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Suppressive Effect of a Proanthocyanidin-rich Extract from Longan (*Dimocarpus longan* Lour.) Flowers on Nitric Oxide Production in LPS-Stimulated Macrophage Cells

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Anthocyanidins found in certain flowers have been shown to act as strong antioxidants in various systems, exhibiting multiple biological actions. The antioxidative effects of water extract and ethanolic extract of longan (*Dimocarpus longan* Lour.) flowers were evaluated by radical scavenging activity and compared to those of gallic acid, myricetin, and epigallocatechin gallate. In this study, the suppressive effects of longan flower extracts on nitric oxide and prostaglandin E_2 production were investigated using a lipopolysaccharide-stimulated RAW 264.7 cell model. Abundant levels of phenolic compounds including flavonoids, condensed tannins, and proanthocyanidins were found in water or ethanolic extracts prepared from dried longan flowers. The antioxidative effect of longan flower extract was similar to the effect exhibited by pure antioxidants. Moreover, longan flower extract showed prominent inhibitory effects on prostaglandin E_2 production. Significant concentration-dependent inhibition of nitric oxide production was detected when cells were cotreated with lipopolysaccharide and various concentrations of longan flower extracts. These inhibitory effects were further attributed to suppression of inducible nitric oxide synthase protein expression and not to reduced enzymatic activity. These results suggest that longan flower crude extracts, especially ethanolic extract, have antioxidant and anti-inflammatory effects, and the probable mechanism involves inhibition of inflammation by proanthocyanidins. Preliminary observations suggest that longan flower extract, especially alcoholic extract, could be another potential source of natural dietary antioxidant and anti-inflammatory agent.

KEYWORDS: Antioxidant; anti-inflammatory; longan flowers; nitric oxide; proanthocyanidin; RAW 264.7 macrophages

INTRODUCTION

Inflammation has long been recognized as a localized protective reaction of tissue to irritation, injury, or infection that is characterized by pain, redness, swelling, and sometimes loss of function (*1*). During inflammation, reactive oxygen species (ROS) produce high levels of nitric oxide (NO) to exert a defense against pathogens (*2*). However, abnormal excess NO produced by inducible nitric oxide synthase (iNOS) is believed to act as a toxic radical that can damage cellular macromolecules such as proteins, DNA, and lipids, triggering several unfavorable cellular responses (*3*, *4*).

Naturally occurring phenolic compounds are bioactive substances in plants that have been reported to possess beneficial effects on inflammatory diseases and are associated with protection against chronic degenerative diseases in humans (*5*). Polyphenols scavenge reactive oxygen species and thereby directly exhibit anti-inflammatory activity by modulating important cellular signaling processes such as cellular growth, differentiation, and a host of other cellular features (*6*). Many kinds of flowers have been used as traditional medicine for treating oxidative stress or inflammatory conditions such as bronchitis or asthma (*7*–*9*). Flower extracts may enhance or inhibit prostaglandin formation and potentially affect inflammation (*10*). Aqueous extract of *Phrygilanthus acutifolius* has been shown to possess significant anti-inflammatory effects in laboratory animals (*11*). The flowers of *Angelica furcijuga* revealed an inhibitory effect on NO production in lipopolysaccharide (LPS)-stimulated mouse peritoneal macrophages (*12*). Tea flowers contain less caffeine but comparable amounts of catechins, which exert strong hydroxyl radical scavenging effects

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and suppress nitric oxide (*13*). In China, *Chrysanthemum* flowers have a long history in the treatment of inflammation, exhibiting anti-inflammatory and cellular immunomodulatory characteristics, probably due to the presence of flavonoids (*14*). Similarly, flavonoids have been identified in the dandelion flower (*Taraxacum officinale*) and were found to exhibit marked antioxidant activity in both biological and chemical models (*15*). Luteolin and luteolin-7-*O*-glucoside from dandelion flowers suppress nitric oxide and prostaglandin E_2 (PGE_2) in bacterial lipopolysaccharide-induced macrophage RAW 264.7 cells without introducing cytotoxicity (*16*). Proanthocyanidins, widely available in fruit, seeds, and flowers, have been reported to exhibit a broad spectrum of biological and pharmacological activities against free radicals and oxidative stress (*17*). The potential beneficial effects of proanthocyanidins on health have been attributed mostly to their strong antioxidant and anti-inflammatory activities (*17–19*). Tannins with higher molecular weight were more effective than simple phenolics in quenching peroxyl radicals (*20*). Moreover, the antioxidant activity of procyanidin dimer was neither lower nor greater than that of flavonol and simple phenolic acid (*21*).

Inflammatory disease is among the most common health problems treated with traditional medicines (*22*). Therefore, it is crucial to evaluate the potential of natural plants or herbs to aid in the discovery of novel bioactive compounds that might serve as leads for the development of potent functional foods or therapeutic agents. Longan (*Dimocarpus longan* Lour), also known as dragon's eye, has been referred to as the "little brother of lychee." The fruit of longan is more widely used than lychee in oriental medicine. Off-season induction of flowering is a desirable economic goal accomplished through the application of gibberellin biosynthesis inhibitors (*23*). To increase the size and quality of the fruits, an important operation is to prune or remove flower spikes in the cluster. Longan flowers are sold in herb markets but are not a part of traditional medicine. The dried longan flower has a fresh and fruity aroma and is mainly used to prepare an infusion that is drunk for pleasure or refreshment in Taiwan. Longan fruit and seed extract, containing high levels of gallic acid, corilagin (an ellagitannin), and ellagic acid, were found to be as effective as Japanese green tea extract in antioxidant activity (*24*, *25*). However, a survey of the literature has revealed that the anti-inflammatory effect of longan flowers has not been studied so far. The purpose of this study was to examine the anti-inflammatory effect of a crude longan flower extract in LPS-stimulated RAW 264.7 macrophages. In addition, the effectiveness of longan flower extracts was also compared to that of epigallocatechin gallate (EGCG), myricetin, and gallic acid. Furthermore, we determined proanthocyanidin content and antioxidant activity to elucidate the relationship between phenolic content, NO suppression, and antioxidant activity.

MATERIALS AND METHODS

Materials and Reagents. The RAW 264.7 cell line was obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured in Dulbecco Modified Eagle's Medium (DMEM) (Gibco BRL Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL Life Technologies Inc.) and maintained at 37 °C in a humidified incubator containing 5% CO_2. Lipopolysaccharide (LPS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), Folin−Ciocalteu reagent, and BCIP/NBT liquid substrate system were purchased from Sigma Chemical Co. (St. Louis, MO). Antioxidants such as gallic acid (98%), myricetin, and (−)-epigallocatechin gallate (EGCG) (95%) were also purchased from Sigma Chemical Co. All other chemicals were of analytical purity grade.

Extraction of Longan Flower. Male longan flowers were harvested and processed by a certified longan farm (Tainan, Taiwan) during early summer. Flowers were separated manually from aerial parts and washed with tap water prior to being air-dried. Dried longan flower was then refluxed with a 50 v/w (mL/g) ratio of distilled water for 30 min. The extract was filtered through no. 1 filter paper. The filtrate was then collected and freeze-dried; the final crude water extract was denoted LFWE. Dried longan flower was also extracted twice with 95% ethanol at 20 mL/g ratio for 24 h at room temperature. The extracts were filtered and combined followed by concentration with rotary evaporator and then freeze-dried; the ethanolic extract was denoted LFEE. The extracting yield of LFWE was 37.42%, and LFEE was 14.1%. For the cell culture experiment, weighed extract was dissolved in DMEM and filtered through a 0.2 μm pore size membrane and then diluted with DMEM to the indicated concentration.

Measurement of Phytochemicals. The amount of total phenolics in the longan flower extract was determined by the Folin−Ciocalteu colorimetric assay described by Julkunen-Tiitto (*26*). The total content of phenolic compounds in each longan flower extract was then determined by a standard curve prepared with gallic acid and expressed in terms of milligrams of gallic acid equivalents per gram of extract-solid. Total flavonoid contents were measured according to the method of Zhishen et al. (*27*). The amount of condensed tannin was determined as described by Julkunen-Tiitto (*26*). Both flavonoids and condensed tannin are expressed as milligrams of catechin equivalents per gram of dry weight. Anthocyanin was determined by pH differential method as described by Fuleki and Francis (*28*); cyanidin-3-glucoside was used as a reference standard. Proanthocyanidin contents were determined by spectrophotometry at 550 nm, on the basis of a colorimetric reaction with 10% $NH_4Fe(SO_4)_2$ after dissolution in hydrochloric acid containing *n*-butanol, as described by Luximon-Ramma et al. (*29*). Proanthocyanidin contents were expressed in terms of milligrams of cyanidin chloride equivalents per gram of extract-solid. Ascorbic acid content was determined according to the AOAC analytical method (*30*). Gallic acid content was determined by HPLC using a Phenomenex Luna C_{18} reverse phase column (25 cm × 0.46 cm i.d., 5 μm) and a UV–vis detector. The mobile phase contained 0.9% acetic acid (solvent A) and acetonitrile (solvent B), with a linear gradient from A/B (92:8) to A/B (76:24) over a period of 40 min with a flow rate of 1 mL/min. The detector was monitored at 280 nm. Tocopherol content was determined according to the method of Podda et al. (*31*). The unsaponifiable fraction was analyzed by using HPLC with a Phenomenex Gemini C_{18} column (25 cm × 0.46 cm i.d., 5 μm) and a UV–vis detector. The isocratic elution was performed by 98% methanol solution with a flow rate of 1.5 mL/min. The detector was monitored at 292 nm.

Evaluation of Antioxidant Activity. The total antioxidant capacity of longan flower extract was determined by using a commercial kit (Randox Laboratories Ltd., Crumlin, U.K.) as described by Sánchez-Moreno (*32*). This assay is based on 2,2′-azinobis(3-ethylbenzothiazoline sulfonate) (ABTS) incubated with metmyoglobin and hydrogen peroxide to produce the radical cation $ABTS^+$. $ABTS^+$ has a stable blue-green color and can be measured at 600 nm. Total antioxidant capacity was calculated relative to the reactivity of Trolox as a standard under the same conditions and with results expressed as millimoles per gram TEAC.

Scavenging Effects on Nitrite Oxide. The scavenging effects of longan flower extract on NO were measured according to the method of Dirsch et al. (*33*). A total of 4 mL of sample solution was added to 1 mL of SNP solution (25 mM) and was incubated at 37 °C for 150 min. An aliquot of the incubation solution was removed and diluted with 0.3 mL of Griess reagent (1% sulfanilamide in 5% H_3PO_4 and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the chromophore was read at 540 nm and compared to the absorbance of standard solutions of sodium nitrite salt treated in the same way with Griess reagent.

Determination PGE_2. The amount of PGE_2 was measured by enzyme immunoassay according to the manufacturer's instruction (Cayman Chemical). PGE_2 level was expressed as picograms per milliliter.

Determination of NO Production. To determine the effect of longan flower extract on NO (nitrite) production, cells were cultured at a density of 6×10^4 cells per well in 96-well culture plates. Following a 24 h incubation, the adherent cells were washed three times with PBS. Cells

Table 1. Contents of Phytochemicals in Water (LFWE) and Ethanolic Extracts (LFEE)[a]

phytochemical	content (mg/g) LFWE	LFEE
total phenols	548.2 ± 12.7	476.8 ± 4.8
total flavonoids	139.3 ± 0.2	156.0 ± 8.7
condensed tannins	94.8 ± 0.5	140.6 ± 7.3
anthocyanins	ND	ND
proanthocyanidins	112.5 ± 5.2	186.7 ± 7.8
gallic acid	10.4 ± 0.3	2.2 ± 0.2
ascorbic acid	ND	ND
γ-tocopherol	ND	3.42 ± 0.14

[a] All data are expressed as mean ± standard deviation of triplicate tests. ND means not detected. Tocopherol content was analyzed by HPLC, and only γ-tocopherol was present.

were then incubated in medium with samples, with or without 1 μg/mL LPS. After 24 h of incubation, the medium was collected and stored at −70 °C until assayed. Cell viability was then evaluated using the MTT method (*34*). Finally, media nitrite concentration was measured as an indicator of NO production by the Griess reaction. The rate of inhibition was calculated as follows: percentage inhibition = $(NO_{LPS} - NO_{sample+LPS})/(NO_{LPS} - NO_{control}) \times 100\%$, where NO_{LPS}, $NO_{control}$, and $NO_{sample+LPS}$ represent NO production of cultural media containing LPS, without LPS, and sample with LPS, respectively.

Evaluation of iNOS Enzyme Activity. Cells were cultured in a 10 cm culture dish and stimulated with LPS (1 μg/mL) for 12 h. Subsequently, cells were harvested and plated in a 24-well culture dish and then were treated with various samples for an additional 12 h. The medium was finally collected and assayed for nitrite.

Evaluation of iNOS Protein Expression. Cells were seeded at a density of 5×10^6 cells per 6 cm culture dish and incubated for 24 h. After three washes, the adherent cells were incubated for 12 h in the presence or absence of 1 μg/mL LPS and the indicated concentration of tea extract. The adherent cells were then washed with PBS, collected, suspended in lysis buffer (50 mM Tris, pH 7.6, 0.01% EDTA, 1% Triton X-100, 1 mM PMSF, and 1 μg/mL leupeptin) and centrifuged at 12000*g* for 20 min at 4 °C. The protein concentration was determined with a BCA kit (Pierce Co., Rockford, IL). Additionally, iNOS protein levels were determined by immunoblot analysis. Briefly, samples with equal protein were loaded and separated on 8% SDS−polyacrylamide gel and then transferred to PVDF filters. Meanwhile, filters were blocked and then probed with antibodies (BD Transduction Laboratories, San Jose, CA). The filters were then incubated with secondary antibody conjugated to alkaline phosphatase and detected using an NBT/BCIP solution. Finally, band intensities were quantified with a software-supported photoimager (ImageMaster VDS; Amersham Pharmacia Biotech Co., Piscataway, NJ).

Statistical Analysis. All data are presented as mean ± SD from three independent experiments. The significance of the differences at each sample concentration was analyzed by ANOVA and Duncan's multiple-range test using SPSS software. The paired *t* test was used to evaluate the differences between the treatments and the control, with significant difference set at $p < 0.05$ (SPSS for Windows 10.0; SPSS Inc., Chicago, IL). Correlation between two variants was analyzed by the Pearson test.

RESULTS

Phytochemical Content. To study the effects of longan flower flavonoids on NO production, the contents of phytochemicals in water extract (LFWE) and ethanol extract (LFEE) of longan flower were measured including total phenolics, total flavonoids, condensed tannins, anthocyanins, proanthocyanidins, ascorbic acid, gallic acid, and tocopherols (**Table 1**). Both of the two longan flower extracts contained high concentrations of phenolic compounds and were rich in flavonoids, condensed tannins, and proanthocyanidins. LFWE appeared to contain more phenolic compounds and gallic acid than LFEE. However, the contents of flavonoids, condensed tannin, proanthocyanidins, and γ-tocopherol in LFEE were higher than in LFWE. Neither LFWE nor LFEE contained α-, β-, or δ-tocopherol and ascorbic acid. It is interesting to note that no anthocyanin is present in LFWE and LFEE as determined by pH differential method. Anthocyanins might be destroyed during the drying process of longan flower. Proanthocyanidins and gallic acid are known to occur in inflorescence (*7*). Water- and ethanol-soluble phenolic compounds had molecular weights between 500 and 3000. Moreover, proanthocyanidin oligomers up to six units are generally soluble in water; those greater than this are insoluble. Therefore, the results suggest that proanthocyanidins in LFEE and LFWE are low molecular weight oligomers.

Table 2. Total Antioxidant Activity Assessed by TEAC[a]

sample	total antioxidant capacity (mmol/g)
gallic acid	13.4 ± 0.7 a
myricetin	13.3 ± 0.3 a
EGCG	12.9 ± 0.4 a
LFWE	8.9 ± 0.2 b
LFEE	8.8 ± 0.1 b

[a] All values are expressed as means ± SEM of triplicate tests. Means not sharing a common letter were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple-range test.

Antioxidant Activity. Proanthocyanidins are defined comprehensively as oligomers and polymers built from polyhydroxy flavan-3-ol units. To investigate the relationship between chemical structure and antioxidant activity, longan flower extracts were compared with gallic acid (side group), myricetin (monomeric flavonoids), and EGCG (dimeric flavanoids). The $ABTS^+$ radical system was used in this study to evaluate antioxidant activity, which was expressed as Trolox equivalent antioxidant capacity (TEAC). The scavenging potencies of LFWE, LFEE, and the pure compounds were assayed by the TEAC method as ranging from 8.8 to 14.4 mmol/g with three antioxidants exhibiting the higher $ABTS^+$ scavenging activity (**Table 2**). Gallic acid, myricetin, and EGCG were characterized by higher TEAC values than the two longan flower extracts in the chemically assessed model.

Longan flower extracts were also evaluated for their scavenging effects on NO (**Figure 1**). The scavenging effects of LFWE, LFEE, and antioxidants are concentration dependent. IC_{50} on NO occurs in increasing order of myricetin <gallic acid < EGCG < LFWE < LFEE. The NO scavenging result is similar with TEAC. That is, the lower molecular weight pure molecule possesses a higher radical scavenging ability than longan flower extracts, which contain oligomeric proanthocyanidins in aqueous phase analytical system.

Cytotoxicity. All of the additives clearly reduced the viability of RAW 264.7 cells in a dose-related manner (dose range from 25 to 1000 μg/mL). Regarding dietary quantities, three pure antioxidants exhibited cytotoxicity when concentrations exceeded 50 μg/mL. Both longan flower extracts, especially LFWE, have lower cytotoxicity when compared to pure antioxidants, except when concentrations exceeded 250 μg/mL (data not shown). It is necessary to mention that new functional foods, which are typically processed to contain high levels of phytochemicals, might be toxic when ingested in large amounts.

Effect of Longan Flowers on PGE_2. PGE_2 is the major prostaglandin produced by macrophage, which is a well-known pro-inflammatory mediator. In cultural supernatant obtained from macrophage cells after LPS addition, PGE_2 production was significantly ($p < 0.05$) increased over a basal level. Production

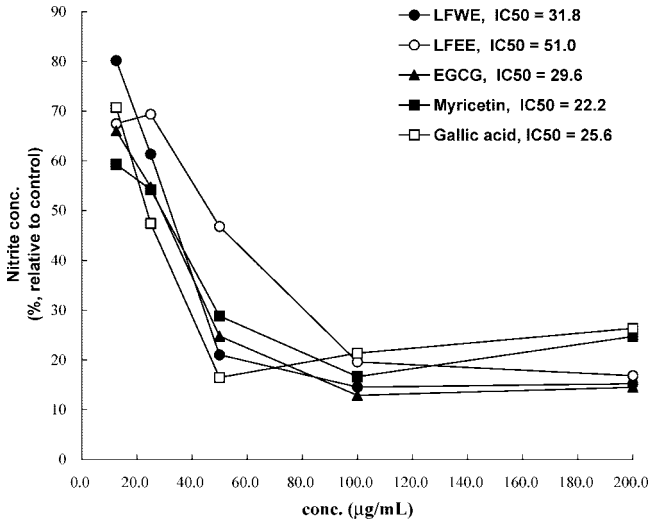

Figure 1. Nitrite scavenging ability of longan flower extracts and antioxidants. Data are the means of triplicate wells from a representative experiment. The experiment was repeated at least three times with similar results.

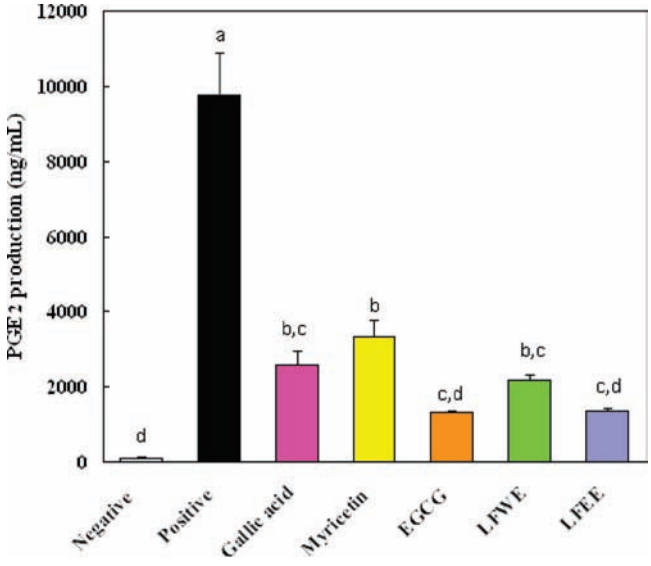

Figure 2. Effects of longan flower extracts and antioxidants on LPS-stimulated PGE_2 production. LFEE was dissolved in a minimal amount of ethanol and then diluted with DMEM. Data are the means ± SD of triplicate wells from a representative experiment. The experiment was repeated at least three times with similar results. A dose of 100 μg/mL were used except for LFWE, for which we used 300 μg/mL.

of PGE_2 was significantly ($p < 0.05$) reduced when treated with LFWE (300 μg/mL), LFEE, and antioxidants (100 μg/mL) (**Figure 2**). Gallic acid, myricetin, EGCG, LFWE, and LFEE evoked significant inhibition of LPS-stimulated PGE_2 release, and inhibition reached 73.6, 66.0, 86.5, 77.6, and 87.0%, respectively. Although LFWE concentration was higher than others, both LFWE and LFEE showed prominent inhibitory effect and lower cytotoxicity. Likewise, LFEE, just as EGCG, showed the highest activity.

NO Production in Unstimulated and LPS-Stimulated Macrophages. Macrophage cells showed an obvious cytotoxicity when incubated with a concentration of longan flower extract exceeding 250 μg/mL. In this study, to avoid possible cytotoxic effects on NO production by longan flower extracts, we treated cells with a concentration below 100 μg/mL. NO production was analyzed by measuring nitrite with the Griess reaction, revealing that placing unstimulated RAW 264.7 cells in culture medium for 24 h produced a basal amount of nitrite (**Figure 3**). When cells were incubated with extracts from these additives in the absence of LPS, medium nitrite concentration was maintained at a background level similar to that in the unstimulated control (data not shown). After treatment with LPS for 24 h, the medium concentration of nitrite increased markedly compared to the control group (**Figure 3**). Significant concentration-dependent inhibition of NO production was detected when cells were cotreated with LPS and various concentrations of these antioxidants (**Figure 3**). The concentrations of 50% inhibition (IC_{50}) by LFEE, LFWE, myricetin, and EGCG were 70.7, 147.7, 40.7, and 54.7 μg/mL, respectively. Gallic acid, with an IC_{50} exceeding 100 μg/mL, showed poor inhibition of NO production in LPS-stimulated RAW 264.7 cells. Notably, although LFWE contained much higher levels of phenolic compounds (**Table 1**), the NO-suppressing activity of LFWE was about 50% of that of LFEE (**Figure 3**).

iNOS Catalytic Activity. This study examined whether inhibitory effects on inducible nitrite production resulted from a direct effect of specific additives on intrinsic enzyme activity of iNOS. We selected a dose of 100 μg/mL to examine the effect of longan flower extracts and antioxidants on iNOS enzyme activity except for LFWE, for which we used 300 μg/mL, the amount at which the additives exerted the greatest inhibitory action on NO production. According to the results of statistical analysis shown in **Table 3**, only myricetin shows a significant inhibitory effect on nitrite production. LFEE and LFWE appeared to have no inhibitory effect on iNOS enzyme activities. This phenomenon was also observed in EGCG and gallic acid (**Table 3**).

iNOS Protein Levels. We next investigated whether these additives might influence iNOS protein levels. RAW 264.7 cells did not express detectable iNOS protein when incubated in the medium without LPS for 12 h (**Figure 4**), whereas LPS induced a marked increase in iNOS protein. At 100 μg/mL of treated concentration, gallic acid, myricetin, and EGCG exhibited inhibitory rates of 37.76, 12.07, and 13.43, respectively, on LPS-induced iNOS expression. Similarly, LFEE (100 μg/mL) and LFWE (300 μg/mL) inhibited iNOS protein levels by 10.91 and 12.72%, respectively, compared with the LPS-stimulated group. All antioxidants demonstrated a significant inhibitory effect.

DISCUSSION

In this study, we produced extracts of longan flowers with water and 95% ethanol and aimed to determine the impact of longan flower proanthocyanidins on suppressing NO and PGE_2. Except for gallic acid, the proanthocyanidins in longan flower extract are diverse and complex and needed further identification. Although LFWE contained more polyphenolics and gallic acid, it seemed obvious to find that LFWE (**Table 1**) had a lower NO and PGE_2 suppressive effect in a cellular model (**Figures 2**, **3**) and higher NO scavenging ability in an aqueous model (**Figure 1**); said another way, LFWE effectively scavenges NO in an aqueous chemical model, but ineffectively inhibits NO production in a macrophage cell model. In contrast, LFEE, with more flavonoids, condensed tannins, proanthocyanidins, and γ-tocopherol, showed a significantly higher activity of NO and PGE_2 suppression in the macrophage cell model when compared to LFWE. Pure gallic acid also showed poor NO-suppressing ability compared to myricetin and EGCG. These results demonstrate that gallic acid may not be the major contributor of NO or PGE_2 inhibitory activity of longan flower extracts in an LPS-stimulated RAW 264.7 cell model, but that

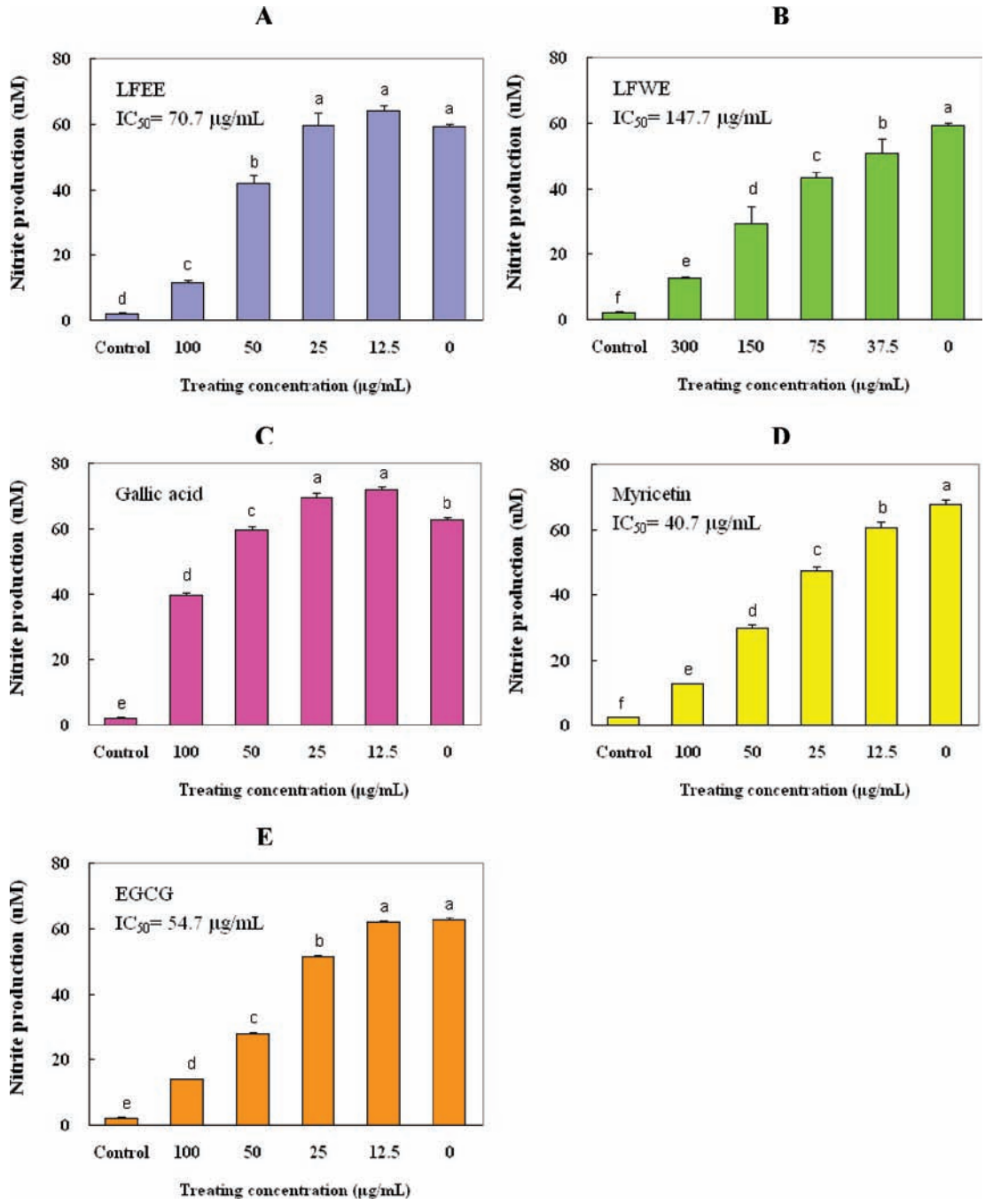

Figure 3. Effects of longan flower extracts and antioxidants on NO production in LPS-stimulated RAW 264.7 cells. Cells were incubated in the presence of different concentrations of (**A**) LFEE, (**B**) LFWE, (**C**) gallic acid, (**D**) myricetin, and (**E**) EGCG. Values are expressed as means ± SEM of triplicate tests. Means not sharing a common letter were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple-range test.

this high activity may be due to other flavonoids or proanthocyanidins present in longan flower extracts.

Galloylation of catechins and dimeric procyanidins increased aqueous phase antioxidant activity as described by Plumb et al. (*35*). Similar results as shown in the current study; pure myricetin and EGCG showed higher antioxidant activity (**Table 2**) and NO-scavenging activity (**Figure 1**) in an aqueous model. According to previous papers, total phenolic compounds correlated with NO-suppressing ability (*36*). Results also strongly suggest that flavonoids, condensed tannins, and proanthocyanidins content relate to NO inhibitory effects (*37*). Additionally, the structure of flavan-3-ol linked to gallic acid seems to be an important determinant in NO, superoxide, and peroxynitrite scavenging activities of catechins (*38*). Proanthocyanidins, which account for about 39% of total phenolics in LFEE, have been revealed to be the important contributor of NO inhibition in a cellular system (**Figure 3**). In addition to proanthocyanidins and flavonoids, several other compounds such as gallic acid, quercetin, rutin, and kaempferol have been identified as potent NO-scavenging agents, iNOS enzyme inhibitors, and iNOS expression suppressors (*37*). The current results demonstrate that the inhibitory effect of NO and PGE_2 production in a cellular system was attributed to the combination of phenolic compounds, flavonoids, and proanthocyanidins. Apparently, the NO-suppressing activity of LFEE is due to the combined activity of flavonoids and proanthocyanidins, rather than being attributable to only gallic acid or other simple phenolic compounds. This opinion is consistent with the results of Paquay et al. (*39*).

Table 3. Inhibitory Effect of Sample on the iNOS Catalytic Activity in RAW 264.7 Cells

LPS induction	sample	nitrite production[a] (μM)	rate of inhibition[b]
control	none	3.1 ± 0.1	
LPS	none	55.6 ± 5.1 a	NA
	gallic acid	54.1 ± 6.0 a	NA
	myricetin	31.6 ± 8.7 ab	46
	EGCG	56.0 ± 2.1 a	NA
	LFEE	61.0 ± 1.7 a	NA
	LFWE	53.4 ± 4.0 a	NA

[a] a and b indicate $p < 0.05$, significantly different from control and LPS alone, respectively. [b] The rate of inhibition was calculated as percentage inhibition = $(NO_{LPS} - NO_{sample+LPS})/(NO_{LPS} - NO_{control}) \times 100\%$. NA indicates the sample was not applied.

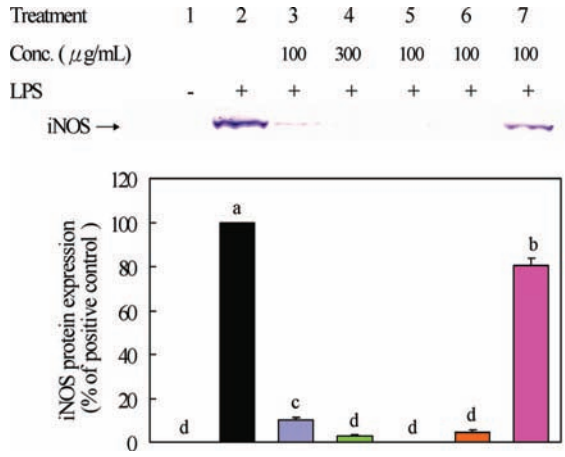

Figure 4. Effects of longan flower extracts and antioxidants on LPS-induced iNOS protein levels in RAW 264.7 cells. The lanes represent cells treated without (2) or with 100 μg/mL of LFEE (3), 300 μg/mL of LFWE (4), 100 μg/mL of myricetin (5), EGCG (6), or gallic acid (7), respectively, in the presence of LPS (1 μg /mL) for 12 h. Lane 1 presents results of cells without any treatment. Values are expressed as means ± SEM of triplicate tests. Means not sharing a common letter were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple-range test.

Our results suggest that the gallo group in the proanthocyanidins did not contribute the NO-suppressing activity. However, flavonoids (myricetin) or dimeric flavanoids (EGCG) could play an important role in increasing NO-suppressing activity in LPS-stimulated RAW 264.7 cells. More flavonoids and proanthocyanidins in LFEE were revealed to be the primary contributor of NO-scavenging activities. The content of these compounds fluctuates according to the extracted solvent; hence, they might contribute some effects to NO-suppressing activities of longan flower extract. Further investigation is needed of the NO-suppressing effect of γ-tocopherol of LFEE in macrophage cells.

Undesired effects of reactive nitrogen species have been found to be controlled by the antioxidant and/or anti-inflammatory effects of dietary polyphenols (*5*). In addition, these polyphenols modulate the signal transduction pathway or the scavenging effect of reactive oxygen species directly and, as a consequence, regulate inflammatory genes in macrophages cells. Phytochemicals can exert NO-suppressing effect through three routes: direct scavenging of NO radicals, inhibition of NOS catalytic activity, and suppression of iNOS expression (*38*). The NO- and PGE_2-suppressing effects of phytochemicals in longan flower extract were not attributed to inhibition of iNOS catalytic activity (**Table 3**), but were contributed by the inhibition of iNOS protein expression (**Figure 4**). Both longan flower extracts, just as EGCG and myricetin, can completely block iNOS expression through down-regulation of the NF-κB transcription factor, and therefore longan flower extract is considered to be a dominant phytochemical against NO production by LPS-stimulated cells. Recently, proanthocyanidins were also found to be potent inhibitors of iNOS expression (*40*). Furthermore, the effects of many procyanidins are based on their ability to form complexes with proteins and polysaccharides. Procyanidins can undergo both intra- and intermolecular hydrogen bonding (*41*). Therefore, we suggest that longan flower extracts inhibit inflammation by the signal transduction pathway without introducing cytoxicity.

Although the NO-suppressing effects of various flowers or their components have been extensively investigated (*8*, *12–17*), this is the first study to investigate the inhibitory effects of longan flower extracts on NO and PGE_2 production. According to the results of this study, we conclude that the alcoholic extract possesses higher proanthocyanidin and flavonoid contents than the hot water infusion of longan flowers, therefore enhancing the anti-inflammatory activity. The antioxidant activity and anti-inflammatory effects of longan flower extract were not greater than those of pure phenolic compounds; however, it showed no cytotoxicity. The demonstrated safety of longan flower makes it a potential natural nutraceutical for anti-inflammatory activity.

ABBREVIATIONS USED

ABTS, 2,2′-azinobis(3-ethylbenzothiazoline sulfonate); EGCG, (−)-epigallocatechin gallate; iNOS, inducible nitric oxide synthase; LFEE, ethanolic extract of longan flower; LFWE, water extract of longan flower; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, nitric oxide; PGE_2, prostaglandin E_2.

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