Inhibitory Effects of Hydrolyzable Tannins from *Melastoma dodecandrum* LOUR. on Nitric Oxide Production by a Murine Macrophage-Like Cell Line, RAW264.7, Activated with Lipopolysaccharide and Interferon- γ

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An extract of *Melastoma dodecandrum* LOUR. with 80% aqueous acetone (MDL) inhibited nitric oxide (NO) production by a murine macrophage-like cell line, RAW264.7, activated with lipopolysaccharide (LPS) and recombinant mouse interferon- γ (IFN- γ). On further fractionation of the extract, the majority of the inhibitory activity was recovered in the 50% methanol extracts, which contained hydrolyzable tannins. Among the latter, casuarinin, casuarictin, pedunculagin and nobotannin B exhibited strong inhibitory activities toward NO production, with ID₅₀ values between 2.0 and 5.1 μ M. Both MDL and the purified tannins significantly reduced the induction of the inducible nitric oxide synthase (iNOS) protein in the course of macrophage activation with LPS and IFN- γ . In addition, the NO production by macrophages preactivated with LPS and IFN- γ for 16 h was also inhibited by these tannins, with IC₅₀ values around 30–130 μ M, but not by MDL. These results suggest that MDL has the pharmacological ability to suppress NO production by activated macrophages and that the hydrolyzable tannins have major inhibitory activities.

Key words hydrolyzable tannin; *Melastoma dodecandrum*; macrophage; nitric oxide (NO); lipopolysaccharide (LPS); interferon- γ (IFN- γ)

Melastoma dodecandrum LOUR. is a perennial plant which is found throughout the southern part of China. The whole plant of *Melastoma dodecandrum* LOUR. has been used as a traditional antipyretic, antitoxic, diuretic, hemostatic and anti-rheumatic medicine.^{1,2)} However, the physiological and pathological functions of this plant have not been fully clarified. We previously found that an 80% aqueous acetone extract (MDL) of whole plants of *Melastoma dodecandrum* LOUR. exhibited anti-allergic activity in the Passive Cutaneous Anaphylaxis test and toward histamine release from rat peritoneal mast cells with an antigen antibody reaction *in vitro*. The hydrolyzable tannins isolated from this plant, such as casuarinin (1), casuarictin (2), pedunculagin (3), and nobotannin B (4), were considered to be the major inhibitors of this anti-inflammation activity.³⁾ These tannins have been reported to show interesting biological and pharmacological properties, such as anti-oxidation activity,^{4,5)} anti-tumor activity,^{6,7)} and anti-viral activity.^{8–10)}

Macrophages play major roles in inflammation and host-defense mechanisms against bacterial as well as viral infections.¹¹⁾ The nitric oxide radical (NO), together with oxyradicals, is known to be one of the effector molecules for the anti-tumor and anti-microbial effects of macrophages. The enzyme responsible for the synthesis of nitric oxide in macrophages is an inducible nitric oxide synthase (iNOS), which does not exist ordinarily but is strongly induced upon exposure to bacterial endotoxins (lipopolysaccharide, LPS) and inflammatory cytokines.¹²⁾ Synergistic activity of interferon- γ (IFN- γ) is known to induce higher amounts of iNOS in the LPS-treated macrophages, while IFN- γ alone does not induce it in macrophages or macrophage-like cell line.¹³⁾ However, the excessive production of NO may also lead to severe damage to host cells and tissues during acute and

chronic inflammation.¹⁴⁾ In a variety of inflammatory diseases, like rheumatoid arthritis, excessive NO production by activated macrophages was observed.¹⁵⁾ We therefore investigated the effects of MDL and its components on the NO production by macrophages in order to clarify the mechanisms of the anti-inflammatory and anti-rheumatoid activities of *Melastoma dodecandrum* LOUR. In this paper, we report that MDL and hydrolyzable tannins inhibit NO production by activated macrophages treated with LPS and IFN- γ .

MATERIALS AND METHODS

Materials The compounds casuarinin (1), casuarictin (2), pedunculagin (3), nobotannin B (4), vitexin (5), gallic acid 3-*O*- β -D-(6'-*O*-galloyl)-glucopyranoside (6), and methyl gallate (7) (Fig. 1) were isolated from *Melastoma dodecandrum* LOUR.¹⁶⁾ In brief, dried whole plants of *Melastoma dodecandrum* LOUR. were extracted with 80% aqueous acetone. The extract (MDL) was applied to a column of Diaion HP-20, then the column was eluted with either water (fraction A), 50% methanol (fraction B), 75% methanol (fraction C), 100% methanol (fraction D), or 80% acetone (fraction E). Fraction B was fractionated further by Sephadex LH-20, MCI-gel CHP-20P and Toyopearl HW-40 column chromatographies, and the hydrolyzable tannins shown in Fig. 1 were obtained from this fraction. Ham's F12 medium was obtained from Sigma (St. Louis, MO, U.S.A.), fetal bovine serum from Cansera International, Inc. (Rexdals, Ont., Canada), IFN- γ from Biosource International (Camarillo, CA, U.S.A.), LPS (*Escherichia coli*, O55: B5) from Difco (Detroit, MI, U.S.A.), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-3-oxide-1-oxyl (carboxy-PTIO) from Dojindo Laboratories (Kumamoto, Japan), and L-arginine and N^G-(1-

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Fig. 1. The Structures of the Compounds in *Melastoma dodecandrum* LOUR

iminoethyl)-L-ornithine hydrochloride (L-NIO) from Wako Pure Chemical Industries (Osaka, Japan). ^{125}I -Labeled protein A was prepared by the iodination of protein A (Sigma) with ^{125}I -labeled NaI (NEN; Du Pont, Lincoln Park, NJ, U.S.A.) according to the chloramine-T method.¹⁷⁾ All other chemicals and reagents were of commercial grade.

Cell Culture The murine macrophage-like cell line, RAW264.7, was obtained from the American Type Culture Collection (Rockville, MD). The cells were maintained by culturing them in Ham's F12 medium, containing 10% (v/v) heat-inactivated fetal bovine serum, in a CO_2 incubator (5% CO_2 95% humidified air) at 37°C . The cells were seeded onto a plastic petri dish (Falcon, #1001; Becton Dickinson) and passaged twice a week.

NO Production Assay and Induction of iNOS in Macrophages The cells were seeded at 1.2×10^6 cells/ml onto a 35 mm petri dish (Sumitomo Bakelite, #1135R; Tokyo), and then incubated at 37°C for 2 h. Then, MDL or a test compound was added to the culture simultaneously with both 100 ng/ml LPS and 10 U/ml $\text{INF-}\gamma$, and the cells were incubated at 37°C , usually for 8 h. After incubation, the cells were chilled on ice, scraped from the dish with a cell scraper, collected in a microfuge tube and then immediately centrifuged at 7000 rpm at 4°C . A 700 μl volume of the supernatant was placed in a new microfuge tube and stored at 4°C before assaying for nitrite (NO_2^-). The cells were washed twice with phosphate-buffered saline without divalent cations (PBS) by repeated centrifugation at 7000 rpm. The final cell pellet was extracted with 40 μl of a lysis buffer, comprising

1% (v/v) Triton X-100, 0.1 mM EDTA and 1% aprotinin (Sigma) in 20 mM HEPES NaOH buffer, pH 7.5, at 4°C for 30 min. The cell lysate was centrifuged at 10000 rpm at 4°C for 1 min, and 30 μl of the resultant supernatant was placed in a new microfuge tube. The final cell extract was stored at -80°C until use.

SDS-PAGE and Immunoblotting As reported previously,¹⁸⁾ a 60 μg protein cell extract was treated with sodium dodecyl sulfate (SDS)-sample buffer, loaded onto a 5–20% gradient slab gel (Pagel®; Atto, Tokyo), and then electrophoresed at 20 mA/gel (Atto, Tokyo). The proteins in the gels were electro-transferred to a PVDF membrane (Immobilon-P®, Millipore, Bedford, MA, U.S.A.) with a Mini-Slab Blotting System (Bio-Rad, Hercules, CA, U.S.A.). The proteins on the membrane were reacted with a monoclonal anti-macrophage iNOS antibody (Transduction Laboratories, Lexington, KY, U.S.A.), then with rabbit anti-mouse IgG (Zymed Laboratories, Inc., San Francisco, CA, U.S.A.), and finally with ^{125}I -labeled protein A. Then, the membrane was placed on an imaging plate (Fuji Photo Film Co., Tokyo, Japan), and the intensity of the band corresponding to iNOS of 130 kDa was determined with a BAS2000 bioimage analyzer (Fuji Photo Film Co.).

Effect on iNOS Activity in Preactivated Macrophages MDL, the compounds in fractions A–E, and the hydrolyzable tannins obtained from fraction B were examined as to whether they inhibit the iNOS activity in preactivated macrophages, as reported previously.¹⁹⁾ In brief, macrophages were treated with 100 ng/ml LPS and 10 U/ml $\text{INF-}\gamma$ at 37°C

Fig. 2. Inhibitory Effects of MDL and the Tannins on NO Production

Cells were treated with various concentrations of MDL (a), 1, 2, 5 and 6 (b), and 3, 4 and 7 (c) in the presence of 100 ng/ml LPS + 10 U/ml IFN- γ at 37 °C for 8 h. The symbols correspond to: (a) MDL ($\circ$), (b) 1 ($\diamond$), 2 ($\square$), 5 ($\blacklozenge$), and 6 ($\blacksquare$), and (c) 3 ($\triangle$), 4 ($\bullet$), and 7 ($\blacktriangle$). The results are the means $\pm$ S.E. of two independent experiments comprising duplicate assays. Statistical analysis was performed by means of Student's *t*-test as to the significance of difference against the control (cells treated with 100 ng/ml LPS + 10 U/ml IFN- γ). **, $p < 0.01$.

for 16 h, then the cells were collected, washed three times with PBS, and finally suspended at 4×10^5 cells/ml in modified Hank's balanced salt solution, (HBSS(+)). A 500 μ l volume of cells was seeded onto a 24-well plate (Sumitomo Bakelite, #8024R; Tokyo), followed by incubation at 37 °C for 10 min to allow the cells to adhere to the plate. Then, MDL and the other compounds were added, followed by incubation at 37 °C for 5 min, and then by the addition of 100 μ M carboxy-PTIO and 1 mM L-arginine to start the reaction. The cells were reincubated at 37 °C for 0–30 min, then the amount of NO_2^- in the culture supernatant was determined by means of the Griess reaction.²⁰⁾

Nitrite Assay One hundred microliters of the culture supernatant were placed in a well in a 96-well flat bottom plate, in duplicate. A standard solution of NaNO_2 was also placed in other wells on the same plate. To quantitate NO_2^- , 50 μ l of Griess reagent, 1% sulfanilamide in 5% H_3PO_4 and 0.1% N-1-naphthylethylenediamine dihydrochloride was added to each well.²¹⁾ After 10 min, the reaction products were colorimetrically quantitated at 550 nm with subtraction of the background absorbance at 630 nm, using a Corona MTP-120[®] microplate reader (Corona Electric Co., Ltd.).

Other Assay Protein concentrations were determined according to Bradford, with bovine serum albumin (BSA) as the standard.²²⁾

RESULTS

Inhibitory Effect on NO Production by Activated Macrophages The release of NO_2^- was first observed 6 h after incubation with 100 ng/ml LPS and 10 U/ml IFN- γ , then the amount of NO_2^- increased almost linearly with time up to 18 h, but the control cells without any addition showed no release. Based on these results, we examined the effects of MDL and its components on NO production by macrophages at 8 or 18 h.

The addition of MDL caused concentration-dependent inhibition of NO production by macrophages during incubation at 37 °C for 8 h (Fig. 2a). Fractions B (IC_{50} : 5.4 μ g/ml) and C (IC_{50} : 7.6 μ g/ml) also caused inhibition of the NO production; however, fractions A, D and E did not (Table 1). The ac-

tive compounds in fraction B, hydrolyzable tannins such as 4 (IC_{50} : 2.0 μ M), 1 (IC_{50} : 2.2 μ M), 2 (IC_{50} : 2.8 μ M) and 3 (IC_{50} : 5.1 μ M), showed strong inhibitory activities. However, 7, a constituent unit of these hydrolyzable tannins, 5 or 6 did not show such inhibitory activity (Fig. 2b,c). Time course experiments showed the continuous inhibition of NO production by MDL (3 μ g/ml), 2 (3 μ M), and 3 (3 μ M) for up to 18 h (Fig. 3). In addition, none of these compounds showed any effect on NO production by the control macrophages without LPS or IFN- γ treatment. Although some of these compounds induced apoptotic cell damage to RAW264.7 macrophages at higher concentrations than were used in this study (data not shown), all of the experiments were performed under conditions under which cell viability was not affected, as revealed by means of the trypan blue dye-exclusion test and morphological observation (data not shown). These results suggest that MDL has a suppressive effect on NO production in the course of macrophage activation, and that a major portion of the inhibitory effect is due to some of the hydrolyzable tannins in fraction B of MDL.

Inhibitory Effect on iNOS Protein Induction Induction of the iNOS protein in RAW264.7 cells was examined in the presence or absence of MDL and the hydrolyzable tannins. MDL and 1, 2, 3 and 4 remarkably decreased the amount of iNOS protein, even at 1 μ M (Fig. 4). However, 5, 6 and 7 had little or no effect on iNOS induction (data not shown). These results suggested that the inhibitory effects of MDL and 1–4 on NO production by activated macrophages are due, at least in part, to the inhibition of iNOS protein induction in LPS+IFN- γ -treated macrophages.

Inhibitory Effect on iNOS Activity in Preactivated Macrophages In order to determine whether MDL and 1–4 inhibit NO production directly in activated macrophages, cells were pretreated with LPS+IFN- γ , washed, reseeded, and then treated with MDL and 1–4 in HBSS(+) containing 1 mM L-arginine and 100 μ M carboxy-PTIO. Carboxy-PTIO converts $\cdot\text{NO}$ to $\cdot\text{NO}_2$, and then to NO_2^- in a fully stoichiometric manner.¹⁸⁾ The NO_2^- production by the non-treated control cells was 140 pmol $\cdot\text{NO}/\text{min}/2 \times 10^5$ cells under these conditions and was linear at 37 °C for up to 30 min. This NO production was completely abolished by 1 mM L-NIO, a

Fig. 3. Time Courses of Inhibition of NO Production by MDL and the Tannins

Cells were treated with MDL or the tannins in the presence of 100 ng/ml LPS+10 U/ml IFN- γ at 37°C for up to 18 h. The results are the means in a typical experiment. The symbols correspond to: (a) control cells (treated with 100 ng/ml LPS+10 U/ml IFN- γ) ($\blacktriangledown$) and 3 μ M MDL ($\circ$), and (b) control cells ($\blacktriangledown$), 3 μ M **2** ($\square$) and 3 μ M **3** ($\triangle$). Similar results were obtained repeatedly three times.

Fig. 4. Inhibitory Activities of MDL and the Tannins toward iNOS Induction

Cells were treated with various concentrations of MDL or the compounds in the presence of 100 ng/ml LPS+10 U/ml IFN- γ , and then cell lysates were prepared and assayed as described in the text. The amounts of iNOS protein in the cell extracts were calculated from the intensities of the bands corresponding to a M.W. of 130 kDa. We used standard iNOS in the immunoblot analysis in order to normalize the iNOS amounts in different gels. The results are expressed as values relative to the amount of iNOS in 60 μ g of the control cell (treated with LPS+IFN- γ , but MDL or other compounds not added) extract being expressed as 100. The symbols correspond to: (a) MDL ($\circ$), **1** ($\diamond$) and **2** ($\square$), and (b) **3** ($\triangle$) and **4** ($\bullet$). In the photographs in the lower panel, the bands corresponding to the iNOS protein of 130 kDa are indicated. The photograph numbers correspond to: 1, standard; 2, control (absence of LPS or IFN- γ); 3, control (presence of LPS+IFN- γ); 4, MDL 0.3 μ g/ml; 5, MDL 1 μ g/ml; 6, MDL 3 μ g/ml; 7, MDL 10 μ g/ml; 8, MDL 30 μ g/ml; 9, MDL 100 μ g/ml; 10, **1** 1 μ M; 11, **1** 3 μ M; 12, **1** 10 μ M; 13, **1** 30 μ M; 14, **2** 1 μ M; 15, **2** 3 μ M; 16, **2** 10 μ M; 17, **2** 30 μ M; 18, **4** 1 μ M; 19, **4** 3 μ M; and 20, **4** 10 μ M. M means molecular weight markers.

strong inhibitor of iNOS (Fig. 5a).¹⁸) All of **1**, **2**, **3** and **4** significantly inhibited NO production with time; however, MDL showed only a slight inhibitory effect, with significant sup-

pression only at 15 min (Fig. 5b, c). Compounds **1**–**4** showed various degrees of inhibition, the order being **4** (IC_{50} : 32.1 μ M)>**2** (IC_{50} : 36.8 μ M)>**1** (IC_{50} : 48.4 μ M)>**3** (IC_{50} :

Fig. 5. Time Courses of the Inhibitory Effects of MDL and the Compounds on the iNOS Activity in Preactivated Macrophages

Assays were performed as described in the legend to Table 1. The concentrations of the examined samples were 100 $\mu\text{g/ml}$ MDL and 100 μM of 1–4. The symbols correspond to: (a) control (+100 μM carboxy-PTIO and 1 μM L-arginine) ($\blacktriangledown$), +NONE (∇) and +L-NIO ($\blacktriangle$), (b) control ($\blacktriangledown$), MDL ($\circ$), 1 ($\diamond$) and 2 ($\square$), and (c) control ($\blacktriangledown$), 3 ($\triangle$) and 4 ($\bullet$). The results are presented as the means $\pm$ S.E. for the two different experiments involving triplicate assays. The significance of differences against the control was statistically determined by means of Student's *t*-test. *, $p < 0.05$; **, $p < 0.01$.

Fig. 6. Dose-Dependence of the Inhibitory Effects of MDL and the Tannins on the iNOS Activity in Preactivated Macrophages

Assays were performed as described in the legend to Table 1. The symbols correspond to: (a) MDL ($\circ$), 1 ($\diamond$) and 2 ($\square$), and (b) 3 ($\triangle$) and 4 ($\bullet$). The results are presented as the means $\pm$ S.E. for two different experiments involving triplicate assays. The significance of differences against the control was statistically determined by means of Student's *t*-test. *, $p < 0.05$; **, $p < 0.01$.

Table 1. Inhibitory Activity of a Fractionated Crude Extract of MDL toward NO Production by Macrophages

crude extract	Assay I			Assay II		
	Dose ($\mu\text{g/ml}$)	Inhibition (%)	IC ₅₀ ($\mu\text{g/ml}$)	Dose ($\mu\text{g/ml}$)	Inhibition (%)	IC ₅₀ ($\mu\text{g/ml}$)
Fraction A	100	9.4 $\pm$ 3.8	>100	300	5.1 $\pm$ 0.3	>300
	10	3.2 $\pm$ 1.8		100	N.D.	
	1	N.D.		30	N.D.	
Fraction B	100	78.4 $\pm$ 1.4	5.4	300	68.9 $\pm$ 4.3	82.7
	10	57.2 $\pm$ 2.3		100	53.4 $\pm$ 3.6	
	1	26.3 $\pm$ 2.4		30	34.9 $\pm$ 4.2	
Fraction C	100	74.8 $\pm$ 2.3	7.6	300	55.3 $\pm$ 1.2	158.9
	10	54.3 $\pm$ 3.4		100	46.4 $\pm$ 3.6	
	1	20.5 $\pm$ 5.6		30	39.4 $\pm$ 0.8	
Fraction D	100	9.2 $\pm$ 1.9	>100	300	28.5 $\pm$ 2.0	>300
	10	7.3 $\pm$ 0.8		100	14.8 $\pm$ 1.3	
	1	3.4 $\pm$ 0.2		30	11.4 $\pm$ 3.0	
Fraction E	100	6.9 $\pm$ 2.0	>100	300	10.1 $\pm$ 0.6	>300
	10	N.D.		100	N.D.	
	1	N.D.		30	N.D.	

Assay I: cells were treated with a crude extract in the presence of 100 ng/ml LPS + 10 U/ml IFN- γ at 37 $^{\circ}\text{C}$ for 8 h. The amount of NO₂⁻ in the culture supernatant was determined as described in the text. The results are the means $\pm$ S.E. for two different experiments involving duplicate assays. Assay II: cells were pretreated with 100 ng/ml LPS + 10 U/ml IFN- γ at 37 $^{\circ}\text{C}$ for 16 h and suspended in HBSS (+). Then, the cells were incubated with a crude extract, and after incubation for 5 min, 100 μM carboxy-PTIO and 1 μM L-arginine were added to the cell culture, followed by incubation for another 30 min. The results are the means $\pm$ S.E. of triplicate assays. N.D. means not detected.

132.3 μM) (Fig. 6a, b). However, MDL showed little or no effect, even at 100 $\mu\text{g}/\text{ml}$ (Fig. 6a) and higher concentrations (data not shown). We guessed that components in MDL other than the hydrolyzable tannins in fraction B might have attenuated the inhibitory activity toward the NO production of **1**–**4**. Further analysis of fractions A–E revealed that fractions B and C caused an inhibition of NO production both in the course of macrophage activation for 8 h and in preactivated macrophages. However, the other fractions, A, D and E, did not cause inhibition (Table 1). These results suggest the possibility that the components in fractions A, D and E might attenuate the inhibitory activity toward NO production in fractions B and/or C. Fraction D was insoluble in the culture medium at 100 $\mu\text{g}/\text{ml}$. So, we investigated whether the addition of fraction A or E suppresses the inhibitory activity in fraction B. Fraction B caused 34.9% inhibition of the iNOS activity in preactivated macrophages. The addition of 30 $\mu\text{g}/\text{ml}$ and 100 $\mu\text{g}/\text{ml}$ fraction A suppressed the inhibitory activity of fraction B to 26.5 and 20.3%, respectively, and that of 100 $\mu\text{g}/\text{ml}$ fraction E suppressed it to 28.4%. These results suggest that MDL contains both strong inhibitors and suppressors of the inhibitors of NO production by preactivated macrophages, and that the former are mainly the hydrolyzable tannins, as shown in Fig. 1, and the latter are distributed in fractions A and E on the separation of MDL by Diaion HP-20 column chromatography.

DISCUSSION

We found that MDL inhibited NO production in the course of macrophage activation, and that the major inhibitory activity was due to hydrolyzable tannins. Study of the structure activity relationships of these tannins, *i.e.*, **4** (IC_{50} : 2.0 μM) > **1** (IC_{50} : 2.2 μM) > **2** (IC_{50} : 2.8 μM) > **3** (IC_{50} : 5.1 μM), showed that **4**, a dimeric tannin, was a stronger inhibitor than the other monomeric tannins. Compound **2** exhibited stronger inhibition than **3**, suggesting that the presence of a galloyl group at the glucose core led to stronger inhibition than the absence of one. Compound **6** showed little or no inhibition, suggesting that the conjugation of functional galloyl or hexahydroxydiphenoyl groups on the glucose core was necessary for manifestation of the inhibitory activity. As for inhibition of the iNOS protein induction in LPS+IFN- γ activated macrophages, all of **1**–**4** caused significant inhibition, even at 1 μM . The direct inhibitory effects on iNOS activity in pretreated macrophages were in the order of **4** (IC_{50} : 32.1 μM) > **2** (IC_{50} : 36.8 μM) > **1** (IC_{50} : 48.4 μM) > **3** (IC_{50} : 132.3 μM). This trend was similar to that of the inhibitory effects on NO production during incubation for 8 h. However, **1** exhibited somewhat weaker inhibition than **2**. It was assumed that the open or close chain form of the glucose moiety in these tannins might affect their inhibitory potency, or an increase in the rigidity of the tannin molecule might decrease the inhibitory activity, because **1** has two hexahydroxydiphenoyl groups, of which one takes part in the C-glucosidic linkage.

Flavones and catechins have been reported to inhibit NO production by activated murine macrophages.^{23,24} Chan *et al.* have reported that catechins in green tea reduce NO production by mouse peritoneal cells activated with LPS and IFN- γ through two mechanisms, *i.e.*, by the reduction of iNOS gene expression and the inhibition of enzyme activity.²⁴ In this

study, hydrolyzable tannins inhibited both the induction of the iNOS protein and the activity of the iNOS in preactivated macrophages. There seem to be some similarities in the biological activities of polyphenols between these two studies. They suggested that the gallate structure in catechins was important for inhibition of the enzyme activity. We also found that the addition of a galloyl group to the glucose core in hydrolyzable tannins increased the inhibitory activity.

iNOS is a flavin adenine dinucleotide (FAD)- and flavin mononucleotide (FMN)- containing flavoprotein, representing a reductase domain with binding sites for NADPH, FAD and FMN. Flavoproteins transfer electrons from NADPH to an endogenous heme group or an associated hemeprotein.²⁵ NO was synthesized from arginine through a reaction involving two sequential mono-oxygenation steps requiring oxygen and electrons in the presence of tetrahydrobiopterin. It was assumed that these tannins might block electron flow in the enzyme molecule. The catechins and hydrolyzable tannins examined in this study are known to act as radical scavengers. Okuda *et al.* have reported that tannins inhibit lipid peroxidation in rat liver microsomes stimulated by adenosine diphosphate (ADP) and NADPH, and inhibit the generation of the superoxide anion radical through the hypoxanthine xanthine oxidase system.^{4,5} They also concluded that these inhibitions by tannins were due to their radical-scavenging activity. However, the results of the present study on these tannins, as to inhibition of the iNOS activity, showed no correlation with the antioxidative activity of these compounds in Okuda's studies of the structure activity relationships. Instead, these results imply that these hydrolyzable tannins might influence other mechanisms such as arginine synthesis²⁶ or the dimer-formation of iNOS,²⁷ both of which have been suggested to be involved in the regulation of iNOS activity in macrophages post-translationally.

Inhibitors of NO production by macrophages act mainly through two mechanisms: one is the inhibition of iNOS expression, and the other is the inhibition of enzyme activity. Most iNOS inhibitors act through either of these mechanisms. The inhibition of both the induction of iNOS and the activity of iNOS seems to be a characteristic of hydrolyzable tannins in MDL and also of catechins in green tea.²⁴

We suggest that MDL and these hydrolyzable tannins play roles in the modulation of inflammatory and immune responses by inhibiting the production of nitric oxide.

Suppressors of the inhibition of iNOS activity by tannins in fractions A and E seem to have another property of MDL, a mild pharmacological drug which has been used for a long time in China due to its moderate effect in therapy to treat pathophysiological disorders. Studies to determine these suppressors of the inhibition of iNOS activity in MDL are underway.

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