Identification of Coumarins from Lemon Fruit (*Citrus limon*) as Inhibitors of in Vitro Tumor Promotion and Superoxide and Nitric Oxide Generation

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Three coumarins were isolated as siginificant inhibitors of tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced Epstein−Barr virus (EBV) activation in Raji cells from the peel of lemon fruit. They were identified as 8-geranyloxypsolaren (LE−1), 5-geranyloxypsolaren (bergamottin, LE−2), and 5-geranyloxy-7-methoxycoumarin (LE−3), respectively, by spectroscopic analysis. Three isolates had no potential O_2-scavenging and markedly suppressed TPA-induced superoxide (O_2^-) generation in differentiated human promyelocytic HL-60 cells. Furthermore, LE−1 and LE−3 reduced both lypopolysaccharide (LPS) and interferone-γ (IFN-γ)-induced nitric oxide (NO) generation in mouse macrophage RAW 264.7 cells. Similarly, they were found to be NO generation inhibitors rather than scavengers by measuring the extracellular L-citrulline levels. The occurrence of these coumarins in a lemon fruit was abundant in the flavedo of the peel based on quantitative analysis using high-performance liquid chromatography (HPLC). The present study suggests that the coumarins in lemon fruit are promising chemopreventive agents by inhibiting radical generation.

Keywords: *Citrus limon; coumarin; psoralen; chemopreventive agent; Epstein−Barr virus; superoxide; nitric oxide*

INTRODUCTION

Cancer chemoprevention is currently regarded as one of the most promising avenues in cancer control (Greenwald et al., 1990; Wattenberg, 1993). In the search for effective chemopreventive agents, inhibition of tumor promotion has been the focus of attention because the tumor promotion process takes a long time to occur and is the only reversible process during the multistages of carcinogenesis (Hennings et al., 1993). Hitherto, the Epstein−Barr virus (EBV) activation test is known to be one of the convenient in vitro assays for the detection of naturally occurring antitumor promoters (Murakami et al., 1996). Most inhibitors, identified by this assay, have been proven to possess marked antitumor-promoting activities in vivo (Tokuda et al., 1986; Ohigashi et al., 1994).

Oxidative stress is one of the critical biological responses induced by tumor promoters (Kensler et al., 1989). In particular, TPA-type tumor promoters are reported to trigger O_2^- generation in leukocytes through the NADPH oxidase system (Cross and Jones, 1991). Nitric oxide (NO), a gaseous free radical, is synthesized in biological systems by a family of enzymes, constitutive NO synthase (cNOS) and an inducible one (iNOS) (Vanvaskas and Schmidt, 1997). For the latter, NO is readily released by bacterial lipopolysaccharide (LPS) or interferon-γ (IFN-γ) to form stoichiometric amounts of L-citrulline from L-arginine in some cell lines, such as macrophage (Nathan and Xie, 1994). The excess generation of NO by iNOS has attracted attention due to its relevance to carcinogenesis (Xie et al., 1997). An important chemical property of NO is that it rapidly and spontaneously reacts with a superoxide anion (O_2^-) to form a peroxynitrite anion ($ONOO^-$) (Ischiropoulos et al., 1992), which is more toxic to biological systems than O_2^- or NO by causing a modification of proteins or nucleic acids (van der Vliet et al., 1995; Yermilov et al., 1995). Suppression of the iNOS-induced NO generation in excessive amounts is now widely accepted as a new paradigm for chemoprevention.

Lemon fruit (*Citrus limon*) is used in various foods, for example, soft drinks, alcoholic drinks, and jams. Lemons contain a number of physiologically functional components such as citric acid, ascorbic acid, minerals, coumarins, and flavonoids. The coumarins and flavonoids in citrus fruit, for a long time, have been regarded as nonnutritional and nonfunctional components in a living system for a long time.

However, some coumarins have recently been reported to have anti-platelet aggregating (Chen et al., 1995), anti-microbial (Nakatani et al., 1987), antimutagenic (Edenharder et al., 1995), and some flavonoids have been found to have antitumor and antioxidant

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properties (Tom and Ulubelen, 1980). They are expected to be available in living systems when they are orally ingested as food (Middleton and Kandaswami, 1994). We have studied the antioxidative flavonoids from lemon fruit and identified eriocitrin (eriodictyol-7-rutinoside) (Miyake et al., 1997a) and *C*-glucosylflavones (Miyake et al., 1997b). Eriocitrin in lemons has been demonstrated to have a suppressive effect toward oxidative stress in vivo using streptozotocine (STZ)-induced diabetic rats (Miyake et al., 1998).

Moreover, the research has been carried out to find chemopreventive agents in citrus fruit. Limonoids and their glucosides had been reported to inhibit benzo[*a*]-pyrene-induced forestomach and lung carcinogenesis in mice, TPA-induced skin tumor promotion in mice (Lam et al., 1994), and D-limonene had been reported to inhibits azoxymethane (AOM)-induced rat colonic aberrant crypt foci (ACF) formation (Kawamori et al., 1996). Hesperidin, one of the flavonoids commonly found in citrus fruit, has been reported to inhibit AOM-induced rat colonic ACF formation (Tanaka and Mori, 1996). As for coumarins in citrus fruit, auraptene (7-geranyloxycoumarin) isolated from *Citrus natsudaidai* HAYATA have been reported to exhibit the inhibition of TPA-induced EBV activation and TPA-induced superoxide (O_2^-) generation in differentiated human promyelocytic HL-60 cells (Murakami et al., 1997). Some coumarins in *Citrus hystrix* DC have been reported to have antitumor-promoting activities which exhibits to reduce both LPS/IFN-γ-induced NO generation in mouse macrophage RAW 264.7 cells (Murakami et al., 1999).

In this study, we attempted to isolate new promising chemopreventers in lemons and paid attention coumarins in lemon fruit. We examined their inhibitory effects on TPA-induced EBV activation, and O_2^- generation and LPS/IFN-γ-induced NO generation.

MATERIALS AND METHODS

Chemicals and Cells. Lemon fruit (*Citrus limon* BURRM. f.) produced in California was purchased at a supermarket. Cytochrome *c*, L-arginine, and (6*R*)-tetrahydro-L-biopterin(BH_4) were purchased from Sigma Chemical Co., Ltd. (MI); RPMI 1640 medium and fetal bovine serum (FBS) from Gibco BRL (Grand Island, NY); LPS (*Escherichia coli* serotype 0127, B8) from Difco Labs (MI); IFN-γ from Genzyme (MA); 12-*O*-tetradecanoylphorbol-13-acerate (TPA) from Research Biochemicals International (Natick, MA); fluorescence isothiocyanate (FITC)-labeled anti-human IgG from Dako (Glostrup, Denmark); and other chemicals were obtained from Wako Pure Chemicals Co. Ltd. (Osaka, Japan). The cold-pressed lemon oil was purchased from Takasago International Co. Ltd. (Tokyo, Japan).

High-titer EBV early antigen (EA)-positive sera from nasopharyngeal (NPC) patients were the gift of Prof. Dr. Ohsato (Health Sciences University of Hokkaido). Human B-lymphoblastoid Raji cells and human promyelocytic leukemia HL-60 cells were the gifts of Prof. Ohsato (Health Sciences University of Hokkaido) and Prof. Dr. Sasaki (Kyoto University), respectively. RAW 264.7 cells were a kind endowment from Ohtsuka Pharmaceutical Co. Ltd. (Ohtsu, Japan).

Isolation of Coumarins from Lemon Fruit. The peel of lemons (1.20 kg) obtained from 18 lemon fruits was chopped (5 mm × 5 mm) in a homogenizer and extracted with methanol (1.5 L) at 37 °C for 3 days. The extract was filtered through a cloth to remove the peel and was concentrated in vacuo. The peel extract (52.0 g) was then dissolved in water (300 mL). The solution was chromatographed on a Cosmosil 75C 18-OPN ODS column (Nakalai Tesque, Inc., Kyoto, Japan, column size; ∅37 × 500 mm). The column was washed with 2 L of water and successively eluted with 60% methanol−water, 80% methanol−water, and methanol (2 L each). The 80% methanol−water portion was concentrated in vacuo, and 0.305 g was obtained after drying. This sample was dissolved in methanol, and preparative high-performance liquid chromatography (HPLC) was conducted using a Shim-pack PREP ODS (L) column (Shimadzu Co., Ltd., Kyoto, Japan, ∅50 × 250 mm) with a UV spectrophotometric detector (305 nm) using 90% methanol as the solvent at a flow rate of 80 mL/min. The LE−1 (6.2 mg), LE−2 (4.1 mg), and LE−3 (10.1 mg) were isolated from the 80% methanol−water portion of the lemon peel extract. The purity of the LE−1, LE−2, and LE−3 was more than 99.0% based on HPLC analysis.

TPA-Induced EBV Activation Test. Human B-lymphoblastoid Raji cells were incubated in 1 mL of RPMI 1640 medium containing sodium *n*-butyrate (3 mM), TPA (50 nM), and the test compound at 37 °C under a 5% CO_2 atmosphere for 48 h. EBV activation was estimated by detection of EA using the indirect immunofluorescence method (Murakami et al., 1995). Smears were made from a cell suspension and stained with high-titer EA-positive sera from anaplastic NPC patients followed by FITC-labeled anti-IgG. The percentage of EA-induced cells was compared to that of a control experiment with only sodium *n*-butyrate and TPA, in which the percentage of EA-induced cells was ordinarily around 50%. Cell viability was measured using the trypan blue-exclusion test. Every test was done in duplicate, and a mean value was obtained.

TPA-Induced O_2^- Generation Test. The test for inhibition of TPA-induced O_2^- generation was done as previously reported (Miller et al., 1994). Human promyelocytic leukemia HL60 cells were inoculated at 5×10^5/mL in RPMI 1640 supplemented with 10% FBS. The cells were preincubated with 1.25% dimethyl sulfoxide (DMSO) at 37 °C in a 5% CO_2 incubator for 4 days to stimulate their differentiation into granulocyte-like cells. The cells were washed with phosphate-buffered saline (PBS) and suspended at a density of 1×10^6/mL. The test compound, dissolved in 5 μL of DMSO, was added to the cell suspension, and the mixture thus obtained was incubated at 37 °C for 15 min. The cells were washed with PBS twice to remove extracellular test compounds. Ninety seconds after stimulation with 5 μL of TPA solution (20 μM), 50 μL of cytochrome *c* solution (20 mg/mL) was added to the reaction mixture, which was incubated for another 15 min. The reaction was stopped by the addition of 5 μL of superoxide dismutase solution (15 000 units/mL). The reaction mixture was centrifuged at 2000*g* for 30 s, and the visible absorption of the supernatant at 550 nm was measured. The level of O_2^- production was calculated by using of the following equation (Markert et al., 1984):

$$O_2^- \text{ (nmol/mL)} = 47.7A_{550\text{nm}}$$

Cytotoxicity was measured with the trypan blue-exclusion test. Every test was done in duplicate, and a mean value was obtained.

NO Generation Test in RAW 264.7 Cell. The isolated coumarins were examined the reduction both LPS/IFN-γ-induced NO generation in mouse macrophage RAW 264.7 cells according to the method of Murakami et al. (1999). Cytotoxicity was measured by an MTT assay (Sladowski et al., 1992). Every test was done in duplicate, and a mean value was obtained.

Quantitative Analysis of Coumarins in Lemons. The lemon fruit was separated into the peel and juice by hand-squeezing the fruit. Part of the peel was used for ethanol extraction. The other part of the peel was further separated into the flavedo (epicarp), albedo (mesocarp), and pulp vesicles using a knife. Samples (2.0 g) of the peel, subdivided peel, and seed were homogenized and extracted with 100 mL of ethanol at 37 °C for 2 days. The organic solution was concentrated in vacuo. The juice was removed from the pulp by filtration. LE−1, LE−2, and LE−3 in the extract of the samples and filtered juice were determined by HPLC analysis. HPLC (LC-10A, Shimadzu Co., Ltd., Kyoto, Japan) was conducted using a Shim-pack CLC-ODS(M) (column size; ∅4.6 × 250 mm,

Table 1. UV, IR, FAB-MS, and ^{1}H NMR Spectral Data for LE−1, LE−2, and LE−3 Isolated from Lemon Fruit

	LE−1	LE−2	LE−3
UV λ_{max} nm (ϵ)	213 (7744), 250 (7773), 264 (6479), 300 (5734)	213 (7803), 250 (7925), 258 (7964), 267 (7861), 304 (7332)	213 (7486), 247 (5184) 255 (5184), 318 (6744)
IR ν_{max} (cm^{-1})	1720, 1624, 1448, 1152	1720, 1624, 1448, 1152	1740, 1608, 1440, 1160
FAB-MS (m/z)	339 $[M + H]^+$	339 $[M + H]^+$	329 $[M + H]^+$
^{1}H NMR (δ)	6.37 (1H, d, J = 9.5, H3)	6.28 (1H, d, J = 9.5, H3)	6.15 (1H, d, J = 9.5, H3)
	7.76 (1H, d, J = 9.5, H4)	8.17 (1H, dd, J = 9.5 and 0.5, H4)	8.01 (1H, dd, J = 9.5 and 0.5, H4)
	7.36 (1H, s, H5)	6.96 (1H, dd, J = 2.5 and 1.0, H6)	6.29 (1H, d, J = 2.0, H6)
	6.81 (1H, d, J = 2.5, H6)	7.60 (1H, d, J = 2.5, H7)	6.41 (1H, dd, J = 2.0 and 0.5, H8)
	7.69 (1H, d, J = 2.5, H7)	7.16 (1H, bs, H8)	4.60 (2H, d, J = 6.5, H1′)
	5.03 (2H, d, J = 7.0, H1′)	4.95 (2H, d, J = 7.0, H1′)	5.48 (1H, tq, J = 6.5 and 1.0, H2′)
	5.60 (1H, dt, J = 7.0 and 1.0, H2′)	5.54 (1H, tq, J = 7.0 and 1.0, H2′)	1.75 (3H, bs, H4′)
	1.69 (3H, bs, H4′)	1.69 (3H, d, J = 1.0, H4′)	2.12 (2H, m, H5′)
	2.01 (2H, m, H5′)	2.10 (2H, m, H5′)	2.12 (2H, m, H6′)
	2.01 (2H, m, H6′)	2.10 (2H, m, H6′)	5.09 (1H, m, H7′)
	4.99 (1H, m, H7′)	5.07 (1H, m, H7′)	1.61 (3H, s, H9′)
	1.56 (3H, s, H9′)	1.60 (3H, s, H9′)	1.68 (3H, s, H10′)
	1.64 (3H, s, H10′)	1.68 (3H, s, H10′)	3.85 (3H, s, OCH_3)

Table 2. 13C NMR Spectra for LE−1, LE−2, and LE−3 Isolated from Lemon Fruit

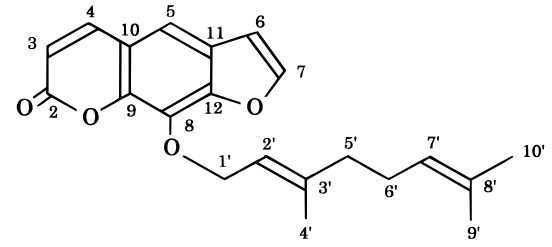
	LE−1 (δ)	LE−2 (δ)	LE−3 (δ)
C3	114.68	112.52	112.52
C4	144.31	139.59	139.59
C5	113.18	148.95	148.95
C6	106.70	105.05	114.17
C7	146.58	144.85	158.10
C8	143.12	94.19	94.19
C9	143.93	152.63	152.63
C10	116.45	107.48	107.48
C11	125.80	114.17	
C12	148.74	158.10	
C1′	70.06	69.73	69.73
C2′	119.41	118.84	118.84
C3′	131.55	143.02	143.02
C4′	16.51	16.65	16.65
C5′	39.54	39.47	39.47
C6′	26.32	26.19	26.19
C7′	123.74	123.47	123.47
C8′	131.69	130.00	130.00
C9′	17.62	17.68	17.68
C10′	25.62	25.65	25.65
OCH_3			55.74

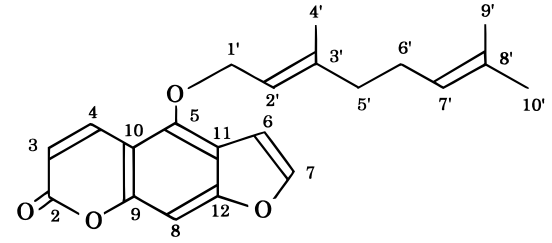
particle size; 5 μm) under 40 °C with a UV detector (λ = 305 nm). The solvent system contained 80% acetonitrile and 20% water. The flow rate was 1.0 mL/min.

Instrumental Analysis. UV-*v*is absorption spectra were recorded on a spectrophotometer with a HITACHI U-2000 in ethanol. IR spectra were recorded on a FT/IR-8200RC (Shimadzu Co., Ltd., Japan), with KBr. The fast-atom bombardment mass spectra (FAB-MS) were recorded on a JEOL JMS-DX-705L with 1 N HCl-glycerol as the mounting matrix. ^{1}H NMR and ^{13}C NMR spectra were obtained using a JEOL JNM-EX-400 NMR instrument (400 MHz for ^{1}H and 100 MHz for ^{13}C) in dimethyl sulfoxide-d_6 containing tetramethylsilane (TMS) as the internal standard.

RESULTS AND DISCUSSION

Characterization of Coumarins Isolated from Lemon Fruit. The UV absorption λ_{max}, IR spectrum, FAB-MS, and ^{1}H NMR spectral data of LE−1, LE2, and LE−3 are shown in Table 1. The ^{13}C NMR spectral data of these isolates are shown in Table 2. The UV absorption maxima (λ_{max}) obtained from LE−1, LE−2, and LE−3 are in good agreement with those found in the literature (Stanley and Vannier, 1957). The spectral properties obtained from LE−2 are also consistent with those in the literature (Dreyer and Huey, 1973; Murakami et al., 1999). The ^{1}H NMR and ^{3}C NMR spectral data of LE−3 are in good agreement with those found in the literature (Herpol-Borremans et al., 1985; Chang et al., 1977). LE−1 was assigned an 8-geranyloxypsolaren from MS, ^{1}H NMR, and ^{3}C NMR spectral data. Thus, LE−1, LE2, and LE−3 were identified as 8-geranyloxypsolaren, 5-geranyloxypsolaren (bergamottin), and 5-geranyloxy-7-methoxycoumarin, respectively. The structures of these compounds are shown in Figure 1. It has been reported that these compounds were isolated as major coumarins from the lemon peel (Stanley and Jurd, 1971; Fisher and Trama, 1979). However, their inhibitory activities of EBV activation, suppression of O_2^-, and NO generation possibly related to antitumor-promoting properties have not been reported yet.





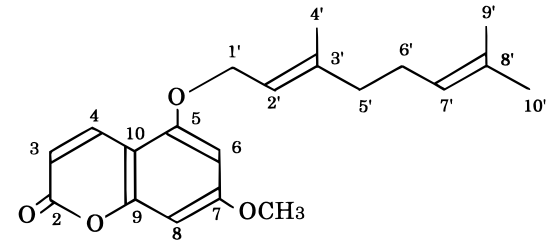


Figure 1. Chemical structures of coumarin-related compounds isolated from lemon peel fruit.

TPA-Induced EBV Activation. The in vitro antitumor promotion activity of the LE series was then examined using a tumor promoter-induced Epstein−Barr virus (EBV) activation test in Raji cells. As shown

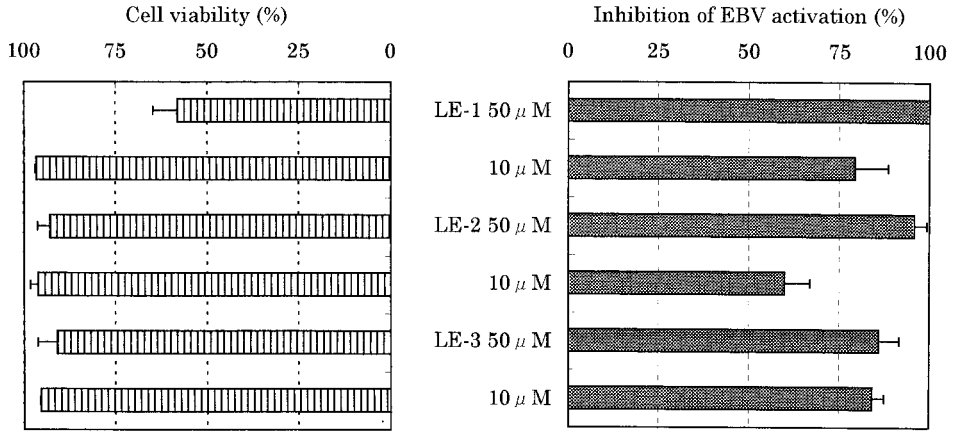


Figure 2. Inhibitory activity of LE−1, LE−2, and LE−3 toward EBV activation. Human B-lymphoblastoid Raji cells latently infected with EBV were incubated with *n*-butyrate (3 mM), TPA (50 nM), and LE−1, LE−2, or LE−3 at 37 °C for 48 h. (Left, cytotoxicity; right, inhibition of EBV activation.)

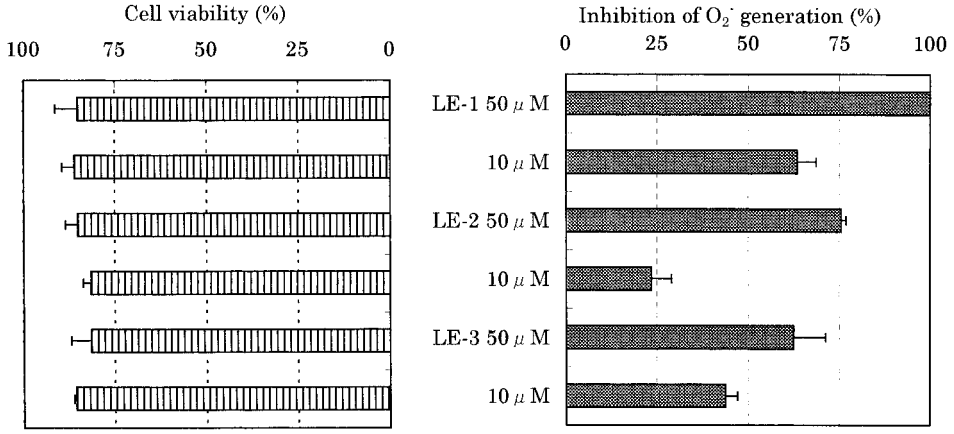


Figure 3. Inhibitory effects of LE−1, LE−2, and LE−3 on superoxide generation in differentiated HL-60 cells. LE−1, LE−2, or LE−3 solution was added to differentiated HL-60 cells suspension, and the mixture was incubated at 37 °C for 15 min. Ninety seconds after stimulation with TPA (100 μM), cytochrome *c* solution was added and incubated at 37 °C for 15 min. (Left, cytotoxicity; right, inhibition of NO_2^-.)

in Figure 2, LE−1, LE−2, and LE−3 exhibited inhibitory activity toward TPA-induced EBV activation at every concentration tested. At a concentration of 10 μM, the inhibitory activities of LE−1 (79.3%) and LE−3 (84.2%) were higher than that of LE−2 (59.9%). LE−1 at a concentration 50 μM, at which 100% inhibitory activity was observed, showed a slightly detectable cytotoxicity using a trypan blue test, while LE−2 and LE−3 exhibited no cytotoxicity at concentrations tested. However, LE−1 at 10 μM did not show cytotoxicity and exhibited a high EBV-activation inhibitory activity. LE−1, LE−2, and LE−3 have geranyloxy groups on the psolaren- or coumarin-ring skeleton (Figure 1). 7-Geranyloxycoumarin (auraptene), a coumarin from *Citrus natsudaidai*, has been reported to inhibit TPA-induced EBV activation, and umbelliferone, lacking the geranyl group of auraptene, was shown to have much lower inhibitory activity (Murakami et al., 1997). The inhibitory activity of EBV activation of LE−1 and LE−3 was comparable to that of auraptene. The reason for the notable activity of LE−1 and LE−3 may be due to the presence of a geranyl group, similar to the case auraptene (Murakami et al., 1999). The importance of the geranyl group in inhibitory activity may be related to the higher cellular uptake ability of LE−1, LE−2, and LE−3 or more favorable hydrophobic interactions with the target site.

TPA-Induced Superoxide Generation. Auraptene has recently been reported to inhibit O_2^- generation in differentiated HL-60 cells (Murakami, et al., 1997). Thus, LE−1, LE−2, and LE−3 were examined for their ability to inhibit TPA-induced O_2^- generation in differentiated HL-60 cells (Figure 3). The O_2^- concentration in the control test treated only with 100 μM TPA was 18 nmol/mL, after 15 min of TPA stimulation. As shown in Figure 3, LE−1, LE−2, and LE−3 at a concentration of 10 and 50 μM significantly inhibited O_2^- generation. Their inhibitory activities of O_2^- generation were slightly lower than that of auraptene (Murakami, et al., 1997). The inhibitory activity of LE−1 was stronger than that of LE−2 and LE−3. They showed no detectable cytotoxicity using a trypan blue test at every concentration tested. NADPH oxidase is known to play a major role in O_2^- generation in phagocytes such as macrophages, neutrophils, or granulocytes (Cross and Jones, 1991). Since the LE series has no O_2^- -scavenging potential (data not shown), they may inhibit the multicomponent NADPH oxidase system.

NO Generation Test in RAW 264.7 Cell. NO is readily released by LPS/IFN-γ stimulation to form stoichiometric amounts of L-citrulline from L-arginine in mouse macrophage RAW 264.7 cells. NO is then simultaneously converted to NO_2^- under physiological conditions. It has been reported that LE−2 suppressed

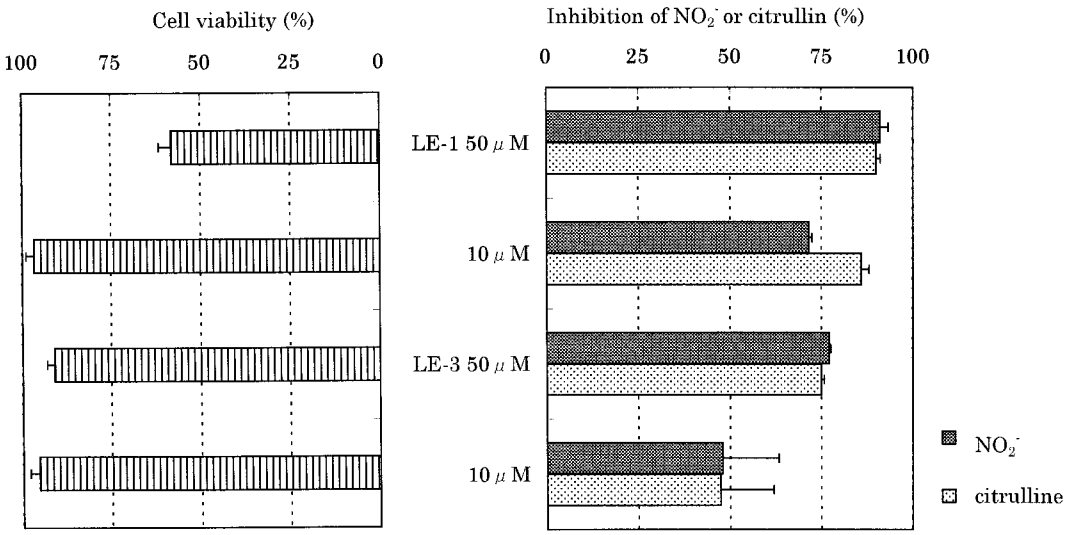


Figure 4. NO generation inhibitory activities of LE−1, LE−2, and LE−3 in RAW 264.7 cells. The RAW 264.7 cells were incubated with LPS (100 ng/mL), BH_4 (10 mg/mL), IFN-γ (100 units/mL), L-arginine (2 mM), and LE−1, LE−2, or LE−3 at 37 °C for 24 h. (Left, cytotoxicity; right, inhibition of NO_2^- and L-citrulline.)

NO generation (Murakami et al., 1999). Then, we tested LE−1 and LE−3 for inhibition toward both lypopolysaccharide (LPS) and interferone-γ (IFN-γ)-induced NO generation in RAW 264.7 cells. Both LE−1 and LE−3 inhibited the formation of L-citrulline and NO_2^- to a similar extent (Figure 4), clearly indicating that they have no NO scavenging potential, similar to the case in the O_2^- assay. They probably inhibit the LPS/IFN-γ-triggered iNOS expression pathways and/or iNOS enzyme activity. They showed no detectable cytotoxicity at a concentration of 10 μM, while LE−1 showed a slightly detectable cytotoxicity at 50 μM. LE−1 at 10 μM exhibited inhibitory activity toward NO generation but had no detectable cytotoxicity.

Overall, LE−1 was indicated to have higher inhibitory activities toward EBV activation, O_2^- and NO generation than LE−2 or LE−3. The reason LE−1 has higher in vitro inhibitory activities as compared with LE−2 and LE−3 is unclear and needs further exploration. Moreover, it has been reported that coumarins bearing a geranyl group such as bergamottin (LE−2) are highly active in the NO test because of its higher celluar uptake ability (Murakami et al., 1999). There are structurally diverse coumarins in the plant kingdom (Gray and Waterman, 1978), and categorization of their various potentials for antitumor promoting properties based on chemical structure are important and useful in arranging and predicting the antitumor properties of the various coumarins. The present data steadily support that coumarins or psolarens bearing a geranyloxy group are potent NO generation inhibitors (Murakami et al., 1999). Auraptene (7-geranyloxycoumarin) has been reported to be an effective chemopreventer in mouse skin (Murakami et al., 1997), rat colon (Tanaka et al., 1997a), and tongue (Tanaka et al., 1997b). The present study thus suggests that LE−1, LE−2, and LE−3, isolated from lemon fruit have chemopreventive activity in vivo because of the structure and activity similarity to auraptene. Chemopreventive studies of the LE series using experimental rodents are currently ongoing.

Quantitative Analysis of Coumarins. The quantitative levels of LE−1, LE2, and LE−3 in lemon fruit were examined using HPLC analysis (Table 3). They were more abundant in the peel than in the juice or seed. The coumarins were more abundant in the flavedo rather than in the albedo or pulp vesicles of the peel. Citrus coumarins have been reported to occur abundantly in citrus oil (Fisher and Trama, 1979). LE−1, LE−2, and LE−3 were suggested to be accumulated in the flavedo of the peel because the flavedo carries many oil glands containing coumarins. We analyzed the contents of LE−1, LE2, and LE−3 in commercial cold-pressed lemon oils. They abundantly existed in cold-pressed lemon oil at the levels of 1850, 1700, and 1,700 ppm, respectively. As the cold-pressed oils of citrus fruits are produced on an industrial scale throughout the world, coumarins in citrus oil are readily available.

Table 3. Occurrence of LE−1, LE−2, and LE−3 in Lemon Fruit

		LE−1 (ppm)	LE−2 (ppm)	LE−3 (ppm)
peel		33.5	34.7	21.5
	flavedo	116	138	83.2
	albedo	18.4	17.8	10.0
	pulp vescles	2.3	3.0	1.9
juice		0.2	0.1	0.1
seed		0.4	0.4	0.3

Coumarins are generally known to be found in various foods. It is tempting to speculate, thus, that these coumarins may have a generally low toxicity for dietary foods since it occurs in the edible peel, the oils of which are used as food additives. While the oral intake of coumarins at low levels suggest to be safe in vivo and exhibit antitumor activity, their intake at high level may cause harmful to health because furanocoumarins in celery root have been shown to be phytophototoxic dermatitis (Ljunggren, 1990). On the other hand LE series did not exhibit the mutagenic activity according to the Ames method (Ames, 1975) using *Salmonella typhimurium* TA98 or TA 100 (data not shown). But they should be examined for further safety in vivo. We anticipate that the designer foods used citrus coumarins containing effective chemopreventive properties were developed in the future. They may be expected to enhance human health and prevent cancer when eaten daily, although their availability in vivo must be examined further.

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