded on a polygraph (Gould RS 3400, Valley View, Ohio, USA). The animal was ventilated mechanically (Harvard 683, UK) to maintain end-tidal  $\text{CO}_2$  within 4.0–4.5%, as monitored by a capnograph (Datex Normocap, Helsinki, Finland). This procedure was conducted to minimize possible confounding cardiovascular changes secondary to respiratory perturbation. All data were collected from animals with a steady baseline MSAP above 90 mmHg before administration of the test agents.

#### *Preparation of Test Agents*

The WSE of *M. calabura* was dissolved in isotonic normal saline (0.9 % w/v) to make a stock solution with concentration of 100 mg/ml. Five different doses at 10, 25, 50, 75 or 100 mg/kg were systemically injected into the animals. Additional test agents used in this study included a cholinergic receptor agonist (Furchgott and Zawadaki, 1980), acetylcholine chloride (Sigma Chemical Co., St. Louis, MO, USA); a nonselective NOS inhibitor (Rees *et al.*, 1990),  $N^G$ -nitro-L-arginine methyl ester ( $\text{L}$ -NAME) (Sigma Chemical); a selective eNOS inhibitor (Rees *et al.*, 1990), N5-(1-Iminoethyl)-L-ornithine ( $\text{L}$ -NIO) (Tocris Cookson Inc., Bristol, UK); a selective nNOS inhibitor (Moore *et al.*, 1993), 7-nitroindazole (7-NI) (Tocris Cookson); a selective iNOS inhibitor (Szabo *et al.*, 1994), S-methylisothiourea (SMT) (Tocris Cookson), or a sGC inhibitor (Garthwaite *et al.*, 1995), 1H-[1,2,4]oxadiazole[4,3- $\alpha$ ]quinoxalin-1-one (ODQ) (Tocris Cookson). All chemicals were freshly prepared before use and administered in a volume of 1 ml/kg body weight, while acetylcholine was given in a volume of 100  $\mu\text{l}$ . They were dissolved in isotonic normal saline, with the exception of ODQ, which was dissolved in dimethyl sulfoxide (DMSO). The normal saline served as the vehicle and volume control for all agents. The only exception was ODQ, for which DMSO served as the vehicle control. Control experiments showed that these vehicles had no significant effect on baseline MSAP or HR during an observation period of 180 min.

#### *Measurement of Blood pH/Gas/Electrolytes*

The effect of WSE on baseline blood pH/gas/electrolytes was analyzed before and 10, 30, 60, 120 and 180 min after a single dose (50 mg/kg) of plant extract treatment. At each post-injection interval, 0.2 ml of arterial blood from animal was collected. Any blood withdrawn was immediately replaced by intravenous injection of an equal amount of normal saline. Blood pH, sodium ( $\text{Na}^+$ ), potassium ( $\text{K}^+$ ), calcium ( $\text{Ca}^{2+}$ ), hematocrit (Hct), partial pressure of carbon dioxide ( $\text{pCO}_2$ ) and oxygen ( $\text{pO}_2$ ) were determined using Rapidlab 348 automatic Blood Gas/Electrolytes Analyzer (Bayer Co., East Walpole, MA, USA).

*Chemiluminescence Assay of Plasma Nitrate Levels*

NO concentration in plasma was determined using a chemiluminescence analyzer. At various post-injection time intervals, 0.5 ml of blood samples were collected from the femoral artery. Any blood withdrawn was immediately replaced by intravenous injection of an equal amount of saline. The blood samples were centrifuged (3000 rpm for 12 min) to prepare plasma and these plasma were subsequently centrifuged for 12 min at 6900 rpm (4°C) through a 30 kDa molecular weight cut-off filter using a microfuge ultrafiltration device (Millipore Co., Bedford, MA, USA) to remove the presence of hemoglobin and improve detection sensitivity. The ultrafilter samples were stored at -80°C for no more than 2 weeks before assay. The nitrate concentration in plasma depicted in this study is actually the total nitrite and nitrate (NO<sub>x</sub>) concentration in plasma. In plasma, NO reacts with molecular oxygen to form nitrite and with oxyhemoglobin and superoxide anion to form nitrate. The nitrite and nitrate were reduced to NO using 0.8% vanadium (III) and 1 N hydrochloric acid at 92°C. The NO was stripped from the plasma (5 µl) using a helium purge gas and then drawn into the NO Analyzer (Sievers NOA 280, Sievers Inc., Boulder, Colorado). NO<sub>x</sub> contents were calculated by comparison with standard solutions of sodium nitrate (Sigma Chemical).

*Protein Extraction and Western Blot Analysis*

For biochemical experiments, rats were killed with an overdose of pentobarbital sodium and perfused intracardiacally with 150 ml of warm (37°C) saline containing heparin (100 U/ml). The apical heart or a 1-cm segment of thoracic aorta was rapidly removed, placed on dry ice and then trimmed adipose and connective tissues. The same samples thus obtained from 3–4 rats under the same experimental condition were pooled and stored at -80°C until further analysis.

Western blot analysis of iNOS, eNOS, nNOS, or β-actin was carried out as reported previously (Chan *et al.*, 2001). A rabbit polyclonal antiserum against iNOS, eNOS, nNOS, or β-actin (Santa Cruz Biotechnology) was used as the primary antiserum, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson). Specific antibody-antigen complex was detected with an enhanced chemiluminescence Western Blot detection system (NEN Life Science Products) (Chan *et al.*, 2001; Shih *et al.*, 2003). The amount of iNOS, eNOS, nNOS, or β-actin protein was quantified by Photo-Print Plus software (ETS Vilber-Lourmat), and was expressed as the ratio (%) to β-actin protein, which served as an internal control to demonstrate equal loading of the proteins.

*Experimental Protocol*

In the first set of experiments, the change of MSAP, HR or blood pH/gas/electrolytes after systemic administration of WSE at the dose of 10, 25, 50, 75 or 100 mg/kg was examined

for at least 3 hours. Each injection was given at a fixed volume of 0.2 ml to reduce possible volume effects. Same volume of isotonic normal saline served as vehicle control. In a separate series of experiments, time-course of changes in MSAP or HR elicited by an intravenous injection of acetylcholine (5 µg/kg) was included as a positive control. Changes in plasma NOx levels or the expression of eNOS or iNOS protein in the heart or aorta after the extract injection were also evaluated for 180 min.

To delineate the causative relationship between NO and *M. calabura*-induced cardiovascular responses, temporal change in MSAP or HR elicited by intravenous injections of WSE (50 mg/kg) were evaluated for 180 min in rats subjected to pre-treatment with L-NAME, L-NIO, SMT, 7-NI or ODQ, administered 20 min prior to WSE application. Each agent (L-NAME, 0.65 mg/kg/min; L-NIO, 1 mg/kg/min; SMT, 0.5 mg/kg/min; 7-NI, 6 mg/kg/min; ODQ, 0.2 mg/kg/min) was administered by continuous intravenous infusion for 5 min. The doses chosen were adopted from studies (Moore *et al.*, 1993; Szabo *et al.*, 1994; DeWitt *et al.*, 1997; Lahlou *et al.*, 2002; Preiser *et al.*, 2003) that employed the same test agents for the same purpose as in this study. The time lag was adopted to ensure a complete recovery from the cardiovascular response induced by the test agents alone before intravenous injections of plant extract or vehicle. To avoid confounding effects of drug interactions, each animal received only one treatment scheme or vehicle.

### *Statistical Analysis*

All values are expressed as mean ± SEM (standard error of mean). For functional experiments, the temporal effects of various treatments on MSAP, HR, blood pH/gas/electrolytes or plasma NOx concentrations were assessed using two-way analysis of variance (ANOVA) with repeated measures. For biochemical experiments, differences between treatment groups were assessed using one-way ANOVA. This was followed by the Scheffé multiple-range test for post-hoc assessment of individual means. Statistical significance was accepted at  $p < 0.05$ .

## **Results**

### *Effects of M. calabura on SAP, HR or Blood pH/Gas/Electrolytes*

Baseline MSAP or HR before drug treatment was similar among control and experimental groups ( $p > 0.05$ , one-way ANOVA). Averaged baseline MSAP and HR for the animals was  $107 \pm 5$  mm Hg and  $348 \pm 6$  beats/min, respectively (pooled data from total of 37 rats). In comparison to saline control, intravenous injections of WSE of *M. calabura* (10, 25, 50, 75 or 100 mg/kg) resulted in significant decreases in MSAP dose-dependently without appreciable changes in HR (Fig. 1). The crude extract promoted depressor response was characterized by an immediate decrease in MSAP (initial phase) that returned to pre-injection baseline within 10 min post-injection, followed by a delayed hypotensive effect (delayed phase) that commenced at 90 min and lasted for at least 180 min post-injection.

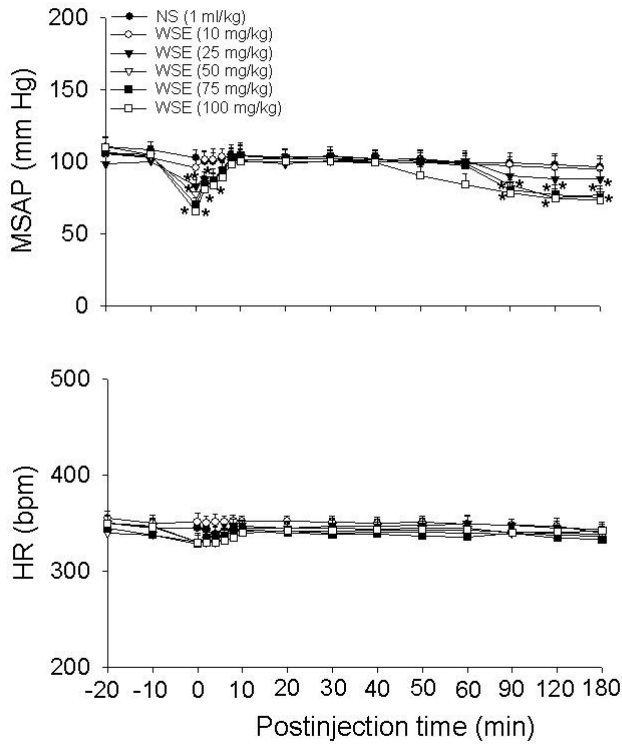


Figure 1. Time-course of the changes in mean systemic arterial pressure (MSAP) and heart rate (HR) in anesthetized animals that received (at time 0) intravenous injection of normal saline (NS, 1 ml/kg), or water-soluble extract (WSE) of *M. calabura* leaf (10–100 mg/kg). Values are presented as mean  $\pm$  SEM, n = 7–9 animals per experimental group. \*p < 0.05 vs. NS group at corresponding time points in the Scheffé multiple range test.

**Table 1. Temporal Effects of Water-Soluble Extract of *M. calabura* Leaf (50 mg/kg, i.v.) on pH/Blood Gas/Electrolytes**

	Post-injection Time (min)					
	Control	10	30	60	120	180
pH	7.4 $\pm$ 0.2	7.45 $\pm$ 0.1	7.45 $\pm$ 0.1	7.5 $\pm$ 0.1	7.5 $\pm$ 0.1	7.5 $\pm$ 0.1
pCO <sub>2</sub> (mmHg)	34 $\pm$ 4	33.7 $\pm$ 3	34.7 $\pm$ 4	32.9 $\pm$ 2	37.3 $\pm$ 3	37.9 $\pm$ 3
pO <sub>2</sub> (mmHg)	98.5 $\pm$ 7	98.3 $\pm$ 6	98 $\pm$ 8	98.7 $\pm$ 9	96.3 $\pm$ 7	99 $\pm$ 8
Na <sup>+</sup> (mM)	131 $\pm$ 4	135 $\pm$ 3	134 $\pm$ 3	130 $\pm$ 5	130 $\pm$ 4	129 $\pm$ 4
K <sup>+</sup> (mM)	3.6 $\pm$ 0.3	3.7 $\pm$ 0.3	3.4 $\pm$ 0.3	3.4 $\pm$ 0.3	3.6 $\pm$ 0.4	3.6 $\pm$ 0.3
Ca <sup>2+</sup> (mM)	1.2 $\pm$ 0.3	1.2 $\pm$ 0.2	1.2 $\pm$ 0.2	1.2 $\pm$ 0.3	1.2 $\pm$ 0.2	1.2 $\pm$ 0.2
Hct (%)	37 $\pm$ 4	38 $\pm$ 4	36 $\pm$ 3	37 $\pm$ 4	36 $\pm$ 3	38 $\pm$ 4

Values are presented as mean  $\pm$  SEM, n = 5–6 animals per experimental group. No significant difference was detected among experimental groups by two-way ANOVA.

Control injection of acetylcholine (5  $\mu\text{g}/\text{kg}$ ) also induced a significant decrease in MSAP that reached its peak ( $-40 \pm 3$  mm Hg) within the first 30 sec and lasted for less than 5 min after drug treatment.

In a separate series of experiments, we found that treatment with a single dose (50 mg/kg) of plant extract, similar to other doses of the extract (25, 75 or 100 mg/kg) or saline (data not shown), resulted in no discernible alteration in baseline systemic arterial blood gases, electrolytes, Hct or pH detected at 10, 30, 60, 120 or 180 min post-injection (Table 1).

#### *Effect of NO Synthase Inhibitor on M. calabura-Induced Hypotension*

In contrast to saline control, pre-treatment with the nonselective NOS inhibitor L-NAME (0.65 mg/kg/min, i.v.), 20 min prior to WSE administration, significantly attenuated both the initial and delayed phases of hypotension promoted by the plant extract (Fig. 2). A similar blockade of *M. calabura*-induced hypotension was also observed in animal who

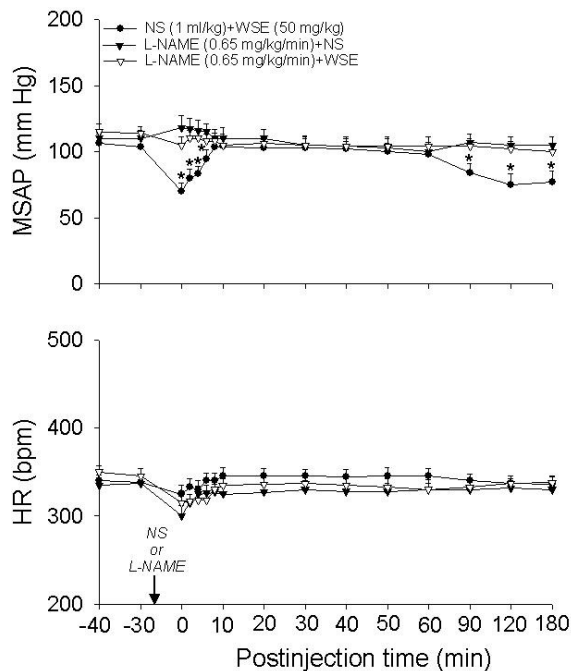


Figure 2. Time-course of the changes in mean systemic arterial pressure (MSAP) and heart rate (HR) in anesthetized animals that received (at time 0) intravenous injection of normal saline (NS, 1 ml/kg), or water-soluble extract (WSE) of *M. calabura* leaf (50 mg/kg). These animals received (arrow) an additional intravenous pre-treatment with NS (1 ml/kg), or L-NAME (0.65 mg/kg/min for 5 min) 20 min before plant extract administration. Values are presented as mean  $\pm$  SEM,  $n = 8-9$  animals per experimental group. \*  $p < 0.05$  vs. L-NAME + WSE group at corresponding time points in the Scheffé multiple range tests.



were pre-treated with a lower dose of  $L$ -NAME (0.15 or 0.35 mg/kg/min for 5 min, i.v.) (data not shown). Of the three isoforms of NOS, we found that the eNOS inhibitor,  $L$ -NIO (1 mg/kg/min, i.v.), significantly suppressed the initial (Fig. 3A), but not the delayed (Fig. 3B), phase of *M. calabura*-induced hypotension. On the other hand, the selective iNOS inhibitor, SMT (0.5 mg/kg/min, i.v.), strongly inhibited the delayed (Fig. 3B), but not the initial (Fig. 3A), phase of the same response. Pre-treatment with the nNOS inhibitor, 7-NI (6 mg/kg/min, i.v.), on the other hand, did not affect either phases of *M. calabura*-induced hypotensive responses (data not shown). We also noted that at the high dose (0.65 mg/kg/min)  $L$ -NAME by itself evoked a transient increase in MSAP by 12% ( $105 \pm 5$  vs.  $118 \pm 9$  mm Hg,  $p < 0.05$ ) and a decrease in HR by 11% ( $337 \pm 9$  vs.  $300 \pm 12$  beats/min,  $p < 0.05$ ). The low dose of  $L$ -NAME (0.15 or 0.35 mg/kg/min),  $L$ -NIO (1 mg/kg/min), 7-NI (6 mg/kg/min) or SMT (0.5 mg/kg/min), on the other hand, had no discernible effect on baseline MSAP or HR (data not shown).

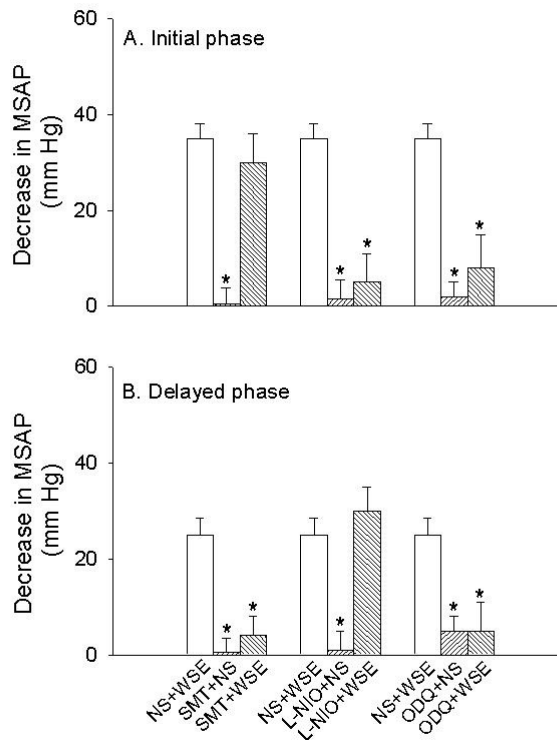


Figure 3. The maximal decreases in mean systemic arterial pressure (MSAP) measured at the initial (A) or delayed phase (B) in anesthetized animals that received intravenous injection of water-soluble extract (WSE) of *M. calabura* leaf (50 mg/kg). These animals received an additional intravenous pre-treatment of normal saline (NS, 1 ml/kg), SMT (2.5 mg/kg),  $L$ -NIO (5 mg/kg) or ODQ (1 mg/kg) 20 min before plant extract administration. Values are presented as mean  $\pm$  SEM,  $n = 8-9$  animals per experimental group. \* $p < 0.05$  vs. NS + WSE group in the Scheffé multiple range tests.

### *Effect of Soluble Guanylate Cyclase Inhibitor on M. calabura-Induced Hypotension*

In comparison to saline pre-treatment, intravenous injections of a sGC inhibitor, ODQ (0.2 mg/kg/min for 5 min), discernibly suppressed both the initial and delayed phases of decreases in MSAP elicited by the plant extract (Figs. 3A, 3B). ODQ alone had no significant effect on baseline MSAP or HR (data not shown).

### *Effects of M. calabura on Plasma Nitrate Levels*

Basal levels of plasma NO<sub>x</sub> for experimental and saline control animals were  $19.5 \pm 0.5 \mu\text{M}$  and  $19.8 \pm 0.6 \mu\text{M}$ , respectively. Treatment with WSE of *M. calabura* (50 mg/kg), increased plasma NO<sub>x</sub> levels in a time-dependent manner (Fig. 4). Such an increase in plasma NO<sub>x</sub> production, detected at 3-hour post-treatment, was significantly antagonized in animals pre-treated with a nonselective NOS inhibitor, L-NAME (0.65 mg/kg/min for 5 min) or a selective iNOS inhibitor, SMT (0.5 mg/kg/min for 5 min) (Fig. 4). We noted that surgical operation alone (sham control) did not cause a significant change in plasma NO<sub>x</sub> (at 1 hour:  $19.6 \pm 0.8 \mu\text{M}$ ; at 2 hour:  $19.8 \pm 0.7 \mu\text{M}$ ; at 3 hour:  $19.8 \pm 0.5 \mu\text{M}$ ,  $p > 0.05$ ).

### *Effects of M. calabura on Nitric Oxide Synthase Expression of the Heart and Aorta*

In comparison to its basal expression, intravenous injection of WSE of *M. calabura* (50 mg/kg) induced a significant increase in iNOS protein expression in the heart or aorta detected at 90 or 180 min post-injection (Figs. 5A, 5B). In both post-treatment time

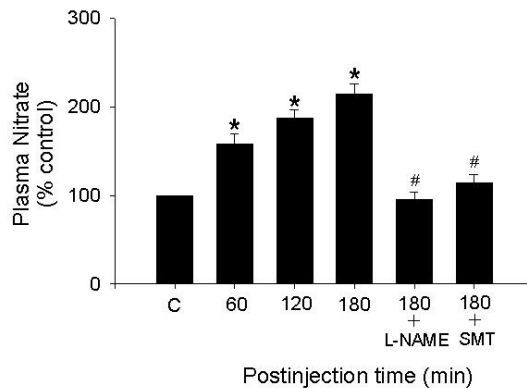


Figure 4. Time-course of the changes in the plasma nitrate/nitrite levels in anesthetized animals that received intravenous injection of water-soluble extract of *M. calabura* leaf (50 mg/kg). These animals received an additional intravenous pre-treatment of, L-NAME (3.25 mg/kg) or SMT (2.5 mg/kg) 20 min before plant extract administration. Values are presented as mean  $\pm$  SEM,  $n = 5-6$  animals per experimental group. \* $p < 0.05$  vs. pre-treatment control (C) group and # $p < 0.05$  vs. plant extract group at 180 min post-injection in the Scheffé multiple range tests.

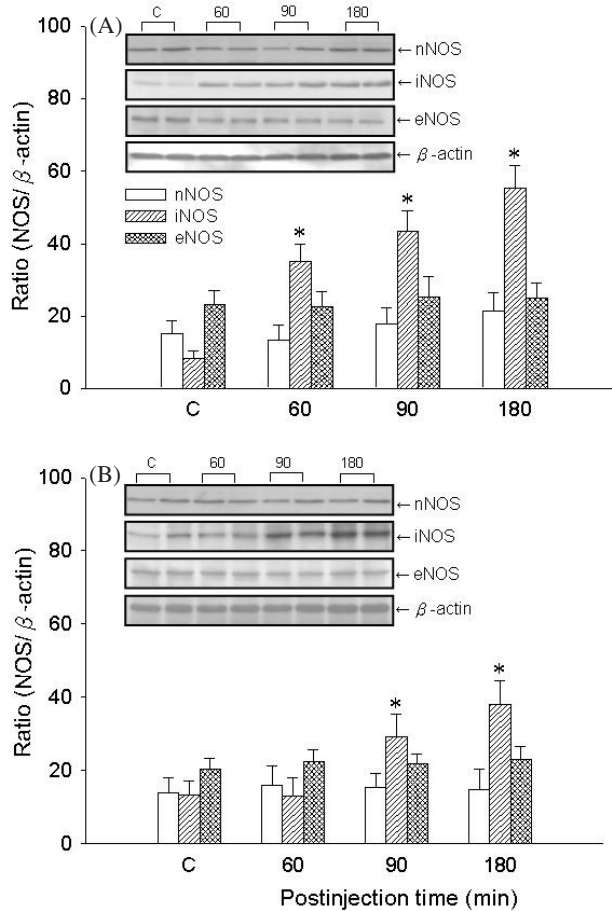


Figure 5. Representative gel (inset) or amount of eNOS, iNOS or nNOS protein relative to  $\beta$ -actin, detected from the aorta (A) or heart (B) 60, 90 or 180 min after animals received intravenous injection of water-soluble extract of *M. calabura* leaf (50 mg/kg). Values are presented as mean  $\pm$  SEM,  $n = 3-4$  animals per experimental group. \*  $p < 0.05$  vs. pre-treatment control (C) group in the Scheffé multiple range tests.

intervals, the *M. calabura*-promoted increases in iNOS were greater in the aorta than those in the heart. The same treatment, on the other hand, had no appreciable effect on the expression of eNOS or nNOS, detected at the same post-injection time intervals, in the heart or aorta.

## Discussion

The novel observations of present study demonstrate, for the first time, that the WSE of the crude leaves methanol extract of *M. calabura* promote a dose-dependent hypotensive effect in anesthetized normotensive rats. The *M. calabura*-induced hypotension manifested

two phases: an initial phase that lasted for less than 10 min and a delayed phase that commenced at 90 min and lasted for at least 180 min post-injection. Whereas the initial hypotension induced by the plant extract was inhibited by eNOS inhibitor, the delayed depressor response was reversed in the presence of iNOS inhibitor. Both phases, however, were inhibited by the non-selective NOS inhibitor or the sGC inhibitor. Biochemical studies further revealed significant increases in the expression of iNOS in the heart and aorta, as well as plasma NO<sub>x</sub> levels after WSE treatment. These results suggest that *M. calabura* leaf extract exerts hypotensive action via cellular processes that may involve production of NO and activation of the NO/sGC/cGMP signaling system.

The *M. calabura* has been widely used in both tropical American and Southeast Asia (Kaneda *et al.*, 1991; Nshimo *et al.*, 1993). In Vietnam and Malaysia, the roots have been employed as an emmenagogue and abortifacient. In East Asia, the flowers are used for treatment of headaches, incipient colds, or as a tranquilizer, antispasmodic or antidiyspeptic. Recently, ethyl acetate-soluble extract from the leaves of *M. calabura*, and its major constituent, flavonoids, have been reported to have cancer chemopreventive effects (Su *et al.*, 2003). Whether the plant extract affects the cardiovascular functions, however, is currently unknown. As such, one of the major contributions of this study is to reveal the cardiovascular depressive action of the WSE from leaf methanol extract of *M. calabura*. Our observations of a delayed long-lasting hypotensive effect of *M. calabura* provide novel evidence for potential therapeutic applications of this plant extract.

Another major contribution of this study is to demonstrate the involvement of NO in both phases of *M. calabura*-induced depressor responses. Both the initial and delayed phases of *M. calabura*-induced hypotension were inhibited by pre-treatment with the nonselective NOS inhibitor, L-NAME. Interestingly, with the application of more selective inhibitor for eNOS or iNOS, we further revealed that eNOS-derived NO contributed to the initial, whereas the iNOS-derived NO mediated the delayed phase of hypotension. The observations that ODQ reversed both phases of hypotension further indicated that *M. calabura* induces hypotension by activating the NO/sGC/cGMP signaling cascade. Many plant extracts or purified drugs derived from botanical medicinal herbs have been reported to affect the NO signaling pathway. For example, the ginsenosides from ginseng have been shown to cause vasodilation in an endothelium-dependent manner in rat aorta (Li *et al.*, 2001) via NO generation from eNOS. These vasodilatory actions of ginsenosides may account, in part, for the antihypertensive effect of ginseng (Gillis, 1997). Activation of eNOS induces short-term releases of NO, whereas iNOS produces prolonged generation of NO (Nathan, 1992; Ueda *et al.*, 2003). These differences in enzyme kinetics may explain why NO generated respectively from eNOS or iNOS participates differentially in *M. calabura*-induced initial and delayed phases of hypotension. Our observations of a lack effect of 7-NI on *M. calabura*-induced hypotension, as well as no significant change in nNOS expression after plant extract treatment also indicate a minor contribution of nNOS to the cardiovascular actions of *M. calabura*.

We demonstrated in this study that NO generated respectively by eNOS and iNOS mediates hypotension induced by *M. calabura* by activating the sGC/cGMP cascade. The release of NO from endothelial cells stimulates sGC, which leads to an increasing production

of cGMP in vascular smooth muscle (Rapoport and Murad, 1983). In this study, we found that the sGC inhibitor, ODQ, inhibited both phases of *M. calabura*-induced hypotension. These data indicate that the formation of cGMP through the activation of NO/sGC/cGMP signaling pathway results in hypotension after plant extract treatment.

To determine whether NO production was altered by *M. calabura*, we directly measured plasma NO levels. Our results showed that the WSE of *M. calabura* increased plasma NO contents in a temporal profile that correlated positively with the increases in iNOS expression. More importantly, such an increase in NO<sub>x</sub> level at late stage of hypotension was significantly inhibited by pre-treatment with a selective iNOS inhibitor. Together these observations indicate that the delayed surge of plasma NO resulted from an up-regulation of iNOS expression after plant extract injection, leading to the delayed phase of hypotension. Although in the present study we did not directly assess the putative endothelium-dependent relaxation mechanisms underlying *M. calabura*-induced hypotension, we found that the time course of the initial hypotensive response induced by the plant extract is similar to that induced by acetylcholine. It is well established that acetylcholine causes generalized vasodilation via release of NO from the vascular endothelial cells (Furchgott and Zawadski, 1980). As such, it is possible that the initial hypotensive effect of *M. calabura* may be due to an active vasodilation mediated by an endothelium-dependent NO signaling pathway. This suggestion is further supported by our findings that the selective eNOS inhibitor, L-NIO, reversed selectively the *M. calabura*-induced initial hypotension.

In addition to inducing an up-regulation of iNOS expression, the plant extract may also increase plasma NO level by directly donating NO. This possibility, however, is deemed unlikely since *M. calabura* derived compounds, such as flavonoids, chalcones, sesquiterpene and phenolic compounds, (Seetharaman, 1990; Kaneda *et al.*, 1991; Nshimo *et al.*, 1993; Wong *et al.*, 1996; Su *et al.*, 2003; Chen *et al.*, 2005) do not decompose to NO. Moreover, *M. calabura* may increase plasma NO level by prolonging the half-life of NO. One cellular process for breakdown of NO is to react with superoxide to form peroxynitrite (Gryglewski *et al.*, 1986). Compounds that are capable of scavenging superoxide are therefore effective in increasing the bioavailability of NO (Faulkner *et al.*, 1994). Whether *M. calabura* increases plasma NO level via an action on the superoxide, and its functional significance on hypotension induced by the plant extract, however, awaits further investigation.

The *M. calabura* leaf extract causes hypotension without a significant change in HR. These observations are interpreted to suggest that the cardiovascular depressive action of the plant extract is attributable mainly to its vasodilative action on the vascular smooth muscles rather than a direct action on the heart. This suggestion is supported by our observations in which *M. calabura*-induced up-regulation of iNOS expression was greater in the aorta than in the heart. Alternatively, no discernible changes in HR in response to the hypotension induced by the plant extract may suggest a suppression of the baroreceptor reflex control mechanism. A lack of effect on the systemic arterial blood gases (pCO<sub>2</sub> and pO<sub>2</sub>), electrolytes (Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>), hematocrit or pH further deems unlikely the possibility of a nonspecific action of the plant extract on general properties of the circulatory blood. A slight rise in pH from 7.4 to 7.5, though statistically insignificant, might indicate a potential

effect of the plant extract to decrease blood acidity. The hemodynamic significance of this effect, however, awaits further investigation.

The constituents responsible for the hypotensive effects of *M. calabura* have not yet been elucidated. Bioflavonoids detected in phytochemical analysis appear to be a potential candidate, since these compounds are known to exert NO-dependent vasorelaxation (Duarte *et al.*, 2001). Recent phytochemical investigations also showed that flavonoids, such as pinocembrin, pinobanksin, pinostrobin, chrysin, isoliquiritigenin and gnaphaliin, are the other major components of the leaves of *M. calabura* (Su *et al.*, 2003; Chen *et al.*, 2005). In a recent study (Duarte *et al.*, 2001), flavone chrysin was reported to induce endothelium- and NO-dependent vasorelaxation *in vitro*. This compound also exerts hypotensive effect in *in vivo* experiments (Villar *et al.*, 2002). Further study will be required to isolate the active compounds from the extract.

The selectivity of SMT as an iNOS inhibitor has been documented (Southan *et al.*, 1995). At the doses we used, SMT has a relative selectivity toward iNOS *in vitro* and *in vivo* when compared with another selective iNOS inhibitor, aminoguanidine (Szabo *et al.*, 1994) or other L-arginine-based NOS inhibitors (Szabo *et al.*, 1994). L-NIO is reported to be a useful experimental tool with which to study the roles of endothelially derived NO (Rees *et al.*, 1990). That SMT and L-NIO produced two distinct effects in the present study also points to the differentiating capacity of these iNOS and eNOS inhibitors. We also recognize the potential influence of anesthesia on *M. calabura*-induced cardiovascular responses. In this regard, the anesthetic maintenance scheme (i.e., propofol at 25–30 mg/kg/h) used in this study has been documented to induce minimal depressive action on the neurogenic sympathetic vasomotor outflow, and hence baseline MSAP and HR (Yang *et al.*, 1995; Shih *et al.*, 2003).

In summary, our results demonstrate for the first time that the WSE from the leaf of *M. calabura* exerts a transient followed by a delayed hypotensive effect via activation of NO/sGC/cGMP signaling pathway. We further revealed that whereas NO derived from eNOS induced the initial depressor response; the delayed hypotension elicited by the plant extract was mediated by NO generated by iNOS. These findings provide scientific evidence supporting the therapeutic uses of *M. calabura* in folk medicines.

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