Inhibitors of nitric oxide (NO) production in murine macrophage-like J774.1 cells from Brazilian propolis

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Abstract

Water and MeOH extracts of Brazilian propolis showed dose-dependent inhibition toward nitric oxide (NO) production in lipopolysacchalide (LPS)-activated murine macrophage-like J774.1 cells. From the water extract, 17 phenolic compounds were isolated and among them 15 are new for the water extract of propolis. Moreover, methyl *p*-hydroxydihydrocinnamate (**9**) and 1-(4-hydroxyphenyl)butane-1,3-dione (**11**) were isolated, for the first time, from propolis. Labdane-type diterpenes, flavonoids and some phenolic compounds possessed potent NO inhibitory activity. Coniferyl aldehyde (**23**) and dimeric coniferyl acetate (**33**) showed the strongest NO inhibition with IC_{50} values of 18.0 and 27.1 μM, respectively, which were stronger than the positive control, N^G-monomethyl-L-arginine (L-NMMA; IC_{50}, 44.5 μM).

Key words Brazilian propolis, nitric oxide (NO), coniferyl aldehyde, dimeric coniferyl acetate, labdane-type diterpene.

Abbreviations DPPH, 1,1-diphenyl-2-picrylhydrazyl; IFN-γ, interferon-gamma; IL-1 interleukin-1; iNOS, inducible nitric oxide synthase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; L-NMMA, N^G-monomethyl-L-arginine; NO, nitric oxide; TNF, tumour necrosis factor.

Introduction

Nitric oxide (NO) is an important signaling molecule that acts in many tissues to regulate a diverse range of physiological processes. When certain cells are activated by specific proinflammatory agents such as endotoxin, tumor necrosis factor (TNF), interferon-gamma (IFN-γ) and interleukin-1 (IL-1), NO is produced by inducible nitric oxide synthase (iNOS) and acts as a host defense by damaging pathogenic DNA and as a regulatory molecule with homeostatic activities.[1] However, excessive production has detrimental effects on many organ systems of the body leading to tissue damage, even leading to a fetal development (septic shock).[2] Therefore, effective inhibition of NO accumulation by inflammatory stimuli presents a beneficial therapeutic strategy.

Propolis is sticky material collected by honey bees from various plant sources to protect their hives. It has been widely used as a popular remedy in folk medicine and has been reported to possesses various biological properties including antiinflammatory, antioxidative, antidiabetic, antihepatotoxic and even anticancer activities.[3] These pharmacological properties encouraged several groups all over the world to conduct research work on active constituents of propolis. Regarding the chemical components of propolis, most of its ingredients are soluble in organic solvents, and thus the alcoholic extract of propolis or propolis balsam (90% EtOH extract) have been studied well and more than 100 components have been identified from Brazilian propolis alone.[4] Although the water extract of propolis or micelle extract have equal demand, on the other hand, only little work have been done due to its hydrophilic nature.

In our previous work, we isolated four

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dicaffeoylquinic acid derivatives from a water extract of Brazilian propolis which possessed potent hepatoprotective activity and strong 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity.[5,6] We further examined the hepatoprotective, cytotoxic and DPPH radical scavenging activities of propolis from Brazil, Peru, the Netherlands and China,[7] and identified several active compounds.[8-11] In this paper, we would like to discuss the NO inhibitory activity and chemical constituents of water extract of Brazilian propolis.

Materials and Methods

General: ^{1}H, ^{13}C and 2D NMR spectra were taken on a JEOL JNM LA-400 spectrometer with tetramethylsilane as an internal standard. UV spectra were recorded on a Shimadzu UV-160A UV-visible spectrophotometer and IR spectra were recorded in Shimadzu IR-408 spectrophotometer either in $CHCl_3$ solution or in KBr disks. Column chromatography was performed on silica gel 60 (Nacalai tesque, Inc., Kyoto, Japan) and analytical and preparative TLC were conducted on precoated Merck Kieselgel 60F_{254} and RP-18F_{254} plates (0.5 or 0.25 mm thickness).

Propolis: Green-type propolis was collected at Minas Gerais, Brazil in 1999. A voucher sample (TMPW 19915) is preserved in the Museum for Materia Medica, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, Toyama, Japan.

Extraction and isolation: Brazilian propolis (1 Kg) was extrated with water (3 L×2) at 80℃ for 3 h and the extract was concentrated and lyophilized to give a water extract (116 g). The water extract (110 g) was further fractionated into MeOH soluble (46 g) and insoluble (62 g) fractions. The MeOH soluble fraction (35 g) was subjected to silica gel column chromatography (7×52 cm) with a $CHCl_3$-MeOH gradient system to afford nine fractions [fraction 1: $CHCl_3$ eluate, 2.24 g; fraction 2: 5% MeOH-$CHCl_3$ eluate, 2.11 g; fraction 3: 5% MeOH-$CHCl_3$ eluate 2.56 g; fraction 4: 5%-15% MeOH-$CHCl_3$ eluate 4.39 g; fraction 5: 20-30% MeOH-$CHCl_3$ eluate 8.0 g; fraction 6: 30-40% MeOH-$CHCl_3$ eluate 1.97 g; fraction 7: 40-45% MeOH-$CHCl_3$ eluate 4.30 g; fraction 8: 50% MeOH-$CHCl_3$ eluate 1.24 g; fraction 9: MeOH eluate 4.75 g]. Repeated column chromatography of these fractions over silica gel, followed by preparative TLC, yielded the following compounds: fraction 1, benzoic acid (**1**, 82.0 mg);[12] fraction 2, **1** (22.4 mg), *p*-methoxybenzoic acid (**2**, 12.4 mg),[13] 3-hydroxy-4-methoxybenzoic acid (**4**, 0.8 mg),[12] 3,4-dimethoxycinnamic acid (**6**, 22.4 mg),[14] 1-(4-hydroxyphenyl)butane-1,3-dione (**11**, 12.0 mg),[15] 4-hydroxy-3-prenylcinnamic acid (**12**, 1.3 mg);[8] 4-dihydrocinnamoyloxy-3-prenylcinnamic acid (**13**, 10.0 mg),[8] 3-(2,2-dimethylchromene-6-yl)propenoic acid (**17**, 10.8 mg);[8] fraction 3, *p*-hydroxybenzoic acid (**3**, 20.1 mg),[12] *p*-hydroxycinnamic acid (**5**, 479 mg),[3] methyl *p*-hydroxydihydrocinnamate (**9**, 15.0 mg),[16] 3-(4-hydroxyphenyl)propionic acid (**10**, 12.2 mg),[17,18] **17** (15.3 mg); fraction 4, **5** (10.0 mg); fraction 6, methyl 3,4-*O,O*-dicaffeoylquinate (**15**, 11.1 mg).[5] The MeOH insoluble fraction (40 g) was subjected to Sephadex LH-20 column chromatography (7 × 50 cm) with water, MeOH and then acetone to afford five fractions [fraction 1: water eluate, 24.7 g; fraction 2: water eluate, 1.36 g; fraction 3: MeOH eluate, 4.31 g; fraction 4: acetone eluate, 1.82 g; fraction 5: acetone eluate, 0.16 g]. Repeated column chromatography of these fractions over silica gel and cosmosil 75C_{18}-OPN, followed by preparative TLC, yielded the following compounds: fraction 2, chlorogenic acid (**16**, 5.6 mg);[13] fraction 4, caffeic acid (**7**, 2.2 mg),[5,13] methyl caffeate (**8**, 186 mg), 3,4-*O,O*-dicaffeoylquinic acid (**14**, 7.2 mg).[5] There is no complete spectral data of **9** and **11** in literature and thus they were included herewith.

*Methyl p-hydroxydihydrocinnamate (**9**)*: Light yellow powder. UV λmax ($CHCl_3$) nm (log ε): 280 (3.8). IR ($CHCl_3$) cm^{-1}: 3600, 3350, 1725, 1610, 1515, 1440, 1360, 1260, 1170, 1100, 830, 810, 660. ^{1}H-NMR ($CDCl_3$) δ: 6.74 (2H, d, J = 8.4 Hz, 2-H, 6-H), 7.06 (2H, d, J = 8.4 Hz, 3-H, 5-H), 2.87 (1H, t, J = 7.7 Hz, 8-H), 2.60 (1H, t, J = 7.7 Hz, 7-H), 3.67 (3H, s, OMe), 5.00 (1H, br s, 4-OH). ^{13}C-NMR ($CDCl_3$) δ: 173.6 (C-9), 154.1 (C-4), 132.6 (C-1), 129.4 (C-2, C-6), 115.3 (C-3, C-5), 36.0 (C-7), 30.1 (C-8), 51.6 (OMe). FAB-MS *m/z*: 179 [M-H]$^-$.

*1-(4-Hydroxyphenyl)butane-1,3-dione (**11**)*: Light yellow powder. UV λmax ($CHCl_3$) nm (log ε): 259.2 (2.7). IR νmax ($CHCl_3$) cm^{-1}: 1700, 1600, 1460, 1255, 1150, 810. ^{1}H-NMR ($CDCl_3$) δ: 7.89 (2H, d, J = 8.6 Hz, 2-H, 6-H), 6.89 (2H, d, J = 8.6 Hz, 3-H, 5-H), 2.60 (2H, s, 8-H), 2.20 (3H, s, 10-H_3). ^{13}C-NMR ($CDCl_3$) δ: 197.1 (C-9), 190.8 (C-7) 160.6 (C-4), 132.4 (C-1), 131.0 (C-2,

C-6), 115.3 (C-3, C-5), 30.2 (C-8), 26.2 (C-10). FAB-MS *m/z*: 179 $[M+H]^+$.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay: DPPH radical scavenging activity was measured according to the procedure described by Hatano *et al.*[19] Briefly, sample dissolved in EtOH (500 μL) was mixed with an equal volume of DPPH solution (60 μM). The resulting solution was thoroughly mixed by vortex, and absorbance was measured at 520 nm after 30 min. The scavenging activity was determined by comparing the absorbance with that of the blank (100%) containing only DPPH and solvent.

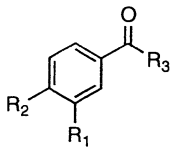
NO inhibitory assay: Macrophage-like J774.1 cell line was purchased from Riken Cell Bank (Tsukuba, Japan) and propagated in 75-cm^2 plastic culture flasks (Falcone, Becton Dickinson, NJ, USA), containing RPMI-1640 medium supplemented with penicillin G (100 units/mL), streptomycin (100 mg/mL) and 10% fetal calf serum. The cells were harvested with trypsin and diluted to a suspension in fresh medium. The cells were seeded in 96-well plastic plates with 1×10^5cells/well and allowed to adhere for 2 h at 37℃ in a humidified atmosphere containing 5% CO_2. Then, the medium was replaced with fresh medium, containing lipopolysacchalide (LPS, 10 μg/mL) and test compounds at indicated concentrations, and the cells were incubated for 24 h. NO production was determined by measuring the accumulation of nitrite in the culture supernatant with Griess reagent.[20] Briefly, 50 μL of the supernatant from 96-well plate was incubated with equal volume of Griess reagent (0.5% sulfanilamide and 0.05% naphthylenediamide dihydrochloride in 2.5% H_3PO_4) and were allowed to stand for 10 min at room temperature. Absorbance at 550 nm was measured using HTS-7000 microplate reader (Perkin Elmer, CT, USA). The blank correction was carried out by subtracting the absorbance due to medium only from the absorbance reading of each well. The percentage inhibition was calculated as followings: % inhibition = $[(A_c-A_b)/A_c]\times100$, where Ac and Ab are absorbance of control group treated with LPS alone and absorbance of the sample, respectively. The data are expressed as mean ± S.D. of four determinations and statistical significance was calculated by student's t-test.

Determination of cell viability: After taking 50 μL of supernatant from 96-well plate for NO determination, the remaining medium was replaced by 100 μL of MTT solution (0.40%) and incubated for 3 h. The formazan formed was dissolved in dimethylsulfoxide (DMSO) and the amount of formazan formed was measured spectrophotometrically at 550 nm using HTS-7000 microplate reader. The cell viability was calculated as follows: % cell viaility = $(A_b/A_a)\times100$, where A_a and A_b are absorbance of LPS-treated group and absorbance of the sample, respectively. The data are expressed as mean ± S.D. of four determinations.

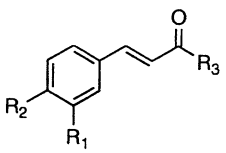
Results and Discussion

Both the MeOH and water extract of Brazilian propolis showed dose-dependent NO inhibition towards LPS-activated NO production in murine macrophage-like J774.1 cells with IC_{50} values of 37.8 and 78.9 μg/mL, respectively (Table I). In our previous work, we isolated 27 compounds from the MeOH extract of Brazilian propolis,[8,21] and thus in the present work we have focused on the water extract. The water extract was further divided into MeOH soluble and insoluble fractions, which possessed nearly equal strength of NO inhibitory activity (data not shown). Thus, both fractions were subjected to chromatographic separation, which resulted in the isolation of 17 compounds including four benzoic acid derivatives (**1-4**), eight cinnamic acid derivatives (**5-10, 12, 13**), three caffeoylquinic acid derivatives (**14-16**), a chromene (**17**) and 1-(4-hydroxyphenyl) butane-1,3-dione (**11**). All these compounds, except for **14** and **15**, were isolated for the first time, from the water extract of Brazilian propolis. Moreover, methyl *p*-hydroxydihydrocinnamate (**9**) and 1- (4-hydroxyphenyl) butane-1,3-dione (**11**) were isolated, for the first time, from propolis.

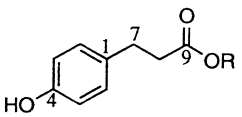
To know active components in Brazilian propolis, 36 compounds (Fig. 1) including **1-17** isolated from water extract and **18-36** having been isolated from a MeOH extract were subjected to NO inhibitory experiment. Almost all compounds showed inhibition against LPS-activated NO production in murine macrophage-like J774.1 cells, with various intensities. Among the 17 compounds isolated from the water extract, methyl caffeate (**8**) showed the strongest inhibition with an IC_{50} value of 78.9 μM. Nearly 75% of compounds from the MeOH extract, on the other hand, possessed potent NO



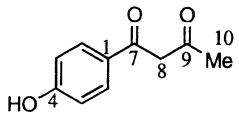
1 $R_1 = R_2 = H$, $R_3 = OH$
2 $R_1 = H$, $R_2 = OMe$, $R_3 = OH$
3 $R_1 = H$, $R_2 = OH$, $R_3 = OH$
4 $R_1 = OH$, $R_2 = OMe$, $R_3 = OH$
22 $R_1 = OMe$, $R_2 = OH$, $R_3 = H$



5 $R_1 = H$, $R_2 = OH$, $R_3 = OH$
6 $R_1 = R_2 = OMe$, $R_3 = OH$
7 $R_1 = R_2 = OH$, $R_3 = OH$
8 $R_1 = R_2 = OH$, $R_3 = OMe$
23 $R_1 = OMe$, $R_2 = OH$, $R_3 = H$



9 R = Me
10 R = H



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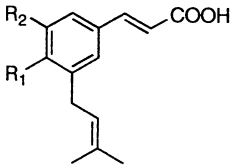
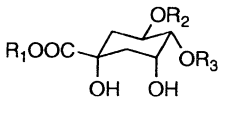
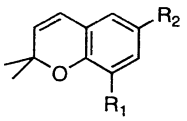


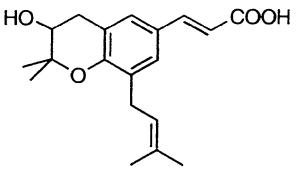
12 $R_1 = OH$, $R_2 = H$
13 $R_1 = OCOCH_2CH_2Ph$, $R_2 = H$
21 $R_1 = OH$, $R_2 = CH_2CH{=}C(Me)_2$



14 $R_1 = H$, $R_2 = R_3 =$ caffeoyl
15 $R_1 = Me$, $R_2 = R_3 =$ caffeoyl
16 $R_1 = H$, $R_2 =$ caffeoyl, $R_3 = H$



17 $R_1 = H$, $R_2 = CH{=}CHCOOH$
18 $R_1 = CH_2CH{=}C(Me)_2$, $R_2 = CH{=}CHCOOH$
19 $R_1 = R_2 = H$



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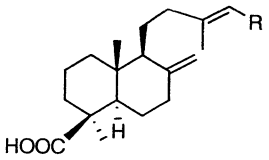
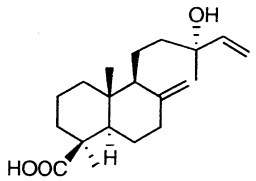


24 R = CH_2OH
25 R = CH_2OAc
26 R = COOH
27 R = COOMe



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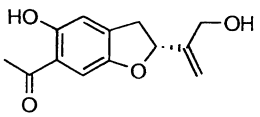

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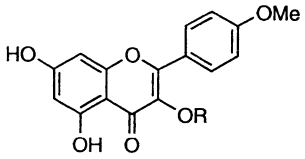
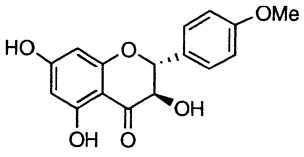


30 R = H
31 R = Me



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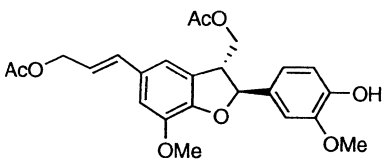

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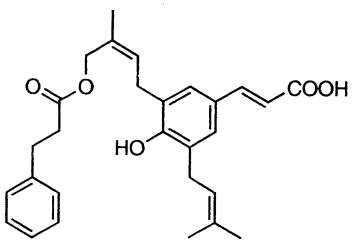

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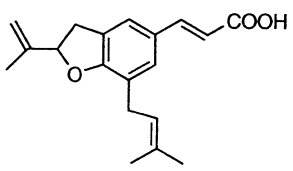

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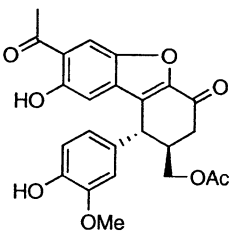

36

Fig. 1 Structures of compounds isolated from Brazilian propolis

Table I Inhibitory effects of constituents from Brazilian propolis on NO production in LPS-activated murine macrophage-like J774.1 cells

Compounds and Extracts	% Inhibition					IC_{50} (μM)
	200 μM	100 μM	50 μM	20 μM	2 μM	
H_2O Extract[a]	93.8 ± 2.1**	65.5 ± 3.5**	28.8 ± 3.0**	15.7 ± 2.5**	2.95 ± 6.2	78.9
MeOH Extract[a]	101 ± 1**	94.7 ± 0.6**	65.4 ± 2.6**	27.4 ± 2.2**	9.8 ± 3.1*	37.8
1	11.9 ± 3.6**	6.2 ± 3.6*	0.0 ± 2.3	0.8 ± 1.8	6.0 ± 4.8	>200
3	29.0 ± 5.4**	13.0 ± 0.9**	10.8 ± 3.0	11.0 ± 5.0	6.0 ± 7.3	>200
4	24.4 ± 6.2**	11.9 ± 3.1**	3.0 ± 6.8	4.1 ± 3.1	4.1 ± 4.2	>200
5	32.2 ± 3.2**	17.6 ± 3.2**	4.9 ± 7.6	8.1 ± 5.9	0.5 ± 6.5	>200
6	51.1 ± 2.3**	27.8 ± 1.2**	8.2 ± 4.7	8.5 ± 4.8	5.6 ± 4.5	196
7	47.0 ± 3.1**	25.2 ± 3.8**	6.9 ± 14.1	-1.7 ± 4.2	1.2 ± 1.8	>200
8	78.2 ± 1.8**	59.9 ± 3.4**	36.5 ± 5.7**	18.7 ± 2.2**	4.7 ± 2.6*	78.9
9	30.7 ± 6.3**	16.4 ± 2.8**	7.9 ± 1.3**	1.6 ± 2.5	1.3 ± 4.0	>200
10	37.0 ± 7.9**	19.3 ± 4.3**	10.6 ± 5.9*	11.1 ± 3.0	9.8 ± 1.9	>200
11	35.2 ± 7.1**	21.2 ± 2.3**	13.2 ± 6.9*	14.3 ± 4.9**	11.6 ± 1.8**	>200
12	76.2 ± 3.0**	39.1 ± 3.7**	19.7 ± 4.7**	14.6 ± 5.8	14.9 ± 3.9	129
13	43.4 ± 6.0**	21.1 ± 4.4**	14.0 ± 5.1**	6.4 ± 7.2	5.5 ± 6.3	>200
14	59.6 ± 2.1**	24.4 ± 2.7**	7.0 ± 3.7*	3.6 ± 7.7	2.3 ± 5.7	173
15	69.0 ± 3.7**	36.7 ± 2.7**	16.6 ± 1.1**	10.2 ± 1.2**	6.1 ± 1.0**	141
16	56.7 ± 3.2**	28.1 ± 1.6**	10.9 ± 10.2*	3.1 ± 5.2**	4.7 ± 2.5**	177
17	31.2 ± 1.5**	17.9 ± 2.2**	8.1 ± 5.3*	6.9 ± 1.5**	5.4 ± 3.1*	>200
18	31.2 ± 6.0**	17.9 ± 2.2**	8.1 ± 5.3*	7.0 ± 1.5**	5.5 ± 3.1*	>200
19	41.5 ± 1.0**	17.9 ± 2.2**	12.2 ± 2.8**	9.6 ± 1.0**	8.4 ± 1.7**	>200
20	59.0 ± 11.0**	31.8 ± 3.5**	12.6 ± 2.0**	4.6 ± 5.1	-1.3 ± 4.6	167
21	44.3 ± 1.0**	21.6 ± 1.9**	8.6 ± 5.3	3.8 ± 2.2	4.1 ± 5.8	>200
22	38.5 ± 3.6**	18.2 ± 4.3**	7.8 ± 1.0**	6.2 ± 4.5*	6.2 ± 1.7*	>200
23	102 ± 3**	99.5 ± 2.6**	79.7 ± 2.0**	54.7 ± 2.0**	12.5 ± 2.4**	18.0
24	99.5 ± 1.1**	72.3 ± 2.1**	33.0 ± 3.6**	15.7 ± 5.4**	12.6 ± 1.9**	71.7
25	90.1 ± 3.4**	71.3 ± 1.4**	27.3 ± 1.3**	6.5 ± 0.6**	1.1 ± 0.0**	75.1
26	65.1 ± 1.9**	25.9 ± 2.5**	9.9 ± 1.7**	9.9 ± 4.1**	5.7 ± 3.3**	162
27	92.3 ⊥ 4.9**	74.1 ± 0.6**	39.2 ± 2.7**	17.0 ± 3.8**	8.0 ± 2.8**	65.4
28	95.2 ± 1.1**	70.5 ± 2.1**	28.7 ± 6.9**	18.5 ± 6.3**	11.9 ± 4.8**	75.5
29	93.5 ± 1.9**	80.3 ± 1.1**	50.3 ± 1.7**	18.5 ± 3.0**	5.6 ± 4.9**	49.7
30	60.1 ± 3.5**	42.7 ± 6.8**	34.8 ± 4.6**	29.5 ± 5.1**	4.2 ± 4.2	142
31	97.5 ± 8.8**	85.7 ± 2.5**	54.2 ± 7.0**	25.3 ± 4.1**	1.1 ± 3.3	45.6
32	31.2 ± 4.4**	15.7 ± 3.3**	7.6 ± 3.7*	4.8 ± 2.7	3.0 ± 5.4	>200
33	93.5 ± 1.5**	101 ± 2**	82.1 ± 1.5**	40.0 ± 2.4**	2.9 ± 4.1	27.1
34	68.5 ± 3.4**	32.6 ± 3.1**	11.2 ± 3.9**	9.4 ± 4.8*	4.7 ± 3.2*	148
35	102 ± 0**	87.4 ± 3.4**	49.1 ± 2.2**	13.8 ± 5.3**	4.7 ± 4.1	51.2
36	101 ± 2**	75.3 ± 3.5**	37.1 ± 2.3**	16.8 ± 3.1**	2.9 ± 5.9	66.9
Polymixin B[a]	95.7 ± 11.6[a,**]	94.0 ± 1.1[a,*]	73.9 ± 6.4[a,*]	36.4 ± 15.5*	19.0 ± 4.5*	30.9
L-NMMA	100 ± 2**	83.3 ± 2.0**	54.0 ± 2.0**	32.1 ± 2.0**	5.1 ± 5.4	44.5

Each value represents the mean ± S.D. of four determinations. Significantly different from the control; *$p < 0.05$, **$p < 0.01$. NO inhibitory activity of **2** was not tested due to meager amount. [a]% inhibition and IC_{50} value are in μg/mL

Table II Cell viability of J774.1 cells on treatment of individual components during LPS-activated NO production

Compounds and Extracts	Cell servival rate				
	200 μM	100 μM	50 μM	20 μM	2 μM
H_2O Extract[a]	105 ± 21	93.9 ± 15.3	96.8 ± 13.7	91.5 ± 24.1	97.5 ± 10.6
MeOH Extract[a]	87.4 ± 15.8	103 ± 22	93.3 ± 11.2	91.5 ± 21.3	69.3 ± 11.6
1	97.4 ± 7.3	95.0 ± 12.3	88.0 ± 8.0	95.6 ± 0.1	91.7 ± 9.2
3	108 ± 1	108 ± 4	111 ± 0	92.9 ± 12.7	80.0 ± 13.7
4	90.6 ± 7.5	79.5 ± 4.3	88.1 ± 13.6	98.4 ± 19.8	96.2 ± 13.9
5	97.7 ± 17.0	92.7 ± 14.6	96.4 ± 17.2	94.8 ± 19.3	85.1 ± 21.4
6	106 ± 6	98.0 ± 14.1	98.7 ± 13.9	95.2 ± 11.4	92.3 ± 14.0
7	104 ± 5	104 ± 10	105 ± 7	105 ± 5	109 ± 4
8	97.1 ± 17.1	104 ± 0	106 ± 4	111 ± 0	108 ± 9
9	102 ± 3	100 ± 3	100 ± 4	100 ± 3	103 ± 8
10	106 ± 15	108 ± 11	98.8 ± 13.7	96.6 ± 20.1	75.9 ± 9.4
11	99.3 ± 21.1	84.4 ± 19.5	81.2 ± 3.7	76.4 ± 18.4	62.5 ± 7.9
12	131 ± 6	137 ± 14	146 ± 13	123 ± 6	121 ± 10
13	98.0 ± 4.5	92.0 ± 27.0	93.6 ± 12.5	107 ± 21	120 ± 26
14	99.1 ± 17.5	110 ± 12	91.7 ± 27.1	92.4 ± 20.7	93.9 ± 20.8
15	87.6 ± 0.0	114 ± 0	108 ± 0	107 ± 5	111 ± 5
16	94.2 ± 12.7	98.9 ± 2.2	95.9 ± 4.0	102 ± 4	114 ± 0
17	114 ± 6	112 ± 4	118 ± 11	122 ± 11	118 ± 15
18	62.0 ± 8.7	93.2 ± 20.6	85.8 ± 12.7	86.0 ± 14.6	83.8 ± 15.6
19	104 ± 9	113 ± 21	102 ± 19	103 ± 16	89.8 ± 4.1
20	73.3 ± 8.3	87.5 ± 17.1	96.3 ± 23.4	94.7 ± 18.9	90.7 ± 20.0
21	145 ± 12	131 ± 10	145 ± 6	129 ± 9	130 ± 12
22	91.0 ± 6.6	83.4 ± 10.9	85.7 ± 8.3	91.8 ± 12.4	104 ± 9
23	79.6 ± 10.0	106 ± 11	113 ± 16	95.2 ± 23.1	95.8 ± 17.2
24	94.9 ± 14.2	92.3 ± 6.3	105 ± 37	101 ± 15	99.1 ± 9.0
25	79.0 ± 4.9	105 ± 9	108 ± 6	98.9 ± 3.0	98.5 ± 5.2
26	109 ± 5	113 ± 5	115 ± 4	115 ± 3	117 ± 0
27	64.8 ± 9.7	89.3 ± 8.4	93.9 ± 8.6	99.2 ± 9.2	98.3 ± 3.5
28	95.6 ± 0.4	104 ± 0	107 ± 0	94.8 ± 0.5	116 ± 0
29	81.0 ± 5.8	94.8 ± 10.0	100 ± 13	101 ± 15	101 ± 9
30	45.5 ± 5.2	57.2 ± 9.1	75.0 ± 10.6	86.9 ± 12.8	85.3 ± 9.8
31	81.0 ± 7.8	94.8 ± 3.1	101 ± 7	101 ± 12	101 ± 10
32	45.5 ± 9.1	57.2 ± 9.3	75.0 ± 15.0	86.9 ± 11.8	85.3 ± 1.4
33	104 ± 9	110 ± 10	110 ± 14	111 ± 9	99.1 ± 12.3
34	93.0 ± 0	117 ± 8	110 ± 9	107 ± 10	94.7 ± 16.1
35	72.8 ± 8.5	88.4 ± 2.3	91.1 ± 9.7	95.2 ± 10.4	93.8 ± 10.1
36	98.1 ± 5.4	90.8 ± 8.3	85.9 ± 2.8	95.0 ± 11.0	80.2 ± 4.5
Polymixin B[a]	9.4 ± 1.4	38.9 ± 8.8	68.1 ± 9.8	85.5 ± 12.0	85.6 ± 11.8
L-NMMA	127 ± 6	111 ± 2	113 ± 14	99.3 ± 8.7	71.6 ± 6.6

Each value represents the mean ± S.D. of four determinations. [a]Cell survival rates are in μg/mL concentration.

inhibitory activity, which is in accordance with the potency of the extracts.

All the labdane-type diterpenes (**24-28**) possessed strong NO inhibitory activity with IC_{50} values less than 100 μM except for agathic acid (**26**; IC_{50}, 162 μM). Coniferyl aldehyde (**23**) and dimeric coniferyl acetate (**33**) possessed more potent activity (IC_{50}, 18.0 and 27.1 μM, respectively) than a positive control N^G-monomethyl-L-arginine (L-NMMA; IC_{50}, 44.5 μM). Flavonoids **30** and **31** showed stronger NO inhibitory activity than flavonol **32**, indicating that the C-2(3) double bond in flavonoid may enhance the NO inhibition. Viscidone (**29**), (*E*)-3-[2,3-dihydro-2-(1-methylethenyl)-7-prenyl-benzofuranyl]-2-propenoic acid (**35**) and propolis-benzofuran B (**36**) also possessed significant NO inhibitory activity with IC_{50} values of 49.7, 51.2 and 66.9 μM, respectively.

Considering all these facts labdane-type diterpenes (**24-28**) and coniferyl aldehyde derivatives (**23, 33**) should be responsible for the NO inhibitory activity of the Brazilian propolis. Moreover, caffeoylquinic acids (**14-16**), flavonoids (**30** and **31**) and some other phenolic compounds (**29, 32, 35, 36**) might also contribute to the NO inhibition. The syngeretic effect which is frequently reported for various other biological activities of propolis can not be ignored,[22] because both water and MeOH extracts of propolis possessed strong NO inhibitory activity compared to most of the individual compounds. The viability of J774.1 cells after treatment of extract and individual compounds are summarized in Table II. Compounds **18, 20, 23, 25** and **30** showed toxic effect at a concentration of 200 μM and **32** at 50 μM. But none of the compounds had a toxic effect at their IC_{50} ranges of NO inhibition.

There are two possibilities regarding to the mechanism of NO inhibition of these compounds; inhibition of iNOS and scavenge of the NO radical. Most of the compounds isolated from Brazilian propolis were phenolics, which were reported to have strong radical scavenging properties. In the present study, we further tested DPPH radical scavenging activity of all isolated compounds from the water extract. Methyl caffeate (**8**) and caffeoylquinic acid derivatives **14-16**, having significant NO inhibitory activity, showed strong DPPH radical scavenging activity with IC_{50} values of 5.26, 2.66, 2.01 and 5.67 μM, respectively. The phenolic compounds **23, 30, 31** and **33** with strong NO inhibitory activities were reported to reveal strong radical scavenging activities.[23] Thus, these compounds would inhibit NO by scavenging NO radical when it was generated. In contrast to them, none of labdane-type diterpenes possessed such DPPH radical scavenging activity.[23] Matsuda *et al.* reported that labdane-type diterpenes inhibit NO production in LPS-activated murine peritoneal macrophages due to their inhibitory activities against induction of iNOS.[24] Considering these facts, the NO inhibitory activity of the labdane-type diterpenes may be due to inhibition of iNOS induction, which is responsible for production of NO in LPS- activated murine macrophage-like J774.1 cells.

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和文抄録

ブラジル産プロポリスの水およびメタノールエキスがLPSで活性化したマウスマクロファージ様J774.1細胞の一酸化窒素（NO）産生を濃度依存的に抑制することを明らかにした。さらに，水エキスの成分検索を行ない17種のフェノール性化合物を単離したが，その中の15化合物はプロポリス水エキスからは初めて単離された化合物であった。また，methyl *p*-hydroxydihydrocinnamate (**9**) と 1-(4-hydroxyphenyl)butane-1,3-dione (**11**) のプロポリスからの単離はこれが最初の例である。次いで各化合物のNO阻害活性を測定したところ，ラブダン型ジテルペン，フラボノイド，数種のフェノール性化合物が強いNO阻害活性を示した。特に，coniferyl aldehyde (**23**; IC_{50}, 18.0 μM) と dimeric coniferyl acetate (**33**; IC_{50}, 27.1 μM) は陽性コントロールのNG-monomethyl-L-arginine (L-NMMA; IC_{50}, 44.5 μM) よりも強い活性を示した。

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