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ORIGINAL ARTICLE

Inhibitory effects of di-*O*-demethylcurcumin on interleukin-1β-induced interleukin-6 production from human gingival fibroblasts

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KEYWORDS
anti-inflammation;
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gingival fibroblasts;
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interleukin-6

Abstract *Background/purpose:* Curcumin is a polyphenolic phytochemical isolated from the medicinal plant *Curcuma longa* L. This phytochemical exhibits anti-inflammatory and antioxidant properties. The aim of this study was to find a curcuminoid compound that has a better effect on the suppression of interleukin-1β (IL-1β)-induced IL-6 production than curcumin in human gingival fibroblasts (HGFs).
Materials and methods: The parent curcuminoids **1**–**3** were isolated from the rhizomes of *C. longa* and 17 curcuminoid analogs **4**–**20** were synthesized from the parent curcuminoids. The condition for IL-6 production by HGFs after inducing the cells with IL-1β was optimized. HGFs were incubated with curcuminoids (0.016–20 μg/mL) for 30 minutes before adding IL-1β (2 ng/mL). After 24 hours of incubation, the culture media were harvested and determined for IL-6 contents using an enzyme-linked immunosorbent assay method. Prednisolone, an immunosuppressive drug, was used as a positive control. Half maximal effective concentration (EC_{50}) is measured and reported as the concentration required for 50% inhibition of the levels found in the control medium.
Results: The maximum IL-6 production was achieved when the HGFs were exposed to an IL-1β concentration of 2 ng/mL for 24 hours; however, addition of the immunosuppressant prednisolone inhibited the production of IL-6. Among the analogs, di-*O*-demethylcurcumin (**5**) exhibited the most potent anti-IL-6 activity with an EC_{50} of 2.18 ± 0.07 μg/mL, which was approximately eightfold more active than the natural curcuminoids **1**–**3**. Cell viability was

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not significantly affected when the concentration of di-*O*-demethylcurcumin was less than 20 μg/mL.

Conclusion: Di-*O*-demethylcurcumin exhibited an inhibitory effect on the production of IL-6 by IL-1β-induced HGFs and can thus serve as a lead compound with its inhibiting property for IL-6 production.

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Introduction

Chronic inflammation is believed to play a predominant role in the pathogenesis of periodontal diseases. Persisting inflammation in the periodontium of patients may be facilitated by the expression of inflammatory cytokines including interleukin-1β (IL-1β), a key mediator of various immunological and inflammatory phenomena,[1] which exhibited a correlation with the severity of periodontal diseases.[2,3] When stimulated with IL-1β, human gingival fibroblasts (HGFs) were showed increasing bone resorption and collagenase activity.[4] IL-1β was also shown to stimulate the expression of IL-6 in HGFs.[5,6] IL-6 plays an important role in the pathogenesis of periodontal diseases because it promotes bone resorption, possibly by stimulating osteoclast precursor recruitment and differentiation.[7,8] Therefore, in IL-1β-induced HGFs, IL-6 seems to stimulate alveolar bone resorption leading to pathogenesis of the periodontal diseases.

Curcumin (**1**) is a naturally occurring active antioxidant,[9] antitumor,[10,11] and anti-inflammatory phytochemical agent.[12] This compound was shown to downregulate the expression of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and IL-1β isolated from lipopolysaccharide (LPS)-stimulated lung cells.[13] Results of an earlier study revealed that long-term oral consumption of compound **1** in low doses activated peroxisome proliferator-activated receptor-γ, deactivated cell-mediated immunity, inhibited inducible nitric oxide synthase, and interfered with adaptive immunity to exacerbate the pathogenesis of *Leishmania donovani* infection *in vivo*.[14] In addition, the treatment led to the downregulation of TNF-α, IL-1β, IL-8, macrophage inflammatory protein-1α, and macrophage inflammatory protein-1 expression by *peripheral blood* mononuclear cells.[15] The production of TNF-α[16] and inducible *nitric oxide* synthase from activated macrophages were also inhibited by compound **1**.[17]

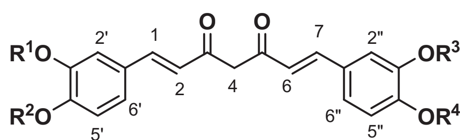
It has been shown that Jun N-terminal kinase (JNK) is required for IL-1-induced IL-6 and IL-8 gene expression in human epithelial carcinoma cell line KB.[18] In addition, curcumin is a potent inhibitor for nuclear factor-κB and mitogen-activated protein kinase activation.[19] Curcumin has also been shown to inhibit the production of thrombin-stimulated connective tissue growth factor (CCN2) by suppressing the activity of JNK in HGFs.[20] Because of the broad anti-inflammatory properties of curcumin, various studies have started investigating other naturally occurring curcuminoids and their analogs to assess their ability to inhibit the suppression of IL-1β-induced IL-6 production. The results of such studies will provide a basic knowledge for future studies to develop such compounds as therapeutic agents for the treatment of periodontal diseases. This study explains the structural modification of the parent curcuminoids **1**–**3** to synthesize analogs **4**–**20** and evaluates their ability to inhibit the production of IL-6.

Materials and methods

Chemicals and structural modification of curcuminoid analogs

The parent curcuminoids **1**–**3** were isolated from the rhizomes of *Curcuma longa* and 17 curcuminoid analogs **4**–**20** (Fig. 1) were synthesized from the curcuminoids **1**–**3**. This is accomplished as follows: curcumin (**1**) is demethylated to the corresponding mono-*O*-demethyl analog **4** and di-*O*-demethyl analog **5** (42% and 33% yield, respectively) by treating it with boron tribromide in dry dichloromethane. Demethylation of curcuminoid **2** was similarly achieved to yield the corresponding *O*-demethyl analog **6** (64% yield). The spectroscopic [infrared (IR), proton nuclear magnetic resonance (^{1}H-NMR), and mass spectra] data of **4**, **5**, and **6** were consistent with the previously reported values.[21] Methylation of compounds **1**, **2**, and **3** was achieved by treating them with methyl iodide in acetone in the presence of potassium carbonate to give the corresponding ether analogs **7**–**13**. The structures of these ether analogs were characterized by spectroscopic (IR, ^{1}H-NMR, and mass spectral) data and by spectroscopic spectral comparison with the previously reported values.[22,23] Acetylation of the parent curcuminoids **1**, **2**, and **3** using acetic anhydride and pyridine furnished the acetates **14**–**20** in good yields. The structures of these acetates were characterized by spectroscopic (IR, ^{1}H-NMR, and mass spectra) data and by spectroscopic spectral comparison with the reported values.[24,25]

Prednisolone (Sigma-Aldrich, St. Louis, MO, USA), an effective anti-inflammatory agent, was used as a positive control to inhibit IL-6 production. Stock solutions of the curcuminoids, prednisolone, and a COX-2 inhibitor, NS-398 (Sigma-Aldrich, St. Louis, MO, USA), were prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 10 mg/mL and stored at −20 °C until further use. At the start of each experiment, all compounds were diluted in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) with the final DMSO concentrations not exceeding 0.1%.



1 $R^1 = R^3$ = Me, $R^2 = R^4$ = H, Curcumin

4 $R^1 = R^2 = R^4$ = H, R^3 = Me, Mono-*O*-demethylcurcumin

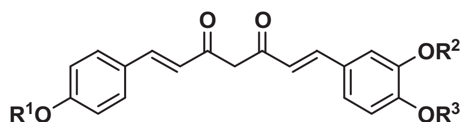
5 $R^1 = R^2 = R^3 = R^4$ = H, Di-*O*-demethylcurcumin

7 $R^1 = R^2 = R^3$ = Me, R^4 = H, Mono-*O*-methylcurcumin

8 $R^1 = R^2 = R^3 = R^4$ = Me, Di-*O*-methylcurcumin

14 $R^1 = R^3$ = Me, R^2 = Ac, R^4 = H, Mono-*O*-acetylcurcumin

15 $R^1 = R^3$ = Me, $R^2 = R^4$ = Ac, Di-*O*-acetylcurcumin



2 $R^1 = R^3$ = H, R^2 = Me, Demethoxycurcumin

6 $R^1 = R^2 = R^3$ = H, *O*-Demethyldemethoxycurcumin

9 R^1 = H, $R^2 = R^3$ = Me, 4″-*O*-Methyldemethoxycurcumin

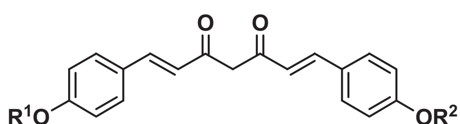
10 $R^1 = R^2$ = Me, R^3 = H, 4′-*O*-Methyldemethoxycurcumin

11 $R^1 = R^2 = R^3$ = Me, Di-*O*-methyldemethoxycurcumin

16 R^1 = H, R^2 = Me, R^3 = Ac, 4″-*O*-Acetyldemethoxycurcumin

17 R^1 = Ac, R^2 = Me, R^3 = H, 4′-*O*-Acetyldemethoxycurcumin

18 $R^1 = R^3$ = Ac, R^2 = Me, Di-*O*-acetyldemethoxycurcumin



3 $R^1 = R^2$ = H, Bisdemethylcurcumin

12 R^1 = Me, R^2 = H, Mono-*O*-methylbisdemethylcurcumin

13 $R^1 = R^2$ = Me, Di-*O*-methylbisdemethylcurcumin

19 R^1 = Ac, R^2 = H, Mono-*O*-acetylbisdemethylcurcumin

20 $R^1 = R^2$ = Ac, Di-*O*-acetylbisdemethylcurcumin

Figure 1 Structures of curcuminoids **1**—**3** and the curcuminoid analogs **4**—**20**.

Primary culture of HGFs

HGF cultures were prepared by harvesting healthy gingival tissues from three volunteers at the time of clinical crown lengthening and were processed as described previously.[26] This study was approved by the *Faculty* of *Dentistry* Committee for *Ethics*, Srinakharinwirot University. Before treatment, the study objectives were explained to each patient, and informed consent was obtained. The gingival tissues were washed two to three times with phosphate-buffered saline (PBS), cut into small pieces, placed on 35-mm tissue culture dishes, and covered with a sterilized glass cover slip. DMEM supplemented with 10% heat-inactivated fetal bovine serum (Seromed-Biochrom, Berlin, Germany) and 1% antibiotic—antimycotic solution (Gibco, Grand Island, NY, USA) was used as a culture medium. The cultures were maintained at 37 °C in a humidified incubator (*ThermoForma Series II Water Jacketed CO_2 Incubator*, Forma Scientific; Marietta, OH, USA) with 5% CO_2 until monolayers of confluent cells were formed. After trypsinization, the HGFs were allowed to regrow and routinely expanded using 0.05% trypsin (Gibco, Grand Island, NY, USA) in PBS containing 0.053 mM ethylenediaminetetraacetic acid in 75-cm^2 tissue culture flasks. These HGFs were used at passages 3—7 in all experiments.

Cytotoxic assay

The effects of the test compounds and control media (DMEM or DMEM—DMSO) on fibroblast viability were determined by sulforhodamine B (SRB) assay.[27] Each well of a 96-well tissue culture plate (Corning Inc., NY, USA) was seeded with 1×10^4 cells in 0.1 mL complete medium and incubated at 37 °C for 24 hours. The medium was then removed and replaced with DMEM or DMEM—DMSO supplemented with curcuminoids or drugs. After 24 hours, cultured cells were fixed with trichloroacetic acid for 60 minutes and then stained for 30 minutes with 0.4% (w/v) SRB sodium salt (Sigma Chemical Co., St. Louis, MO, USA) dissolved in 1% acetic acid. The unbound dyes were removed by washing four times with 1% acetic acid, then the protein-bound dye was extracted with 10 mM unbuffered Tris base (pH 10.5) for 5 minutes. Optical density (OD) values were determined at 490 nm using a microplate reader (Tecan U.S., Durham, NC, USA). Half maximal inhibitory concentration (IC_{50}) values were determined by regression analysis and expressed as mean ± standard error of the mean (SEM), where a minimum of three replicates from two independent experiments were performed at each of the three concentrations per test sample.

Optimized condition for IL-6 production by IL-1β-induced HGFs

To optimize an experimental condition, the condition necessary for stimulating IL-1β to induce secretion of IL-6 from HGFs was evaluated. HGFs were seeded at 1×10^5 cells/mL in 96-well plates (100 μL/well) in complete medium and allowed to adhere overnight at 37 °C. The medium was subsequently removed and each well was washed once with DMEM. In the time-course experiments, cells were incubated with DMEM with or without IL-1β (2 ng/mL; Sigma-Aldrich, St. Louis, MO, USA) for 1—48 hours. In the concentration—response experiments, cells were incubated for 24 hours with 1—8 ng/mL of IL-1β. The culture media were harvested at the indicated times and

stored at −80 °C before analyzing for IL-6 content using an enzyme-linked immunosorbent assay (ELISA) method. This condition was used to evaluate the effects of curcuminoids and its analogs on the production of IL-6 in the subsequent experiments.

Inhibition effects of curcuminoids or its analogs on IL-1β-induced IL-6 production

HGFs (1×10^5 cells/mL) were seeded in 96-well plates (100 μL/well) in the culture medium and the cells were allowed to adhere overnight at 37 °C. The medium was subsequently removed and each well was washed once with DMEM. Cells were incubated with curcumin (**1**) or di-*O*-demethylcurcumin (**5**) (0.016−20 μg/mL) for 30 minutes before adding IL-1β (2 ng/mL). After 24 hours of incubation, the culture media were harvested and determined for IL-6 contents using an ELISA method. The effects of these substances on IL-1β-stimulated IL-6 production by HGFs were calculated in units of ng/mL, and converted to a percentage of the amount in control medium (DMEM−0.1% DMSO) with IL-1β. Prednisolone, the immunosuppressive drug, was used as a positive control. While determining the effective concentration of each compound, half maximal effective concentration (EC_{50}) is measured and reported as the concentration required for 50% inhibition of the levels found in the control medium.

ELISAs for IL-6 determination

To detect cellular production of IL-6, an ELISA method was used according to the method suggested by Shirai and coworkers.[28] In brief, a microtiter plate was coated with 100 μL/well of 0.5 ng/mL monoclonal anti-human IL-6 antibody (Sigma-Aldrich, St. Louis, MO, USA) in carbonate−bicarbonate buffer (pH 9.6) and incubated overnight at room temperature. The wells were washed with 0.05% Tween in PBS (PBS/Tween) and blocked with 1% bovine serum albumin (BSA) in PBS for 2 hours. Samples and standards were diluted with 0.1% BSA in PBS and about 100 μL of sample was added to each well. After washing with PBS/Tween, 100 μL/well of biotinylated anti-human IL-6 antibody (Zymed Laboratories Inc, San Francisco, CA, USA) in 1% BSA was added before incubating the plates for 1 hour. Each well was washed with PBS/Tween, and 100 μL/well of streptavidin horseradish peroxidase (Zymed Laboratories Inc, San Francisco, CA, USA) was added, and the mixture is further incubated for 1 hour before washing again with PBS/Tween. A substrate solution of 100 μL/well was added and incubated for 25 minutes before stopping the reaction with 1M H_2SO_4 (100 μL/well). The absorbance was determined at 450 nm using a microplate reader. In each experiment, the three wells were repeated and the standard deviation was calculated.

Statistical analysis

All experiments were set up three times, with each experiment performed in triplicate, for each cell population of HGFs. Data are presented as means ± SEM. Data were analyzed using SIGMASTAT (SPSS Inc., Chicago, IL, USA). One-way repeated-measurement analysis of variance, followed by *post-hoc* testing using the Holm−Sidak method (when appropriate), and Student paired *t* test were used for statistical analysis. The level of statistical significance was set at $P = 0.05$ and 0.001.

Results

Inhibitory effects of curcuminoids and analogs on IL-1β-induced IL-6 production

Herein, time-dependent and dose-dependent IL-1β stimulation for HGFs have been evaluated. In the time-course experiments, IL-6 was released into the culture medium as early as at 1 hour and continued for up to 24 hours. A significant difference in IL-6 released after stimulating with IL-1β was observed after 8 hours and after 16−24 hours ($P < 0.001$). The amount of IL-6 significantly *decreased after* long-term incubation (48 hours) when compared with 24-hour incubation ($P = 0.025$) (Fig. 2). Because the

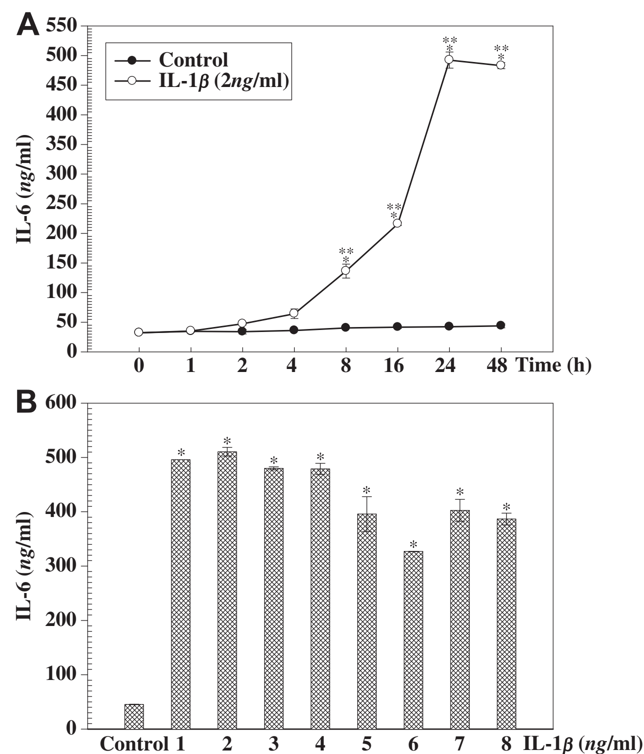


Figure 2 Time course (A) and concentration response (B) of interleukin-1β (IL-1β) on IL-6 production in human gingival fibroblasts (HGFs). HGFs were incubated with IL-1β (2 ng/mL) for the indicated times (0−48 hours) or with increasing concentrations (1−8 ng/mL) of IL-1β for 24 hours. After incubation, the supernatants were evaluated for IL-6 levels by enzyme-linked immunosorbent assay. Data are representative of three experiments and expressed as mean ± standard error of the mean. Analysis of variance with Holm−Sidak tests compare IL-6 production relative to the unstimulated control group (Control), $^{*}P < 0.05$ (A, B). Paired *t* tests compare IL-6 production of IL-1β-stimulated and control groups at a different incubation time, $^{**}P < 0.001$ (A).

highest IL-6 production was obtained after incubating for 24 hours, this duration was used in subsequent experiments. In the concentration–response experiments, HGFs cultured with different concentrations of IL-1β (1–8 ng/mL) produced significantly higher levels of IL-6 as compared with the control (without IL-1β stimulation; Fig. 2). Therefore, we decided to use 2 ng/mL of IL-1β and incubated the cells for 24 hours for the condition used to induce the cytokine production in the subsequent experiments.

The parent curcuminoids **1**–**3** and the chemically modified analogs **4**–**20** were evaluated for the production of anti-IL-6 from IL-1β-induced HGFs. Compounds **1**–**3** exhibited anti-IL-6 production, with EC_{50} values of 16.81 ± 0.09, 16.23 ± 0.22, and 16.02 ± 0.14 μg/mL, respectively (Table 1). The inhibitory activity of the curcuminoid **1** increased at higher doses. However, with IC_{50} values of 25.0 ± 1.11 μg/mL, cytotoxic effects on the HGFs were observed. The monodemethylated analog **4** (Table 1) showed a slightly higher inhibitory activity (EC_{50}: 14.30 ± 0.25 μg/mL) than that of the parent compound **1**. However, the di-*O*-demethylated analog **5** was eightfold more active (EC_{50} 2.18 ± 0.07 μg/mL) than the parent compound **1**. The increase in inhibitory activity was possibly due to the presence of two sets of 1,2-dihydroxyphenyl (catechol) moieties. The high activity of curcuminoid analog with the catechol structure was further evident from the high activity of the demethylated analog **6**, which was about 2.5-fold more active than its parent compound **2**. It was reported that polyhydroxycurcuminoids displayed excellent antioxidant activity, exhibited cytotoxicity to lymphocytes, and tumor-reducing activity on Dalton's lymphoma ascites tumor cells.[29] As expected, the methyl ether analogs **7** and **8** of the parent compound **1** were less active than the corresponding di-demethylated analog **5**, because the former two analogs lacked the catechol moiety. In addition, the methyl ether analogs **9**, **10**, and **11** were less active than their demethylated analog **6** (EC_{50}: 6.64 ± 0.08 μg/mL). Rather surprisingly, the monomethyl and dimethyl ether analogs **12** and **13** were approximately 2.5- and 2-folds more active than the parent compound **3**. Furthermore, the monoacetates **14**, **16** and **17**, and **19** are 2- to 3.5-fold more active than their respective parent compounds **1**, **2**, and **3**. However, the diacetates **15**, **18**, and **20** exhibited low activity. The relatively higher activity of the methyl ether analogs **12** and **13** and the monoacetate ester analogs **14**, **16**, **17**, and **19** than their respective parent compounds clearly indicated that high inhibitory activity was not just due to the contribution from free hydroxyl group in the curcuminoid molecule. It has been reported that some acetate derivatives of curcuminoid exhibited higher antioxidant activity than the parent compounds.[11] However, further study is needed to provide reasonable explanation for this observation.

Cytotoxic effect of curcuminoids

SRB assay was performed to evaluate the toxicity of curcuminoids and prednisolone. Incubation of HGFs with

Table 1 Inhibition of IL-6 production and cytotoxic effects of curcuminoid analogs on HGFs.

Compound	EC_{50} (μg/mL)[a]	HGFs, IC_{50} (μg/mL)[b]
DMEM–DMSO	Inactive	Inactive[c]
Curcumin (**1**)	16.81 ± 0.09	25.0 ± 1.11
Demethoxycurcumin (**2**)	16.23 ± 0.22	24.2 ± 0.57
Bisdemethoxycurcumin (**3**)	16.02 ± 0.14	18.0 ± 0.39
Mono-*O*-demethylcurcumin (**4**)	14.30 ± 0.25	25.0 ± 0.79
Di-*O*-demethylcurcumin (**5**)	2.18 ± 0.07	Inactive[c]
O-Demethyldemethoxycurcumin (**6**)	6.64 ± 0.08	29.6 ± 0.82
Mono-*O*-methylcurcumin (**7**)	13.61 ± 0.2	Inactive[c]
Di-*O*-methylcurcumin (**8**)	8.90 ± 0.32	Inactive[c]
4″-*O*-methyldemethoxycurcumin (**9**)	14.60 ± 0.31	Inactive[c]
4′-*O*-methyldemethoxycurcumin (**10**)	16.04 ± 0.01	Inactive[c]
Di-*O*-methyldemethoxycurcumin (**11**)	16.20 ± 0.32	Inactive[c]
Mono-*O*-methylbisdemethylcurcumin (**12**)	6.10 ± 0.11	Inactive[c]
Di-*O*-methylbisdemethylcurcumin (**13**)	8.01 ± 0.16	Inactive[c]
Mono-*O*-acetylcurcumin (**14**)	7.52 ± 0.08	Inactive[c]
Di-*O*-acetylcurcumin (**15**)	18.84 ± 0.14	Inactive[c]
4″-*O*-Acetyldemethoxycurcumin (**16**)	4.47 ± 0.22	14.2 ± 0.19
4′-*O*-Acetyldemethoxycurcumin (**17**)	8.61 ± 0.31	Inactive[c]
Di-*O*-acetyldemethoxycurcumin (**18**)	12.41 ± 0.13	Inactive[c]
Mono-*O*-acetylbisdemethylcurcumin (**19**)	5.47 ± 0.22	20.02 ± 0.12
Di-*O*-acetylbisdemethylcurcumin (**20**)	Inactive	Inactive[c]

DMEM = Dulbecco's modified Eagle's medium; DMSO = dimethyl sulfoxide; EC_{50} = half maximal effective concentration; HGFs = human gingival fibroblasts; IC_{50} = half maximal inhibitory concentration; IL = interleukin.

[a] Values are the mean concentration of substances ± standard deviations that showed 50% inhibition of the control medium with IL-1β (2 ng/mL).

[b] Values are the mean concentration of substances ± standard deviations that showed 50% inhibition of the control medium.

[c] Inactive at ≥20 μg/mL.

20 μg/mL of the test substances for 24 hours altered cell viability to about 65% of the control. The viabilities of HGFs after incubating the compounds **1**, **2**, **3**, and **4** for a short period (24 hours) were shown to be very toxic at the concentration of 20 μg/mL with viability values of 65.92 ± 0.89%, 61.45 ± 0.56%, 56.42 ± 0.01% and 67.42 ± 0.01%, respectively. Compound **2** was the only curcuminoid that was toxic at a concentration range of 5–20 μg/mL, whereas all other curcuminoids were nontoxic at the concentration range of 5–10 μg/mL (Fig. 3). It was worth noting that increasing the concentration of compound **5** did not decrease the viability of HGFs. The result indicated that there was no dose-dependent effect of compound **5** on the viability of the HGFs. Furthermore, we found that prednisolone was toxic to HGFs in a dose-dependent manner. At concentrations of 5–10 μg/mL, the cell viability of HGFs was reduced by approximately 20–65% (Fig. 3).

Inhibitory effects of prednisolone, compounds 1 and 5 on IL-6 production from IL-1β-induced HGFs

Prednisolone is an anti-inflammatory agent that inhibits pro-inflammatory cytokine production including IL-6.[30] The effects of this drug on IL-1β-stimulated IL-6 production by the HGFs were first determined. From our results, the IL-6 production was reduced by approximately 55% upon treatment with 10 μg/mL prednisolone. However, there were no significant differences in anti-IL-6 production after treatment with the drug at the concentration between 0.16 μg/mL and 5.0 μg/mL (data not shown). The IL-6 production was significantly lower in the prednisolone treatment *as compared with the control* group (Fig. 4).

Effects of curcumin (**1**) and di-*O*-demethylcurcumin (**5**) on IL-6 production from IL-1β-induced HGFs were evaluated. Both compounds **1** and **5** inhibited IL-6 production from IL-1β-induced HGFs. The results indicated that 1.25–20 μg/mL of compound **5** significantly inhibited the secretion of IL-6 from HGFs in a dose-dependent manner (Fig. 5). At 20 μg/mL concentration, compound **5** reduced IL-1β-induced IL-6 production to 40% of the level obtained with IL-1β-treatment alone (Fig. 5). Compound **5** was more active in anti-IL-6 properties than the parent compound **1**. As the EC_{50} value of the compound **5** was eightfold more active (EC_{50} 2.18 ± 0.07 μg/mL) than the parental compound **1** (EC_{50} 16.81 ± 0.09 μg/mL) (Table 1), the inhibitory effect of compound **5** was significantly different from the control media that is induced with IL-1β alone at $P < 0.05$.

Discussion

IL-1β is involved in stimulating the production of protease-like metalloproteinase and a variety of inflammatory mediators, and also in inducing bone resorption.[1] Of these mediators, we have chosen IL-6 as indicators of inflammatory process, because they are involved in the development of inflammation by promoting bone resorption via the stimulation of osteoclast precursor recruitment and differentiation.[31,32] Consequently, regulating gingival inflammation may be one way to prevent and control progression of periodontitis. In this study, we determined the effects of the natural curcuminoids **1**–**3** and their chemically modified analogs **4**–**20** on IL-6 production to investigate their potential as inhibitors of inflammatory process (IL-1β-induced HGFs) by comparing them with an immunosuppressant drug, prednisolone.

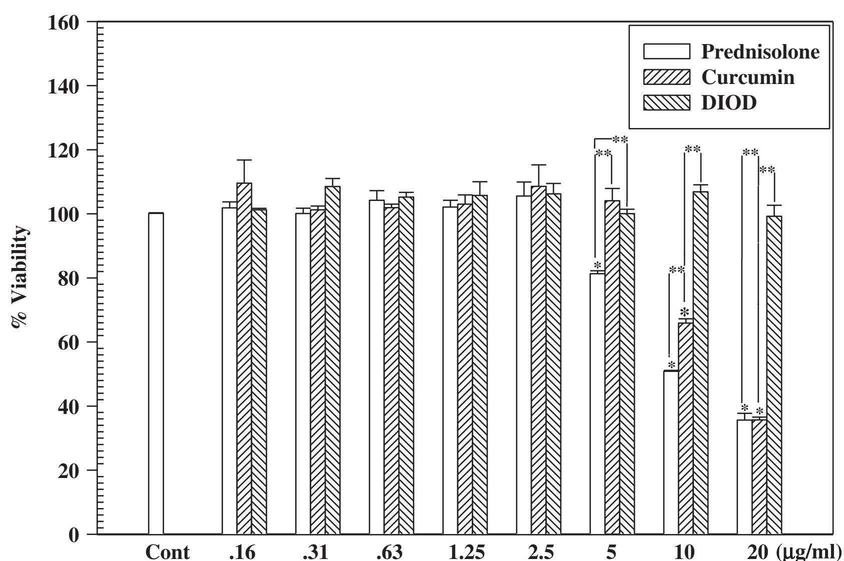


Figure 3 Effect of prednisolone, curcumin, and di-*O*-demethylcurcumin (DIOD) on cell viability of human gingival fibroblasts (HGFs). HGFs were incubated with prednisolone, curcumin (**1**), or di-*O*-demethylcurcumin (DIOD, **5**) (0.016–20 μg/mL) for 24 hours. After incubation, the cell viabilities were measured by sulforhodamine B method. Data are representative of three experiments and expressed as mean ± standard error of the mean. Analysis of variance with Holm–Sidak tests compare % viability of the prednisolone (PDS), curcumin, and DIOD treatment relative to control (Cont), *$P < 0.05$. Paired *t* tests compare % viability of PDS, curcumin, and DIOD treatment at a different concentration, **$P < 0.001$.

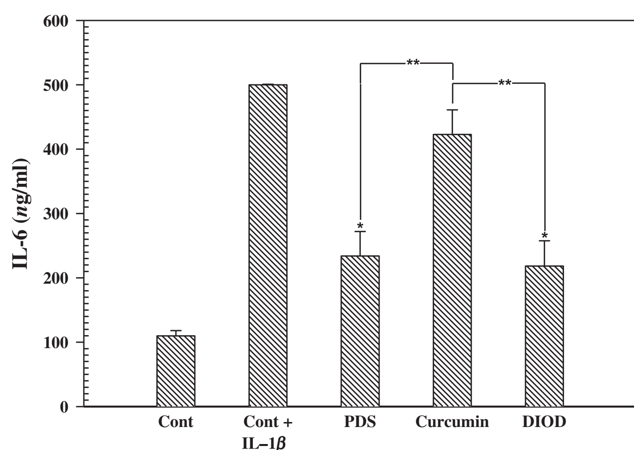


Figure 4 Effect of substances on interleukin-6 (IL-6) production from IL-1β-induced human gingival fibroblasts (HGFs). HGFs were incubated with 10 μg/mL prednisolone (PDS), 10 μg/mL curcumin, or di-*O*-demethylcurcumin (DIOD) for 30 minutes before IL-1β (2 ng/mL) was added. After 24 hours of incubation, the supernatants were evaluated for IL-6 levels by enzyme-linked immunosorbent assay. Data are representative of three experiments and expressed as mean ± standard error of the mean. Analysis of variance with Holm—Sidak tests compare IL-6 production of PDS, curcumin, and DIOD treatment relative to IL-1β-stimulated control (Cont+ IL-1β), **P* < 0.05, ***P* < 0.001.

In the present study, we have shown that IL-1β induced IL-6 production by HGFs in a time-dependent manner. At the concentration of 2 ng/mL, IL-1β could stimulate the secretion of IL-6 within 2 hours of incubation. The concentration of IL-1β can be up to 100 ng/mL for the stimulation; however, a previous study that used peripheral blood monocytes showed that it is not necessary to use a higher amount of IL-1β.[33] Therefore, we used this condition for the experimental model to evaluate the efficacy of the substances to inhibit the inflammatory response in the tested experiments. In another study, this model has been used for triclosan to inhibit the production of inflammation mediators in HGFs.[34]

IL-1β-stimulated IL-6 production from HGFs was reduced by treatment with prednisolone as noted earlier. Glucocorticoids (dexamethasone, prednisolone, and hydrocortisone) were shown to inhibit IL-6 production from human lung fibroblasts with a 50% decrease in IL-1-induced IL-6 gene transcription. This effect does fully account for the potency to induce alterations in IL-6 messenger RNA accumulation and protein production from glucocorticoids.[30] The toxic effect of this drug to HGFs was found at concentrations of 5—10 μg/mL in this study. Prednisolone has been shown to exhibit a dose-dependent anti-proliferative effect on human conjunctival fibroblasts.[35] Recently, prednisolone was coupled to human albumin in order to selectively deliver this steroid drug to macrophages without causing side effects in some pathological condition.[36] Drugs developed by the pharmaceutical industry have thus far been associated with toxicity and side effects, which is why investigating natural substances are of increasing interest. However, we found that curcumin (**1**) was not toxic against HGFs at the concentration less than 10—20 μg/mL after 24 hours of incubation. Our results correlate with the findings of Chen et al who also did not find toxic effect on HGFs at the concentration of up to 20 μM (approximately 7.5 μg/mL).[20] In contrast to the present finding, Syng-Ai et al[37]

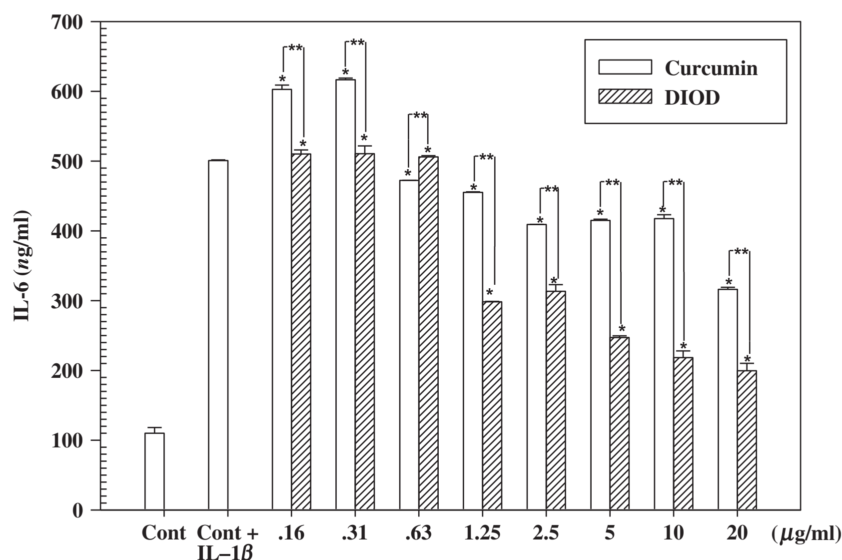


Figure 5 Effect of curcumin (**1**) and di-*O*-demethylcurcumin (**5**) on interleukin-6 (IL-6) production from IL-1β-induced human gingival fibroblasts (HGFs). The HGFs were incubated with curcumin (**1**) or di-*O*-demethylcurcumin (DIOD, **5**) (0.016—20 μg/mL) for 30 minutes before IL-1β (2 ng/mL) was added. After 24 hours of incubation, the supernatants were evaluated for IL-6 by enzyme-linked immunosorbent assay. Data are representative of three experiments. Data are representative of three experiments and expressed as mean ± standard error of the mean. Analysis of variance with Holm—Sidak tests compare IL-6 production of the curcumin and DIOD treatment relative to IL-1β-stimulated control (Cont+ IL-1β) group, **P* < 0.05. Paired *t* tests compare IL-6 production of curcumin and DIOD treatment at a different concentration, ***P* < 0.001.

reported that compound **1** had no effect on normal rat hepatocytes at 50 μM (approximately 15 μg/mL), whereas it had cytotoxic effects on tumor cell lines by inducing apoptosis. Compound **1** is not genotoxic *in vivo* when tested on Balb-C mice, but *in vitro* copper and curcumin interactions induce genetic damage.[38] The cytotoxic effect of curcumin on HGFs and its mechanism of action requires further study.

In HGFs, we found that the anti-IL-6 inhibition of di-*O*-demethylcurcumin (**5**) was more effective compared with curcumin (**1**) and the corresponding controls. Compound **5** had been reported to possess an antioxidant activity and a promising tumor-reducing activity on Dalton's lymphoma ascites tumor cells.[29] However, an *in vitro* study of the anti-inflammatory activity of this compound in HGFs has not been reported. Compound 1 had some ability to reduce IL-6 especially at high concentrations. This compound has been shown to inhibit IL-6, IL-8, and TNF-α from LPS-stimulated macrophages in a dose-dependent manner.[15,39] Alterations in IL-6 production in HGFs by compound **5** may thus be crucial for treating inflammation in patients with periodontitis.

Compound **5** exhibited an inhibitory effect on the production of IL-6 by IL-1β-induced HGFs. Although the activity was not potent compared with conventionally used drugs, this curcuminoid analog appears to have a potential as an anti-inflammatory agent for treating chronic inflammation including periodontal disease, either alone or possibly in combination with other drugs. It may serve as a lead compound with its inhibiting property for IL-6 production.

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