Caffeic Acid Phenethyl Ester (CAPE) Analogues: Potent Nitric Oxide Inhibitors from the Netherlands Propolis

Takema NAGAOKA, Arjun H. BANSKOTA, Yasuhiro TEZUKA, Kiyoshi MIDORIKAWA, Katsumichi MATSUSHIGE, and Shigetoshi KADOTA*

Institute of Natural Medicine, Toyama Medical and Pharmaceutical University; 2630-Sugitani, Toyama 930–0194, Japan.
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The MeOH and water extracts of the Netherlands propolis were tested for their inhibitory activity toward nitric oxide (NO) production in lipopolysaccharide (LPS)-activated murine macrophage-like J774.1 cells. Both of the extract possessed significant NO inhibitory activity with IC_{50} values of 23.8 and 51.5 μg/ml, respectively. Then 13 phenolic compounds obtained from the MeOH extract showing stronger NO inhibition were examined on their NO inhibitory activities. Caffeic acid phenethyl ester (CAPE) analogues, *i.e.*, benzyl caffeate, CAPE and cinnamyl caffeate, possessed most potent NO inhibitory activities with IC_{50} values of 13.8, 7.64 and 9.53 μM, respectively, which were two- to four-fold stronger than the positive control N^G-monomethyl-L-arginine (L-NMMA; IC_{50}, 32.9 μM). Further study on the synthetic analogues of CAPE revealed that both of 3-phenylpropyl caffeate (18; IC_{50}, 7.34 μM) and 4-phenylbutyl caffeate (19; IC_{50}, 6.77 μM) possessed stronger NO inhibitory activity than CAPE (10) and that elongation of alkyl side chain of alcoholic parts of caffeic acid esters enhance the NO inhibitory activity. In addition, it was found that CAPE analogues having longer carbon chain ($>C_5$) in alcoholic part showed toxic effects toward J774.1 cells. This NO inhibitory effect may directly correlate with antiinflammatory properties of the Netherlands propolis.

Key words caffeic acid phenethyl ester; nitric oxide inhibitor; Netherlands propolis; murine macrophage-like J774.1 cell; anti-inflammation

Propolis is a resinous hive product collected by honeybees from various plant sources. It has a pleasant aromatic odor and yellow-green to dark brown color depending on its source and age.[1)] Propolis has a long history of being used in traditional medicine dating back at least to 300 BC[1)] and has been reported to have a broad spectrum of biological activities, *viz.* anticancer, antioxidant, antiinflammatory, antibiotic and antifungal activities.[2)] It has recently gained popularity as a health drink and is used extensively in food and beverages in various parts of the world including Japan, the United States and Europe, where it is claimed to improve health and prevent diseases such as inflammation, heart disease, diabetes and even cancer.

Nitric oxide (NO) is associated with inflammatory reaction and is produced by inducible nitric oxide synthase (iNOS) in certain cells activated by various proinflammotory agents such as lipopolysaccharide (LPS), tumor necrosis factor (TNF), interleukin-1 (IL-1) and interferon-γ (IFN-γ). NO acts as a host defense by damaging pathogenic DNA and as a regulatory molecule with homeostatic activities,[3)] however, excessive production of NO is pathogenic for host tissue itself because NO *per se* as a reactive radical directly damages function of normal tissue.[4)] Thus, effective inhibition of NO accumulation by inflammatory stimuli represents a beneficial therapeutic strategy. In this paper, we will report the NO inhibitory activity of the Netherlands propolis and its active components.

MATERIALS AND METHODS

Chemicals RPMI and Eagle's minimum essential medium (EMEM) were purchased from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). 3-(4,5-Dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT), LPS, penicillin G and sterptomycin were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). N^G-Monomethyl-L-arginine (L-NMMA) was from Funakoshi (Tokyo, Japan). Heat-inactivated fetal calf serum (FCS) and trypsin were from Gibco BRL Products (Gaitherburg, MD, U.S.A.). Falcon primaria surface-modified polystyrene 96-well culture plates (Becton Dickinson, NJ, U.S.A.) were used for NO experiments. Phenylethanol and cinnamyl alcohol were from Nacalai Tesque (Kyoto, Japan). Sodium bicarbonate, glutamine, benzyl alcohol and caffeic acid were from Wako Pure Chemical Industries (Osaka, Japan).

Propolis The Netherlands propolis was collected in the northeast of the Netherlands in 1998, by scraping it off from the frames of beehives belonging to Honeybee Husbandry, Rutten, the Netherlands. The voucher specimen (No. TMPW 19920) is preserved in the Museum for Materia Medica, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, Toyama, Japan, as a reference.

Extraction and Isolation The powder of crude propolis (400 g) was extracted with MeOH (3 l×2) under reflux for 3 h to give a MeOH extract (210 g, 52.5%). The residue was further extracted with water (3 l×2) at 80 °C for 3 h and the filtrate was lyophilized to give a water extract (3.6 g, 0.9%). The isolation procedure of compounds **1**—**13** was described in a previous paper.[5)]

Synthesis of CAPE Analogues Twenty caffeic acid phenyl ester (CAPE) analogues were synthesized by one-pot esterification of caffeic acid and alcohol either with thionyl chloride in dioxane or acid-catalyzed esterification as described in a previous report.[6)]

Nitrite Production in Murine Macrophage-Like J774.1 Cells The J774.1 cell line was purchased from Riken Cell Bank (Tsukuba, Japan) and cultured in RPMI medium supplemented with 0.1% sodium bicarbonate and 2 mM glutamine, penicillin G (100 units/ml), streptomycin (100 μg/ml) and 10% FCS. The cells were harvested with trypsin and di-

* To whom correspondence should be addressed. e-mail: kadota@ms.toyama-mpu.ac.jp

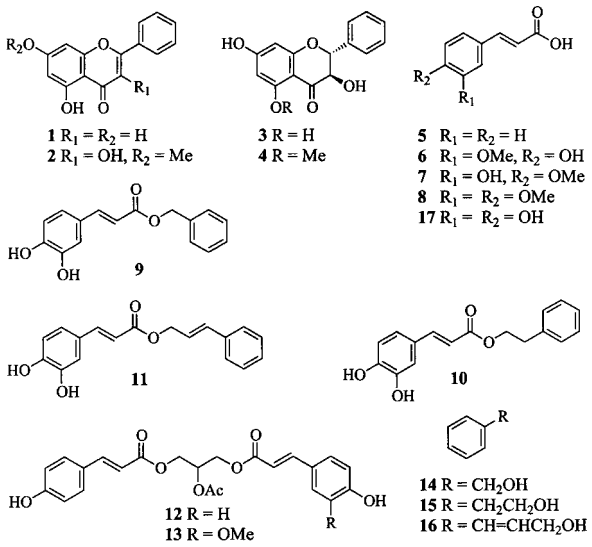


Fig. 1. Structures of the Isolated Compounds from the MeOH Extract of the Netherlands Propolis

luted to a suspension in fresh medium. The cells were seeded in 96-well plastic plates with 1×10^5 cells/well and allowed to adhere for 2 h at 37 °C in a humidified atmosphere containing 5% CO_2. Then the medium was replaced with fresh medium, containing LPS (10 μg/ml) together with test compound at various concentration and then incubated for 24 h. NO production was determined by measuring the accumulation of nitrite in the culture supernatant using Griess reagent.[7] Briefly, 50 μl of the supernatant from incubates were mixed with equal volume of Griess reagent (0.5% sulfanilamide and 0.05% naphthylene-diamide 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, U.S.A.). The nitrite concentration in the medium was determined from the calibration curve obtained by using different concentrations of sodium nitrite ($NaNO_2$) in the culture medium as standard. 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 follows: % inhibition=[(Ac−As)/Ac]×100, where Ac and As 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.

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 formazam formed was dissolved in dimethylsulfoxide (DMSO) and the amount of formazan was measured spectrophotometrically at 550 nm using HTS-7000 microplate reader. The cell viability was calculated as follows: % cell viability=(As/Ac)×100, where Ac and As are absorbance of LPS-treated group and absorbance of the sample, respectively. The cell viability less than 90% with respect to the LPS treated group was considered to be toxic. The data are expressed as mean±S.D. of four determinations. The survival rate of LPS (10 μg/ml)-treated cells were more than 90% of untreated cells.

RESULTS AND DISCUSSION

The Netherlands Propolis and Its Constituents The Netherlands propolis was extracted successively with MeOH and water and tested for their inhibitory effect toward NO production in LPS-activated murine macrophage-like J774.1 cells. Both of these extracts inhibited significantly the production of NO with IC_{50} values of 23.8 and 51.5 μg/ml, respectively (Table 1). The IC_{50} value of the MeOH extract was found to be less than that of polymixin B (IC_{50}, 27.8 μg/ml), a lipopolysaccharide inhibitor,[8] which was used as positive control. The MeOH extract, on chemical examination, led to isolation of 13 phenolic components including flavonoids (**1**—**4**), cinnamic acid derivatives (**5**—**8**), caffeic acid esters (**9**—**11**) and glycerol derivatives (**12**, **13**). These isolated compounds were further tested for their NO inhibitory activity in the same *in vitro* experiment. CAPE analogues (**9**—**11**) possessed the strongest NO inhibitory activity with IC_{50} values equal or less than 10.7 μM, at least three fold stronger than the positive control, L-NMMA (IC_{50}, 32.9 μM). Cinnamic acid derivatives (**5**—**8**) with free carboxylic acid groups, on the other hand, showed weak NO inhibitory activity having IC_{50} values greater than 100 μM, except for 3,4-dimethoxycinnamic acid (**8**; IC_{50}, 50.0 μM). This result led us to conclude that the ester part of caffeic acid should play important role for the NO inhibition of caffeic acid esters. To know the active parts, we further examined the NO inhibitory activity of the both caffeic acid (**17**) and three alcohols (**14**—**16**), which construct the potent NO inhibitors **9**—**11**. Both caffeic acid (**17**) and alcohols **14**—**16** showed much weaker NO inhibitory activity compared to CAPE analogues (**9**—**11**), indicating that the whole must be essential for NO inhibition. Some extent of cytotoxicity toward J774.1 cells was observed in the extract as well as in compound level at higher concentration but not at the concentration around the IC_{50} value (Table 2).

In the case of flavonoids, they showed mixed type of results. Flavonoids **1** and **2** had strong NO inhibitory activity with IC_{50} values of 14.4 and 25.8 μM, respectively, while flavonols **3** and **4** showed moderate NO inhibitory activity with IC_{50} values greater than 70 μM. Thus, the C-2(3) double bond may enhance the NO inhibitory activity. 2-Acetyl-1,3-dicoumaroylglycerol (**12**) also possessed equal strength of the NO inhibitory activity to that of L-NMMA but weaker than CAPE analogues. The NO inhibitory activity of **12** was reduced by a presence of methoxyl group, *i.e.*, 2-acetyl-1-coumaroyl-3-feruloylglycerol (**13**; IC_{50}, 57.6 μM). Moreover, esters **9**—**11** were predominant components of the Netherlands propolis and were contained by 1.0539, 0.0592 and 0.0512%, respectively, in the dry propolis.[5] All these results led us to conclude that the CAPE analogues are the main components responsible for the NO inhibitory activity of the Netherlands propolis.

Synthetic Analogues of CAPE CAPE (**10**) and its analogues (**9**, **11**) had potent NO inhibitory activity, and thus we further tested the NO inhibitory activity of 20 more CAPE analogues (Table 3), which were synthesized in our laboratory to establish the structure–activity relationship.[6] All the CAPE analogues possessed strong NO inhibitory activity with IC_{50} values equal to or less than 20 μM, which is more potent than L-NMMA (IC_{50}, 32.9 μM) and polimixin B (IC_{50},

Table 1. Inhibitory Effects of Compounds **1**—**17** and Propolis Extract on NO Production in LPS-Activated Murine Macrophage-like J774.1 Cells

Compound	% Inhibition of NO production					IC_{50} (μM)
	200 μM	100 μM	50 μM	20 μM	2 μM	
1	109±15**[a)]	135±10**	138±3**	73.8±18.7**	−3.0±7.1	14.4
2	127±11**[a)]	142±7**	120±6**	33.3±11.2**	2.4±12.4	25.8
3	111±7**	66.1±7.4**	38.7±9.8**	20.8±16.5*	10.7±10.4	70.6
4	114±8**	56.5±10.9**	31.5±14.9**	10.7±6.3	14.3±26.7	87.0
5	38.7±14.7*[a)]	19.4±8.3[a)]	−2.4±12.7[a)]	−16.9±17.1[a)]	−11.3±15.0[a)]	>200
6	79.8±8.1**[a)]	45.2±12.6**[a)]	25.0±13.0	0.8±21.6	−13.7±24.0	114
7	65.3±4.8**[a)]	35.5±7.9*	20.2±7.2	17.7±15.0	14.5±19.8	149
8	126±8**	74.2±9.5**	50.0±5.6**	13.7±7.2	2.4±10.3	50.0
9	118±3**[a)]	120±4**[a)]	113±5**[a)]	103±5**	26.0±16.1**	7.61
10	122±4**[a)]	119±8**[a)]	116±5**	116±2**	34.5±7.0**	5.44
11	124±6**[a)]	122±7**[a)]	95.0±8.1**	85.5±8.9**	17.0±4.8**	10.7
12	132±9**[a)]	126±3**[a)]	79.5±3.8**	28.0±5.4**	−8.0±7.1	32.8
13	114±2**[a)]	89.0±2.2**	43.0±2.3**	4.8±4.6	−9.2±2.2	57.6
14	25.0±6.3**	11.0±3.0**	4.4±3.7	−2.2±4.8	−11.0±14.1	>200
15	18.9±6.8**	6.6±5.2	2.2±9.5	−9.6±9.6	−9.2±6.0	>200
16	101±3**	58.8±1.0**	30.7±3.4**	11.0±2.6**	−1.3±8.6	84.3
17	52.7±3.2**	35.5±4.3**	16.4±4.5**	5.5±0.9	−5.5±2.0	184
MeOH ext.[b)]	90.6±1.3**[a)]	98.0±2.0**	88.3±0.9**	44.5±7.3**	−0.8±8.1	23.8
H_2O ext.[b)]	102±2**	89.5±14.5**	48.8±2.7**	16.4±4.5**	1.6±4.6	51.5
Polymixin B[b)]	94.4±11.5**[a)]	93.8±1.1**[a)]	73.9±6.3**[a)]	36.7±15.4**[a)]	19.4±4.4**[a)]	27.8

Each value represents the mean±S.D. of four determinations. Significantly different from the control: $*p<0.05$, $**p<0.01$. *a*) Cytotoxic effect was observed. *b*) Concentrations are in μg/ml.

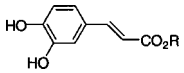
Table 2. Cell Viability of J774.1 Cells on Treatment of LPS and Samples (**1**—**17**) during NO Inhibitory Experiment

Compound	Cell viability (% of Control)				
	200 μM	100 μM	50 μM	20 μM	2 μM
1	71.0±13.8	105±16	112±17	112±19	96.8±3.5
2	75.3±7.6	94.3±21.3	102±13	101±9	105±23
3	95.4±7.5	107±16	97.4±12.4	91.9±9.3	112±13
4	106±9	98.8±11.7	96.7±9.8	95.4±7.8	91.8±9.7
5	63.5±15.2	72.1±22.3	87.9±23.1	78.7±26.7	76.4±20.6
6	88.9±15.0	88.4±17.2	97.1±9.5	99.1±16.1	98.1±9.8
7	84.8±7.9	93.2±2.9	92.9±6.7	98.0±15.0	95.4±6.1
8	109±7	102±5	99.7±9.9	104±4	91.3±10.7
9	15.3±6.8	56.4±10.0	83.0±7.9	103±11	105±9
10	18.5±6.7	67.5±8.5	90.1±6.4	115±6	111±12
11	8.1±1.9	60.4±6.5	94.4±10.8	103±15	108±13
12	4.6±0.7	76.6±9.1	119±12	120±11	124±18
13	26.6±5.3	91.1±8.7	95.2±8.8	101±9	108±15
14	97.1±12.2	94.9±7.8	97.1±11.0	92.3±9.9	84.4±5.1
15	102±10	99.3±7.3	96.0±13.1	108±13	107±10
16	109±11	108±5	103±5	107±8	91.8±11.6
17	114±8	110±11	112±7	106±7	100±12
MeOH ext.[a)]	28.2±5.3	102±12	110±6	108±5	95.7±11.2
H_2O ext.[a)]	117±6	109±5	103±2	105±5	99.4±8.6
Polymixin B[a)]	9.4±1.4	38.9±8.8	68.1±9.8	85.5±12.0	85.6±11.8

Each value represents the mean±S.D. of four determinations. *a*) Tested concentration and IC_{50} values are in μg/ml.

27.8 μg/ml) used as positive controls (Tables 1 and 3). The LPS alone produced 20—25 μM nitrite, which was reduced to 2 μM by the treatment of CAPE at 50 μM (data not shown) and other CAPE analogues also had similar extent of reduction of nitrite. The esters are mainly divided into four different groups according to the nature of their alcoholic part: esters having an alkyl group with a phenyl group at the end of the alkyl chain (**9**, **10**, **18**—**23**), esters having an alkyl group with a stylyl group at the end of the alkyl chain (**11**, **24**, **25**), an ester having an alkyl group with a cyclohexyl group at the end of the alkyl chain (**26**) and esters having a straight alkyl chain (**27**—**35**). It was observed that elongation of alkyl side chain of the alcoholic part of esters enhance the NO inhibitory activity: IC_{50} values decrease from **9** to **21** (**9**>**10**>**18**>**19**>**20**>**21**) and from **27** to **32** (**27**>**28**>**29**>**30**>**31**>**32**). This was also observed in the esters having an alkyl chain with a stylyl group at the end (**11**>**24**). But almost all the CAPE analogues having longer carbon chain (>C_5) in alcoholic part showed toxic effects toward J774.1 cells (Table 4), indicating the possibility that the NO inhibitory activity of these compounds (**20**—**23**, **31**—**35**) may be due to their toxic effects. Accordingly the most potent NO inhibitory

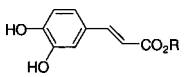
Table 3. Inhibitory Effects of CAPE Analogues on NO Production in LPS-Activated Murine Macrophage-like J774.1 Cells



Compd.	R	% Inhibition of NO production					IC_{50} (μM)
		100 μM	50 μM	20 μM	10 μM	1 μM	
9	$-CH_2Ph$	90.2±2.9**,a)	85.3±5.1**,a)	67.3±5.5**	39.5±0.5**	−4.9±4.5	13.8
10	$-(CH_2)_2Ph$	92.8±1.6**,a)	94.2±0.9**,a)	88.6±2.5**	65.2±4.1**	7.2±7.6	7.64
18	$-(CH_2)_3Ph$	90.2±6.0**,a)	93.7±1.6**,a)	90.9±0.9**,a)	66.1±4.8**	11.7±8.4*	7.34
19	$-(CH_2)_4Ph$	93.5±0.0**,a)	94.9±0.5**,a)	91.4±1.4**,a)	67.1±1.9**	19.4±4.8**	6.77
20	$-(CH_2)_5Ph$	86.9±5.3**,a)	90.6±1.9**,a)	90.1±2.9**,a)	78.2±1.6**,a)	10.4±6.2*	6.26
21	$-(CH_2)_6Ph$	89.9±7.9**,a)	91.1±2.9**,a)	93.1±3.6**,a)	86.9±2.0**,a)	23.0±10.6**	4.80
22	$-(CH_2)_8Ph$	94.6±1.0**,a)	89.9±5.3**,a)	92.6±3.1**,a)	85.6±5.4**,a)	16.1±12.2*	5.39
23	$-(CH_2)_{12}Ph$	91.6±2.4**,a)	88.9±2.2**,a)	80.9±5.5**,a)	45.0±5.7**	9.7±5.5*	11.4
11	$-CH_2CH=CHPh$	87.8±2.4**,a)	89.7±2.1**,a)	78.5±2.6**	52.7±7.5**	0.5±7.0	9.53
24a	$-(CH_2)_6CH=CHPh$	70.0±5.2**,a)	77.0±2.0**,a)	87.0±5.0**,a)	83.0±2.0**,a)	19.0±3.8**	5.36
24b	$-(CH_2)_6CH=CHPh$	94.0±8.3**,a)	96.0±3.3**,a)	93.0±9.5**,a)	94.0±4.0**,a)	35.0±9.5**	3.29
25a	$-(CH_2)_{10}CH=CHPh$	78.0±10.6**,a)	85.0±6.0**,a)	87.0±5.0**,a)	76.0±5.7**,a)	28.0±5.7**	5.13
25b	$-(CH_2)_{10}CH=CHPh$	88.0±8.0**,a)	92.0±6.5**,a)	81.0±8.2**,a)	59.0±3.8**,a)	34.0±2.3**	6.76
26	$-(CH_2)_2$-*c*-Hex	94.3±1.9**,a)	91.8±3.7**,a)	88.0±2.8**,a)	62.0±3.8**	9.8±7.6	7.93
27	$-CH_3$	90.8±1.1**	79.3±3.2**	49.5±6.0**	22.8±14.5**	−5.4±8.1	20.5
28	$-CH_2CH_3$	91.3±1.5**	78.3±8.2**	56.8±4.6**	39.1±6.1**	9.5±8.5	16.2
29	$-(CH_2)_2CH_3$	91.4±1.9**,a)	86.4±1.1**	65.3±1.4**	43.6±11.7**	1.7±7.1	12.9
30	$-(CH_2)_3CH_3$	93.9±1.1**,a)	90.3±3.7**,a)	75.3±5.4**	56.1±2.3**	8.1±6.0	8.86
31	$-(CH_2)_7CH_3$	90.3±2.1**,a)	94.2±0.6**,a)	91.9±1.4**,a)	73.1±6.6**,a)	20.6±4.2*	6.04
32	$-(CH_2)_9CH_3$	93.6±2.5**,a)	91.4±3.2**,a)	91.9±4.4**,a)	80.3±2.1**,a)	29.2±9.6**	4.66
33	$-(CH_2)_{11}CH_3$	89.7±2.7**,a)	90.7±2.5**,a)	86.3±2.6**,a)	81.7±1.0**,a)	9.8±10.0	6.03
34	$-(CH_2)_{13}CH_3$	90.6±1.4**,a)	85.3±1.7**,a)	78.6±5.8**,a)	57.5±8.9**,a)	13.1±19.3*	8.48
35	$-(CH_2)_{15}CH_3$	86.7±3.3**,a)	84.4±3.3**,a)	61.9±2.8*,a)	32.6±8.9**	13.9±11.8*	15.9
L-NMMA		78.1±4.0**	58.3±3.4**	43.8±7.2**	20.8±14.0	19.8±5.2*	32.9

Each value represents the mean±S.D. of four determinations. Significantly different from the control: $*p<0.05$, $**p<0.01$. **24a** and **25a** are *Z*-isomer, and **24b** and **25b** are *E*-isomer. *a*) Cytotoxic effect was observed.

Table 4. Cell Viability of J774.1 Cells on Treatment of LPS and CAPE Analogues during NO Inhibitory Experiment



Compd.	R	Cell viability (% of Control)				
		100 μM	50 μM	20 μM	10 μM	1 μM
9	$-CH_2Ph$	76.7±2.7	93.7±5.4	111±7	113±2	103±6
10	$-(CH_2)_2Ph$	67.4±6.4	82.3±2.9	110±5	115±0	99.4±2.5
18	$-(CH_2)_3Ph$	54.7±12.8	63.5±4.3	81.4±7.9	111±7	107±5
19	$-(CH_2)_4Ph$	56.4±7.5	45.0±2.9	67.9±6	107±5	100±6
20	$-(CH_2)_5Ph$	39.1±4.7	31.7±4.2	38.6±4.6	75.4±4.3	103±3
21	$-(CH_2)_6Ph$	5.9±0.4	20.2±3.7	16.6±2.6	32.2±6.4	103±3
22	$-(CH_2)_8Ph$	7.2±2.0	10.6±0.6	10.0±1.6	21.3±7.6	106±0
23	$-(CH_2)_{12}Ph$	14.5±1.9	14.3±3.3	37.7±5.7	92.1±6.3	102±3
11	$-CH_2CH=CHPh$	51.9±2.0	69.2±5.3	98.8±3.1	107±0	102±4
24a	$-(CH_2)_6CH=CHPh$	4.8±0.3	4.4±0.2	4.4±0.2	10.5±2.7	98.7±2.9
24b	$-(CH_2)_6CH=CHPh$	4.3±0.3	4.4±0.4	4.0±0.2	5.7±0.7	99.8±4.7
25a	$-(CH_2)_{10}CH=CHPh$	4.3±0.8	4.0±0.3	4.6±0.5	13.7±2.1	101±2
25b	$-(CH_2)_{10}CH=CHPh$	4.4±0.3	4.4±0.6	9.9±1.5	62.7±7.6	99.6±2.5
26	$-(CH_2)_2$-*c*-Hex	42.6±3.0	34.0±1.5	60.7±2.8	105±2	103±4
27	$-CH_3$	104±6	105±2	107±0	104±5	102±4
28	$-CH_2CH_3$	104±3	106±2	107±0	107±0	101±4
29	$-(CH_2)_2CH_3$	86.3±2.2	102±2	104±0	103±2	103±1
30	$-(CH_2)_3CH_3$	76.6±5.8	89.3±9.7	104±0	104±0	101±4
31	$-(CH_2)_7CH_3$	38.9±4.5	25.9±4.0	30.8±6.4	80.4±12.2	104±0
32	$-(CH_2)_9CH_3$	28.7±2.5	21.7±6.8	15.6±4.9	32.6±13.6	103±1
33	$-(CH_2)_{11}CH_3$	11.0±1.5	17.5±1.6	18.4±2.0	30.2±3.4	104±2
34	$-(CH_2)_{13}CH_3$	15.7±3.4	17.1±2.3	30.9±7.4	65.2±15.1	103±1
35	$-(CH_2)_{15}CH_3$	22.5±5.9	31.8±2.4	64.4±8.0	94.2±0.5	102±1
L-NMMA		101±3	100±3	99.4±2.1	97.2±1.0	98.2±1.7

Each value represents the mean±S.D. of four determinations. **24a** and **25a** are *Z*-isomer, and **24b** and **25b** are *E*-isomer.

compounds were considered to be 4-phenylbutyl caffeate (**19**; IC_{50}, 6.77 μM) and butyl caffeate (**30**; IC_{50}, 8.86 μM) in their respective group. Of course these caffeates were also toxic at higher concentration but did not have any cytotoxicity at the concentration around the IC_{50} value (Table 2). Esters with phenyl ring at the end of side chain in their alcoholic part possessed stronger NO inhibitory activity than the straight chain counterparts (IC_{50} values, **9**<**27**; **10**<**28**; **18**<**29** and **19**<**30**), indicating that the phenyl ring may play important role for the NO inhibition. 2-*cyclo*Hexylethyl caffeate (**26**; IC_{50}, 7.93 μM), on the other hand, possessed similar extent of NO inhibitory activity to that of CAPE (**10**; IC_{50}, 7.64 μM), which indicates that six-member ring is enough for enhancement of the NO inhibitory activity.

The present study could not explain the exact mechanism of the NO inhibition of the Netherlands propolis. But propolis and its various components were reported to possess strong antioxidative properties and strong scavenging activities toward 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide and hydroxyl radicals.[9—11] They suggested that the NO inhibition by the Netherlands propolis may be due to scavenging of NO radical generated in the cell broth. The other possible mechanism is the blocking of iNOS induction. It is well established that CAPE strongly and selectively inhibits the activation of nuclear transcription factor (NF)-κB induced by TNF and other inflammatory agents including phorbol, ceramide, hydrogen peroxide and okadaic acid.[12] In addition, Song *et al.* demonstrated that CAPE inhibits the iNOS promoter activity induced by LPS and IFN-γ through the NF-κB sites of the iNOS promoter.[13] Thus, the active principles of the Netherlands propolis, *i.e.*, CAPE and its analogues, should block the activation of iNOS through the suppression of NF-κB activation and resulted in potent NO inhibition.

CONCLUSION

The MeOH extract of the Netherlands propolis possessed potent NO inhibitory activity in LPS-activated murine macrophage-like J774.1 cells, which led to isolation of benzyl caffeate (**9**), CAPE (**10**) and cinnamyl caffeate (**11**) as potent NO inhibitors. Further study on the synthetic analogues of CAPE led us to conclude that on elongation of alkyl side chain of alcoholic part of caffeic acid esters enhance the NO inhibitory activity. Both of 3-phenylpropyl caffeate (**18**) and 4-phenylbutyl caffeate (**19**) possessed stronger NO inhibitory activity than CAPE (**10**). But CAPE analogues having longer carbon chain (>C_5) in alcoholic part showed toxic effects toward J774.1 cells. The NO inhibitory effect of the CAPE analogues and the Netherlands propolis were probably due to blocking of the activation of iNOS. This NO inhibitory effect by the CAPE analogues, main components of the Netherlands propolis, may directly correlate with antiinflammatory properties of the Netherlands propolis.

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REFERENCES

1) Ghisalberti E. L., *Bee World*, **60**, 59—84 (1979).
2) Banskota A. H., Tezuka Y., Kadota S., *Phytother. Res.*, **15**, 561—571 (2001).
3) Kou P. C., Schroeder R. A., *Annal. Sur.*, **221**, 220—235 (1995).
4) Moncada S., Palmer R. M. J., Higgs E. A., *Pharm. Rev.*, **43**, 109—142 (1991).
5) Banskota A. H., Nagaoka T., Sumioka L. Y., Tezuka Y., Awale S., Midorikawa K., Matsushige K., Kadota S., *J. Ethnopharmacol.*, **80**, 67—73 (2002).
6) Nagaoka T., Banskota A. H., Tezuka Y., Saiki I., Kadota S., *Bioorg. Med. Chem.*, **10**, 3351—3359 (2002).
7) Green L. C., Wagner D. A., Glogowski J., Skipper P. L., Wishnok J. S., Tannenbaum S. R., *Anal. Biochem.*, **126**, 131—138 (1982).
8) Xiong Q., Tezuka Y., Kaneko T., Li H., Tran L. Q., Hase K., Namba T., Kadota S., *Eur. J. Pharmcol.*, **400**, 137—144 (2000).
9) Matsushige K., Kusumoto I. T., Yamamoto Y., Kadota S., Namba T., *J. Trad. Med.*, **12**, 45—53 (1995).
10) Banskota A. H., Tezuka Y., Adnyana I K., Midorikawa K., Matsushige K., Message M., Alfredo A. G., Huertas A. A. G., Kadota S., *J. Ethnopharmacol.*, **72**, 239—246 (2000).
11) Banskota A. H., Tezuka Y., Adnyana I K., Ishii E., Midorikawa K., Matsushige K., Kadota S., *Phytomedicine*, **8**, 16—23 (2001).
12) Natarajan K., Singh S., Burke T. R., Grunberger D., Aggarwal B. B., *Pro. Nat. Acad. Sci. U.S.A.*, **93**, 9090—9095 (1996).
13) Song Y. S., Park E.-H., Hur G. M., Ryu Y. S., Lee Y. S., Lee J. Y., Kim Y. M., Jin C., *Cancer Lett.*, **175**, 53—61 (2002).