Antioxidant and Vascular Protective Activities of *Cratoxylum formosum*, *Syzygium gratum* and *Limnophila aromatica*

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Phytochemicals contained in dietary plants provide a variety of health benefits and may reduce the risk of cardiovascular diseases. The aqueous extracts from three popular Thai dietary and herbal plants, *Cratoxylum formosum*, *Syzygium gratum*, and *Limnophila aromatica*, were investigated for the antioxidant and vascular protective activities in the *in vitro* and *in vivo* models. The free radical scavenging and antioxidant activities of plant extracts were evaluated *in vitro* by the 1,1-diphenyl-2-picrylhydrazyl assay, the ferric reducing antioxidant power assay, the intracellular antioxidant activity in rat peritoneal macrophages by dihydrofluorescein assay, and the inhibition of nitric oxide (NO) production in RAW 264.7 macrophages. In an animal model of oxidative stress and vascular dysfunction, male Sprague-Dawley rats were orally administered with aqueous plant extracts (1 g/kg/d) or *N*-acetylcysteine (NAC; 300 mg/kg/d) as a control for 6 d. On day four, all animals except the normal control group, were administered with phenylhydrazine (PHZ) intraperitoneally. It was demonstrated that the plant extracts possessed high free radical scavenging and antioxidant activities. PHZ induced severe hemolysis and hemodynamic disturbances and treatment with the extracts and NAC significantly improved the hemodynamic status. Vascular responsiveness to bradykinin, acetylcholine, and phenylephrine in PHZ-control rats was markedly impaired, and the plant extracts or NAC largely restored the vascular responses. Moreover, the plant extracts prevented loss of blood reduced glutathione and suppressed formation of plasma malondialdehyde, plasma NO metabolites and blood superoxide anion. It was concluded that the plant extracts possess antioxidants and have potential roles in protection of vascular dysfunction.

Key words oxidative stress; vascular dysfunction; *Cratoxylum formosum*; *Syzygium gratum*; *Limnophila aromatica*; antioxidant

A body of evidence suggests that free radicals play an important role in the development of tissue damage and pathological events in living organisms. Oxidative stress is found to implicate in several cardiovascular diseases, including septic shock, ischemic reperfusion injury, hear failure, atherosclerosis, hypertension and diabetes.[1,2] Epidemiological studies have shown that dietary intake of polyphenol-rich foods such as fruits and vegetables is inversely associated with the incidence of cardiovascular diseases.[3—6] Supplementation of antioxidants has been shown to alleviate vascular dysfunction in hemolytic anemia, inflammatory response syndrome, coronary heart disease, hypertension and atherosclerosis.[7—10] Several population-based studies have suggested that consumption of plants containing bioactive phytochemicals reduces the risk of developing cardiovascular diseases and cancer.[11—14] The association between risk reduction and intake of dietary herbal plants may be partly explained by the antioxidant protection, however, the mechanisms underlying such protection are not fully understood.

Diet rich in bioactive phytochemicals has long been recognized essential to maintaining healthy body systems. A large number of plant species which are important source of traditional medicine are widely cultivated throughout the kingdom of Thailand. Many of them are used as dietary plants in Southeast Asian countries and claimed for health promotion in traditional medicine. This work describes the ethnopharmacological activity of the three popular dietary and herbal plants, including *Cratoxylum formosum* (Guttiferae), *Syzygium gratum* (Myrtaceae), and *Limnophila aromatica* (Scrophulariaceae). The selected plants are mostly grown in the Northeast of Thailand and commonly consumed in daily diet. The plants are used in Thai traditional medicine as herbal remedies for the treatments of dyspepsia, indigestion, peptic ulcer, diarrhea, bacterial infection, inflammation, asthma, blood disorders, and cardiovascular disease.[15,16] To date, there are very limited scientific evidences supporting these health benefits beyond their nutrient contributions. It is, therefore, still required to verify their biological effects. Previous studies of these plants demonstrated some active compounds. The main compound in *C. formosum* is identified as chlorogenic acid which comprises 60% of the alcoholic extracts and follows by dicaffeoylquinic acid and ferulic acid derivatives.[17] *L. aromatica* contains several flavonoids, such as nevadensin, nevadensin-7-*O*-β glycopyranoside, gardenin B and other flavones, which are polyphenolic compounds with antioxidant activity.[18] Although there is no report about active compounds in *S. gratum*, several plants from *Syzygium* species contain high total phenolic contents, such as gallic acid, proanthocyanidins, and conjugated flavonoids, which suggest they are rich sources of antioxidant compounds.[19] Previous studies on biological activities of *C. formosum*, *S. gratum* and *L. aromatica* demonstrated that the crude extracts from these plants possess antimutagenic, antibacterial, and antioxidant properties.[17,18,20,21] However, scientific data supporting the antioxidant activity relating with cardiovascular diseases of these plants are still lacking. The present study was designed to investigate the antioxidant and vascular protective properties of the three Thai herbal plant extracts in both *in vitro* and *in vivo* animal model of phenylhydrazine (PHZ)-induced oxidative stress and vascular dys-

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function. The model of PHZ-induced vascular injury may be related to some clinical cardiovascular diseases including, hemolytic anemia and systemic inflammatory response syndrome, for instance septic shock.

MATERIALS AND METHODS

Preparation of Plant Extracts Fresh leaves of *Cratoxylum formosum* DYER (*C. formosum*), *Syzygium gratum* (*S. gratum*, synonym: *Eugenia grata* WIGHT), and *Limnophila aromatica* MERR (*L. aromatica*) were collected between January and March from local agricultural field in and around Khon Kaen province, Thailand. Voucher specimens, herbarium No.PSH-KKU04/10, PSH-KKU04/13 and PSH-KKU04/11, respectively, were authenticated by Dr. Srisomporn Preeprem, and deposited in the Department of Pharmaceutical Botany and Pharmacognosy, Faculty of Pharmaceutical Sciences, Khon Kaen University, Thailand.

The fresh leaves of plant samples were weighed, chopped and boiled in deionized water for 0.5 h, then filtered. The filtrates were combined, and then freeze-dried yielding residues of 3.25%, 3.34% and 1.85% per wet weight of *C. formosum*, *S. gratum*, and *L. aromatica*, respectively. There were no major differences in yield between different preparations. The crude extract was collected in a tight, light-protected container and stored at −20 °C until used. The extract was resuspended in distilled water just before use.

DPPH Radical Scavenging Assay The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of the extracts was determined according to a previous described method.[22] The test compound solutions (1.25—1250 μg/ml were added to DPPH (0.1 mM) solution (4 : 1 ratio). The mixture was incubated for 20 min at room temperature and the absorbance at 515 nm was measured. The percent of radical scavenging activity was calculated against DPPH solution without test compound. The free radical scavenging activity of ascorbic acid (0.1—100 μg/ml) was also measured, so as to compare the relative potency of the extracts. The DPPH radical scavenging ability was calculated as percentage of inhibition of DPPH absorbance, by the following equation as [(absorbance of control−absorbance of test samples/absorbance of control)×100].

Ferric Reducing Antioxidant Power (FRAP) Assay The FRAP assay was performed by using Benzie and Strain's method[23] with some modifications. The freshly prepared FRAP reagent was comprised 300 mM acetate buffer, pH 3.6; 10 mM 2,4,6-tripyridyl-s-triazine in 40 mM HCl; and 20 mM $FeCl_2$ at a 10 : 1 : 1 ratio. 300 μl of working FRAP reagent was mixed with 10 μl of water solutions of the extracts of *C. formosum*, *S. gratum* and *L. aromatica* (0.125—2.5 mg/ml) or ascorbic acid (10—500 μM). The latter was used to calibrate the FRAP assay. The mixtures were incubated for 4 min at 30 °C. The change in absorbance was monitored at 610 nm, and was translated into a FRAP value. Antioxidant activity was calculated and expressed as ascorbic acid equivalent per mg of extract (μg/mg or μmol/mg extract).

Dihydrofluorescein (DHF) Assay The intracellular antioxidant activity of the extracts was performed in rat peritoneal macrophages according to the method previously described[24] with some modifications. Briefly, 400 μl of peritoneal macrophage suspension from male Sprague-Dawley rats (230—250 g) with cell concentration of 10^6 cell/ml in Hank's buffer supplemented with 15 mM HEPES was incubated with catalase (1000 U) for 10 min at 37 °C, followed by the plant extracts, dihydrofluorescein diacetate, and phorbol-12-myristate-13-acetate (0.6 μM) to make up volume of 1.0 ml. After incubation for 45 min, fluorescent readings were made with excitation and emission wavelength at 485 and 520 nm, respectively.

Nitric Oxide Production in RAW 264.7 Macrophages RAW 264.7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 100 unit/ml penicillin and 50 μg/ml gentamicin at 37 °C in a humidified atmosphere with 5% CO_2. For evaluating the possible effect of the tested plant extracts on iNOS, the cells in DMEM without phenol red media were plated at a density of 2×10^5 cells/well in 96-well culture plate and grown for 2 h to allow them to attach to the plate. The plant extracts at final concentrations of 10—1000 μg/ml or distilled water as vehicle were added to the cells and incubated for 1 h before stimulation with 200 ng/ml lipopolyscaccharide (LPS) and 100 units/ml murine IFN-γ. The cells were further incubated for 24 h.[25] Control cells were grown under identical conditions except without tested compounds. After that, the supernatants were collected for determination of nitrite as an index for nitric oxide (NO) production by Griess reagent (0.1% naphthylethylenediamine and 1% sulfanilamide in 5% H_3PO_4 solution). One hundred microliters of Griess reagent was added to 100 μl of culture supernatant, incubated for 20 min, and read the absorbance at 540 nm. Inhibition of NO production by the extracts was expressed as the IC_{50}.

Animals and Treatments Adult male Sprague-Dawley rats (230—250 g) were obtained from the Animal Care Unit of Faculty of Medicine, Khon Kaen University (Khon Kaen, Thailand). The animals were maintained in a 12 h-dark/light cycle at an average temperature of 25 °C. The animals received standard rat chow diet (Chareon Pokapan Co. Ltd., Bangkok, Thailand) and water *ad libitum*. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Khon Kaen University. Rats were randomly allocated to control and treatment groups (n=6—10/group). Each treatment group received one of the plant extracts (1 g/kg body weight/d), or *N*-acetylcysteine (NAC; 300 mg/kg body weight/d), as a positive drug, for 6 d. The control groups, including normal and anemic rats, were given the saline vehicle. On day four, all animals, except for normal group, were given a single intraperitoneal injection of PHZ (125 mg/kg body weight) to induce hemolysis.[26] From our acute toxicity test, dosage of the plant extracts up to 5 g/kg body weight was previously shown to be safe.

Blood Sample Collection Forty-eight hours after PHZ injection, blood samples were collected from the tail vein into a microcentrifuge tube containing 50 mM EDTA for the determinations of hematocrit, whole blood reduced GSH, plasma malondialdehyde (MDA) and plasma nitric oxide metabolites (nitrite/nitrate or NOx). Blood samples were immediately reacted with ice-cold 10% sulphosalicylic acid (1 : 1), centrifuged at 10000×g for 10 min at 4 °C, and the supernatants were collected for the analysis of reduced GSH. For the MDA and NOx assays, the plasma was collected after blood samples were centrifuged at 2500×g for 15 min at

4 °C.

Biochemical Assays for Reduced GSH, MDA, NOx and $O_2^{\cdot-}$ Production Whole blood reduced GSH was determined using Tieze's method.[27] Plasma lipid peroxide measured as MDA, was estimated by thiobarbituric acid reactive substances according to the method of Draper *et al.*[28] Plasma NOx was measured using the method of Verdon *et al.*[29] with some modifications. Briefly, plasma samples were deproteinized by ultrafiltration using centrifugal concentrators (Nanosep™, Pal Filtron, U.S.A.). The supernatant was reacted with 1.2 μM NADPH, 4 mM glucose 6-phosphate (G6P), 1.28 U/ml G6P-dehydrogenase and 0.8 U nitrate reductase, and then incubated at 30 °C for 30 min. The mixture was then reacted with a Griess solution (4% sulfanilamide in 0.3% naphthylenediamine dihydrochloride) for 15 min. The absorbance of samples was measured on a microplate reader with a filter wavelength of 540 nm. Assay of $O_2^{\cdot-}$ production in blood cells were measured in a separate set of experiments using the lucigenin-enhanced chemiluminescence assay, as previously described[30] with some modifications. Briefly, 300 μl of blood samples were collected from animals into the EDTA tubes. After removal of the plasma, blood cells were washed twice with 1 ml of phosphate buffer saline (PBS) by centrifugation at 2500×***g*** for 10 min and adjusted to a hematocrit of 10% with PBS. Two hundred and forty microliters of washed blood cell suspensions were incubated at 37 °C for 15 min, and 20 μl of lucigenin (100 μM) was then added. The lucigenin-enhanced chemiluminescence intensity was measured continuously for a total of 80 s using a luminometer (Turner BioSystems Inc., CA, U.S.A.). The assay was performed in duplicate for each sample. The rate of $O_2^{\cdot-}$ production was expressed as lucigenin-enhanced chemiluminescence counts/s.

Hemodynamic Study The animals were anesthetized with sodium pentobarbital (60 mg/kg; i.p.). The animal's body temperature was monitored with a rectal probe and maintained at 37±2 °C by a heating pad. A tracheostomy was performed for spontaneous breathing and the left femoral artery was cannulated with polyethylene tubing connected to a pressure transducer for continuous monitoring of heart rate and arterial blood pressure using the Acknowledge data acquisition and analysis software (BIOPAC System Inc., California, U.S.A.). Additional polyethylene tubing was inserted into the femoral vein for infusion of vasoactive agents. The hindlimb blood flow was continuously measured by placing an electromagnetic flow probe around the abdominal aorta connected to the electromagnetic flowmeter (Carolina Medical Electronics, North Carolina, U.S.A.). Hindlimb vascular resistance was calculated from the mean arterial pressure and hindlimb blood flow. After obtaining a stable baseline measurement, vascular reactivity was assessed by testing responsiveness to the endothelium-dependent vasodilators, bradykinin (0.3, 1, 3 nmol/kg) and acetylcholine (3, 10, 30 nmol/kg), and to the endothelium-independent vasoconstrictor, phenylephrine (0.01, 0.03, 0.1 μmol/kg). Each vasoactive agent was infused in a stepwise fashion at 5-min intervals.

Statistical Analyses Results are expressed as mean± S.E.M. The IC_{50} values of each sample was calculated and defined as the concentration of a tested sample required to scavenge 50% of the DPPH free radicals, to 50% inhibition of the intracellular oxygen radical formation as detected by fluorescent signal, or to 50% inhibition of NO production in activated RAW 264.7 cells by nitrite assay. One-way analysis of variance followed by *post-hoc* Duncan's multiple range test was used to analyze differences among groups. A p value of less than 0.05 was considered statistically significant.

RESULTS

Free Radical Scavenging and Antioxidant Activities *in Vitro* To determine the free radical scavenging activity of the plant extracts, DPPH assay was performed and results are shown in Table 1. The water extracts of *C. formosum*, *S. gratum*, and *L. aromatica* showed relatively strong radical scavenging activities when compared with ascorbic acid. Among three extracts, *L. aromatica* was relatively the least potent. Similarly, the ability to scavenge intracellular oxygen radical by the plant extracts in DHF assay revealed a similar potency order among the test compounds. The total antioxidant power of the extracts, measured as ascorbic acid equivalence, was of comparable, except for *L. aromatica*. Their reducing powers were about 19—26% of ascorbic acid on weight basis (Table 2).

Effect on NO Production by Activated RAW 264.7 Macrophages To evaluate whether the plant extracts could inhibit NO production by macrophages, different concentrations of the plant extracts were incubated with LPS/IFN-γ-activated RAW 264.7 cells. The IC_{50} values for *C. formosum*, *S. gratum*, and *L. aromatica* to inhibit NO formation were 451±24, 432±44, and 553±51 μg/ml, respectively. This result indicates that the plant extracts have modest inhibitory effects on NO production.

Improvement of the Hemodynamic Status and Vascular Responsiveness PHZ induced rapid hemolytic anemia in animals of all treated groups. There was a remarkable decline in hematocrit, MAP and HVR, while there was a slight increase in heart rate in the PHZ control group ($p<0.05$, Table 3). The peak effects were attained about 24—48 h after the induction. Treatment with *C. formosum*, *S. gratum*, and *L.*

Table 1. DPPH Radical Scavenging Activity and DHF Assay of the Plant Extracts and Ascorbic Acid

Compounds	DPPH assay IC_{50} (μg/ml)	DHF assay IC_{50} (μg/ml)
C. formosum	3.63±0.08*	8.47±0.39*
S. gratum	4.08±0.50*	9.89±2.90*
L. aromatica	10.78±0.31*	17.52±2.57*
Ascorbic acid	1.63±0.19	1.04±0.18

Values are mean±S.E.M. from 3—4 separated assays. * $p<0.05$ *vs.* ascorbic acid.

Table 2. The Total Antioxidant Capacity (FRAP Assay) of the Plant Extracts Compared to Ascorbic Acid

Compounds	Ascorbic acid equivalence μg/mg extract (μmol/mg extract)
C. formosum	257±25* (1.46±0.14*)
S. gratum	261±25* (1.48±0.14*)
L. aromatica	188±7 (1.07±0.04)

Values are mean±S.E.M. from 3—4 separated determinations. Antioxidant activity was expressed as ascorbic acid equivalent per mg of extract. * $p<0.05$ *vs. L. aromatica*.

Table 3. Effects of Plant Extracts on Hemodynamic Status in PHZ-Induced Hemolytic Anemia in Rats

Parameter measurements	Normal control	PHZ				
		Control	NAC	*C. formosum*	*S. gratum*	*L. aromatica*
Hematocrit (%)	45.9±0.9	23.9±0.7*	22.8±0.6*	23.8±0.8*	22.9±0.6*	22.8±0.8*
Mean arterial pressure (mmHg)	127.3±2.5	62.9±1.7*	93.3±1.6*†	90.7±4.2*†	78.4±3.1*†‡	86.9±4.5*†
Heart rate (beat/min)	364±14	383±10	364±7	360±13	358±9	366±11
Hindlimb blood flow (ml min^{-1}/100 g tissue)	9.5±0.6	17.4±1.9*	13.6±0.6†	11.4±1.0†	11.9±1.2†	12.9±0.7†
Hindlimb vascular resistance (mmHg·min·100 g tissue ml^{-1})	14.6±0.9	4.1±0.4*	6.9±0.4*†	7.9±0.7*†	6.6±0.8*†	6.7±0.5*†

Values are mean±S.E.M. Study groups included: normal control (n=7), PHZ control (n=8), PHZ-treated with NAC 300 mg/kg/d (n=10), PHZ-treated with the plant extracts at dose of 1 g/kg/d (n=7—8/group). *p<0.05 *vs.* normal control; †p<0.05 *vs.* PHZ control; ‡p<0.05 *vs.* PHZ with NAC treatment.

aromatica extracts or NAC showed an improvement of hemodynamic status (p<0.05, Table 3). There was a decrease in MAP in PHZ-treated rats by 50% of normal control rats, while treatment with plant extracts and NAC raised the MAP to about 62—73% of normal controls. Moreover, hindlimb vascular resistance (HVR), measured as functions of blood flow and blood pressure, was dramatically decreased (28% of normal control), indicating the presence of hyperdynamic state with increased blood flow and decreased peripheral vascular resistance. All animals treated with the extracts and NAC restored HVR to 50—60% of normal controls (p<0.05), suggesting an increase in peripheral vascular resistance.

Treatment with PHZ caused a severe impairment in vascular to bradykinin and acetylcholine, and also a blunted vasopressor response to phenylephrine (Fig. 1). The vascular responses of PHZ-control rats were decreased by 90—75%, 50—40%, and 30—45% for bradykinin, acetylcholine, and phenylephrine, respectively, when compared to those found in normal control rats. These results indicate that PHZ-treated rats have an impairment of endothelial-dependent vasodilation and endothelial-independent vasoconstriction. Interestingly, treatment with the plant extracts and NAC resulted in a partial restoration of vascular responsiveness in a dose-dependent manner (p<0.05). *C. formosum*, *S. gratum* and NAC showed a large protection of vascular responsiveness, especially to acetylcholine and phenylephrine (p<0.05, Figs. 1B, C), whereas *L. aromatica* is probably the least active in this study.

Alleviation of the Oxidant Stress To assess whether an improvement in hemodynamic status and vascular responsiveness in the PHZ-treated rats was due to antioxidant effects of the extracts, we measured four parameters that related to body oxidative status: blood reduced GSH, plasma MDA, plasma NOx and blood $O_2^{\cdot-}$ production. Treatment of rats with PHZ increased oxidative and nitrosative stresses, since there was a reduction in blood reduced GSH and increases of plasma lipid peroxidation, plasma NOx and blood $O_2^{\cdot-}$ production when compared with normal control rats. All plant extracts provide a partial protection for GSH levels in all treated rats (68—72% of normal control) when compared to PHZ control rats (58% of normal control), although they were still less than in the normal controls (803.4±16.2 μM; Fig. 2A). Plasma levels of MDA, an index of lipid peroxidation, were markedly increased in PHZ controls when compared with normal controls (14.1±0.57 *vs.* 3.26±0.41 μM; p<0.05, Fig. 2B). Treatment with plant extracts showed a small but significant protection against lipid peroxidation by a reduction of MDA levels in all treated groups (p<0.05). Moreover, plasma concentrations of NOx

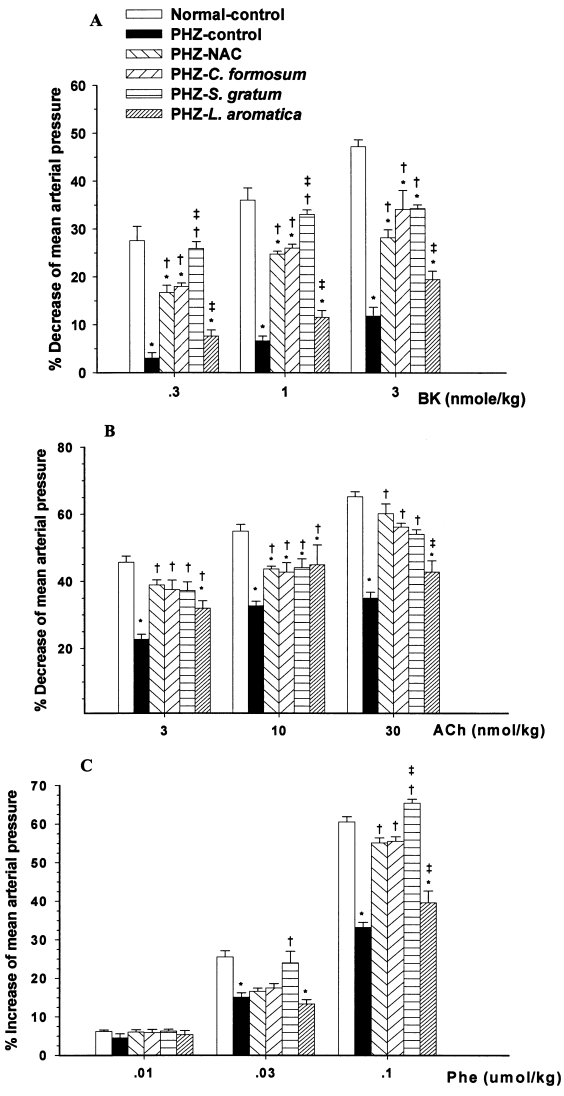

Fig. 1. Effects of Plant Extracts on Mean Arterial Pressure Responses to Various Vasoactive Agents Including Bradykinin (A), Acetylcholine (B), and Phenylephrine (C) in All Study Animals

Rats were treated with plant extracts (1 g/kg), NAC (300 mg/kg) or vehicle for 6 d. On the day four, rats were injected with PHZ (125 mg/kg, i.p.), and vascular reactivity was measured on day six. Values are mean±S.E.M. (n=7—10 rats/group). *p<0.05 *vs.* normal control; †p<0.05 *vs.* PHZ control; ‡p<0.05 *vs.* PHZ with NAC treatment.

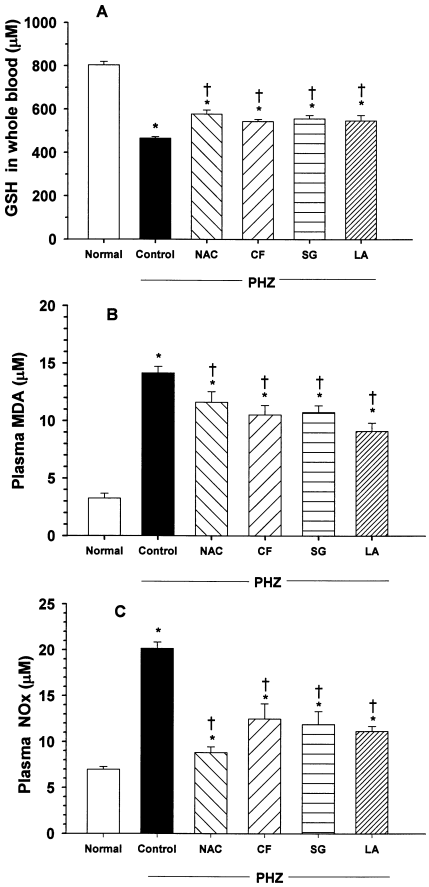

Fig. 2. Effects of Plant Extracts on Antioxidant and Oxidant Status, Including Whole Blood Reduced GSH (A), Plasma MDA (B) and Plasma NOx Concentrations (C) in All Study Animals

Rats were treated with plant extracts (1 g/kg), NAC (300 mg/kg) or vehicle for 6 d. On the day four, rats were injected with PHZ (125 mg/kg, i.p.), and blood samples were taken for biochemical assay on day six. Values are mean±S.E.M. (n=7—10 rats/group). $*p<0.05$ *vs.* normal control; $\dagger p<0.05$ *vs.* PHZ control. CF, *C. formosum*; SG, *S. gratum*; and LA, *L. aromatica*.

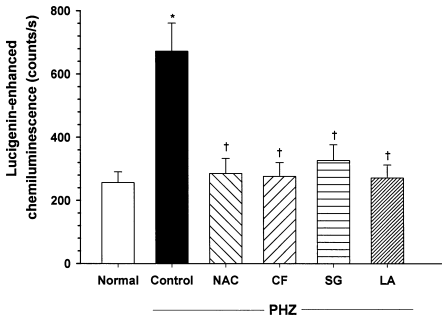

Fig. 3. Effects of Plant Extracts on Superoxide Anion Radical Production in Blood Cells of Tudied Animals

Rats were treated with plant extracts (1 g/kg), NAC (300 mg/kg) or vehicle for 6 d. On the day four, rats were injected with PHZ (125 mg/kg, i.p.), and washed blood cells were taken for lucigenin-enhanced chemiluminescence assay on day six. Values are mean±S.E.M. (n=5—7 rats/group). $*p<0.001$ *vs.* normal control; $\dagger p<0.001$ *vs.* PHZ control. CF, *C. formosum*; SG, *S. gratum*; and LA, *L. aromatica*.

were increased about three-fold in PHZ-treated rats (7.0 ± 0.30 *vs.* $20.2\pm0.68\,\mu$M). The plant extracts and NAC suppressed an elevation of plasma NOx levels with potency in the following order: NAC, *L. aromatica*, *S. gratum* and *C. formosum* (Fig. 2C). Interestingly, the rate of $O_2^{\cdot-}$ production was markedly increased in blood cells obtained from PHZ-control rats compared to the normal control rats, and treatment with plant extracts and NAC could normalize $O_2^{\cdot-}$ production to near basal rates (Fig. 3). There was a correlation between the levels of MDA or NOx with the antioxidant activities by DPPH, DHF and FRAP assays. Moreover, antioxidant activity of the plant extracts was correlated with the improvement of hemodynamic status and vascular responsiveness of the PHZ-treated rats.

DISCUSSION

Many plants have been used as sources of potentially safe natural antioxidants for food and nutraceuticals. Currently, there is continuing research on screening of medicinal plants as new sources of natural antioxidants. Our present study reported for the first time that Thai indigenous dietary and herbal plants, including *C. formosum*, *S. gratum* and *L. aromatica*, possess strong antioxidant and vascular protective activities in both *in vitro* and *in vivo* study models. The first two extract, *C. formosum* and *S. gratum*, were consistently more potent than *L. aromatica* in terms of antioxidant activities in the *in vitro* assays. And, this is well correlated with vascular protective activity in the *in vivo* study in that *C. formosum* and *S. gratum* extracts are more pronounced than *L. aromatica* extract.

In this study, the model of oxidative stress and vascular dysfunction in rats is induced by PHZ. It is well recognized that PHZ is a strong oxidant agent, which is used in laboratories to induce hemolytic anemia by oxidizing the alpha-globin chain of hemoglobin, and consequently, leads to enhanced erythropoietic activity, increased oxidative damage, and increased iron absorption and tissue iron overload.[31—34] The auto-oxidation of PHZ can generate various radicals such as $O_2^{\cdot-}$, hydroxyl radical, and a complex array of PHZ-derived radicals, *i.e.* phenylhydazyl radical, phenyldiazene and benzenediazonium ions.[33,35,36] Not only reactive oxygen species (ROS), PHZ metabolites can also react with plasma membrane to cause lipid peroxidation and protein oxidation resulting in the destruction of red blood cells (RBCs) and hemolytic anemia.[33] Data from this study demonstrate that PHZ causes severe oxidative stress, marked hypotension, low systemic vascular resistance, and impaired vascular reactivity, which is the principal signs of vascular dysfunction. All of these conditions resemble to a systemic inflammatory response syndrome where NO and other radicals are implicated.[37—39] In this study, plausible explanations for the vascular dysfunction caused by PHZ, including PHZ causes injury to erythrocytes and other various cells with subsequent release of free iron and increased production of ROS,[34,40] resulting in a diminution of NO bioactivity for the vascular cells. Moreover, PHZ and its derived-radicals probably have a direct effect on the vascular tissues, and then caused vascular dysfunction.[36]

Since hematocrits of PHZ-treated rats in all groups were rapidly declined and essentially the same levels, even in the concurrent groups treated with plant extracts or NAC. This indicates that plant extracts and NAC cannot protect red cells from lysis directly induced by PHZ, but can protect the subsequent oxidative stress-induced vascular dysfunction. As PHZ action is of short half life and measurement of hemodynamics and vascular reactivity was performed 48 h after ad-

ministration of PHZ, it is likely that PHZ induces hypotension and vascular dysfunction are intensified by oxidant species and inflammatory mediators released from the inflammatory cells.[26,41] This suggestion is consistent with our findings that there was a large flux of $O_2^{\cdot-}$ from blood cells. Moreover, a marked increase in NOx levels may be primarily due to the activation of monocytes. However, the plant extracts are shown to possess negligible effect on iNOS expression in cultured macrophages. It is, therefore, implied that plant extracts primarily inhibit formation of ROS and release of inflammatory mediators from various activated cells whereas these mediators, in turn activate iNOS to produce NO. This is supported by our finding that excessive formation of $O_2^{\cdot-}$ from blood cells is largely suppressed in rats treated with the extracts. Moreover, suppression of $O_2^{\cdot-}$ and NO radicals formation may also avoid the formation of peroxynitrite, a powerful damaging oxidant,[42] which probably damage vascular and other tissues resulting in vascular dysfunction. At last, our results support the concept that antioxidants from these plant extracts play an important role in free radical-mediated vascular dysfunction in cardiovascular diseases.[2,7,43]

Inhibition of lipid peroxidation and increased production of GSH are another protective effect of the plant extracts against oxidative stress induced by PHZ. A small increase in blood GSH levels in rats treated with plant extracts compared with PHZ-treated only group may be attributable from the antioxidant effect of the extracts to scavenge ROS, which otherwise GSH has to be used for neutralizing the ROS. Collectively, these results suggest a potential effect of the study plants on an induction of endogenous defense mechanism of RBCs against ROS.

In conclusion, our study provide the first evidence that Thai dietary herbal plants, including *C. formosum*, *S. gratum* and *L. aromatica*, possess strong antioxidants and prevent the oxidative and nitrosative stresses, which lead to restoration of arterial blood pressure and vascular responsiveness in the rat model of PHZ-induced oxidative stress and vascular dysfunction. These results may lend support a consumption of plants with high antioxidant properties as supplement nutrients for prevention and reduction the risk of developing various diseases related to oxidant stress. Further studies are needed for the isolation and characterization of the active compounds of these dietary plants in order to elucidate their various antioxidant and vascular protective mechanisms.

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