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Anti-inflammatory effect of curcuminoids and their analogs in hyperosmotic human corneal limbus epithelial cells

Ngamjit Kasetsuwan^{1,2*}, Usanee Reinprayoon^{1,2}, Lita Uthaithammarat¹, Amornpun Sereemasun³, Nutchanart Sae-liang³, Waraluck Chaichompoo⁴ and Apichart Suksamrarn⁴

Abstract

Background To assess the efficacy of curcuminoids (curcumin, demethoxycurcumin, bisdemethoxycurcumin [BDC]) and their analogs (tetrahydrocurcumin [THC], tetrahydrodemethoxycurcumin [THDC], tetrahydrobisdemethoxycurcumin) in reducing inflammatory cytokines and their toxicity to primary human corneal limbal epithelial cells, these cells were cultured and exposed to these compounds.

Methods The PrestoBlue assay assessed cell viability after treatment. Anti-inflammatory effects on hyperosmotic cells were determined using real-time polymerase chain reaction and significance was gauged using one-way analysis of variance and Tukey's tests, considering p-values < 0.05 as significant.

Results Curcuminoids and their analogs, at 1, 10, and 100 μM , exhibited no effect on cell viability compared to controls. However, cyclosporin A 1:500 significantly reduced cell viability more than most curcuminoid treatments, except 100 μM curcumin and BDC. All tested curcuminoids and analogs at these concentrations significantly decreased mRNA expression levels of tumor necrosis factor- α , interleukin (IL)-1 β , IL-6, IL-17 A, matrix metalloproteinase-9, and intercellular adhesion molecule-1 after 90 mM NaCl stimulation compared to untreated cells. Furthermore, proinflammatory cytokine levels from hyperosmotic cells treated with 1, 10, and 100 μM curcumin, 100 μM BDC, 100 μM THC, 1 and 100 μM THDC mirrored those treated with cyclosporin A 1:500.

Conclusion The anti-inflammatory efficiency of 1 and 10 μM curcumin, 100 μM THC, 1 and 100 μM THDC was comparable to that of cyclosporin A 1:500 while maintaining cell viability.

Keywords Curcuminoids, Curcumin, Anti-inflammatory, Cytokines, Cell viability, Primary human corneal limbal epithelial cells

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Background

Turmeric (*Curcuma longa*) is a plant in the ginger family. Curcuminoids are yellow chemical compounds found in turmeric, comprising curcumin (diferuloylmethane) (77%), demethoxycurcumin (DC) (17%), and bisdemethoxycurcumin (BDC) (3%) [1]. Historically, curcuminoids have demonstrated numerous biological activities at the molecular level, such as anti-inflammatory, antioxidant, proapoptotic, and anti-cancer properties. These effects have been beneficial in many human disease studies, including rheumatoid arthritis, psoriasis, inflammatory bowel disease, irritable bowel syndrome, gastric ulcer, Alzheimer's disease, and cardiovascular disease. Furthermore, curcuminoids have also shown positive effects in trials related to ophthalmologic diseases such as chronic anterior uveitis [2], age-related macular degeneration [3], diabetic retinopathy [2], central serous chorioretinopathy [2], retinal ischemia [2], and dry eye disease [4], among others.

Tetrahydrocurcumin (THC), a major plasma metabolite of curcumin and a curcuminoid analog, is colorless and more hydrophilic and stable than curcumin due to its non-planar form and benzene rings located at the end of the heptane chain. THC also exhibits a higher antioxidant activity than curcumin and possesses various biological properties, including anti-inflammatory, chemopreventive, antibacterial, antidiabetic, antiviral, cytotoxic, antiangiogenic, neurological, antihistamine,

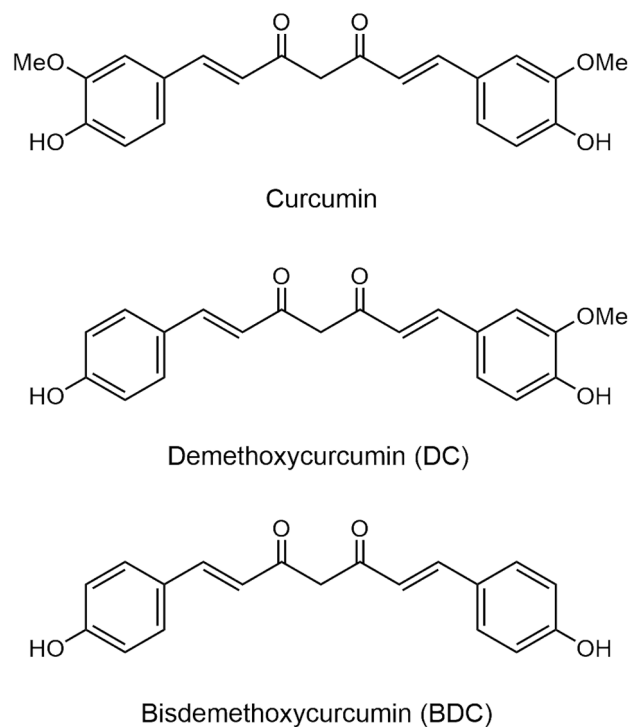


Fig. 1 Curcumin (diferuloylmethane), demethoxycurcumin, and bisdemethoxycurcumin

immunological, and anti-aging activities [5]. Modifications of curcuminoids' structural motifs [6], such as THC, tetrahydrodemethoxycurcumin (THDC), and tetrahydrobisdemethoxycurcumin (THBDC), have been introduced to grant these curcuminoid analogs similar biological effects to curcuminoids with enhanced stability and solubility [5].

Dry eye disease (DED) is an ocular surface ailment causing various symptoms such as photophobia, gritty eyes, and visual disturbances. Inflammation is a critical component in the DED cycle [7]. Currently, there are several treatment options for DED, including topical eye drops, oral medications, lid hygiene combined with warm compression, and device-based treatments. Artificial tear drops are typically the initial treatment choice. Topical corticosteroids and cyclosporin eye drops can effectively reduce inflammation in DED [8]. However, topical corticosteroids may lead to secondary glaucoma and cataract formation, whereas topical cyclosporin can cause a burning and stinging sensation during application, paired with a slow onset of action and a higher price [9].

Due to their biological activity, curcuminoids and their analogs have potential as treatments for numerous diseases in the future, including DED. In this study, we aim to examine the capability of curcuminoids (curcumin, DC, and BDC) and curcuminoid analogs (THC, THDC, and THBDC) in diminishing inflammatory cytokines and to observe the toxicity of these substances on primary human corneal limbal epithelial cells. This research could pave the way for innovative eye drops as a treatment for DED.

Methods

Curcuminoids and Curcuminoid Analogs

The parent curcuminoids (curcumin, DC, and BDC), illustrated in Fig. 1, were isolated from the rhizomes of *Curcuma longa* L. (Zingiberaceae) [10]. The plant materials were purchased from Chatuchak local market, Bangkok, Thailand in January 2014 and were identified by Assoc. Prof. Nopporn Dumrongsiri, Department of Biology, Faculty of Science, Ramkhamhaeng University. A voucher specimen (Apichart Suksamrarn, No. 073) was deposited at the Department of Chemistry, Faculty of Science, Ramkhamhaeng University. These curcuminoids were chemically modified to produce the corresponding tetrahydro analogs THC, THDC, and THBDC, as depicted in Fig. 2, utilizing the method previously described [11]. Starting with curcumin, catalytic hydrogenation, employing palladium on charcoal as a catalyst, yielded 76% THC and 9% hexahydrocurcumin. The spectroscopic data for these isolated analogs aligned with previously reported values [12, 13]. For DC, the tetrahydro analog THDC and hexahydrodemethoxycurcumin were produced in yields of 70% and 8%,

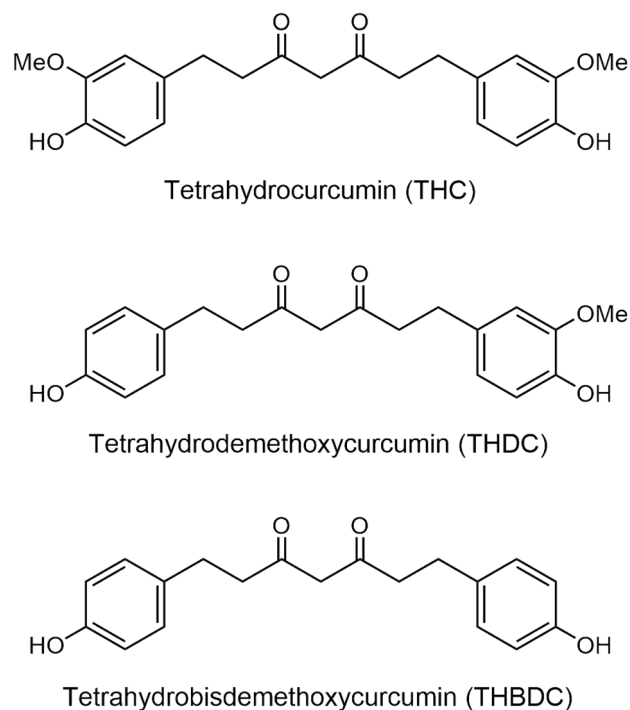


Fig. 2 Tetrahydrocurcumin, tetrahydrodemethoxycurcumin, and tetrahydrobisdemethoxycurcumin

respectively. The spectroscopic data of these synthesized analogs matched previously reported values [14]. From BDC, the tetrahydro analog THDC and hexahydrobisdemethoxycurcumin were obtained in 71% and 8% yields, respectively. Similarly, the spectroscopic data for these synthesized analogs were consistent with the reported values [14]. The purity of both the isolated curcuminoids (curcumin, DC, and BDC) and the synthesized analogs (THC, THDC, and THBDC) was ascertained via thin-layer chromatography (TLC). This was conducted using Merck's precoated silica gel 60 F254 plates, and dichloromethane–methanol and *n*-hexane–ethyl acetate were used as the developing solvent systems. Spots on the TLC were detected under UV light and subsequently by spraying with anisaldehyde–sulfuric acid reagent and heating. The compounds' purity was further validated by nuclear magnetic resonance spectroscopy. All the compounds were dissolved in dimethyl sulfoxide to prepare a 30 mM stock solution and then added to the medium in varying concentrations. The curcuminoid solution exhibited a yellow hue, whereas the solution derived from curcuminoid analogs was colorless.

Feeder cell preparation

Murine 3T3 fibroblast feeder cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 1% GlutaMax

(Gibco, Grand Island, NY, USA), and 1% antibiotic-antimycotic (Gibco, Grand Island, NY, USA). They were incubated at 37 °C in an atmosphere containing 5% CO₂. Once the cells reached approximately 80–90% confluence, they were treated with media containing 10 µg/mL mitomycin C (Sigma, St. Louis, MO) at 37 °C for 2 h. Subsequently, the media was discarded, and the cells were washed with 1X phosphate-buffered saline (PBS). These cells were then trypsinized and plated in 60-mm dishes at a density of 1 × 10⁶ cells, followed by incubation at 37 °C under 5% CO₂. The feeder cells were used 4–24 h post-seeding. The 3T3 feeder cells were used as a feeder layer for the growth and expansion of limbal epithelial cells.

3T3-J2-conditioned medium preparation

3T3-J2 fibroblast feeder cells were cultured in DMEM with 10% bovine calf serum (Gibco, Grand Island, NY, USA). This was done at 37 °C in a 10% CO₂-humidified incubator. The 3T3-J2-conditioned medium was prepared by inactivating the 3T3-J2 fibroblast feeder cells using 10 µg/mL of mitomycin C (Sigma, St. Louis, MO) at 37 °C for 2 h. Then, these cells were rinsed twice with sterile PBS and subsequently treated with epithelial cell culture medium lacking growth factors for 24 h. The 3T3-J2-conditioned medium was then obtained by centrifugation, filtered through a 0.45 µm filter, and stored at 4 °C. The medium was used within 7 days after preparation.

Primary human corneal limbal epithelial cell culture

Primary human corneal limbal epithelial cells, which were derived from human donor cornea, were sourced from the Excellence Center for Stem Cell and Cell Therapy, Faculty of Medicine, Chulalongkorn University, Thailand. The marker of these cells were p63 alpha. These cells were co-cultured with dishes containing 3T3 feeder cells in DMEM: Nutrient Mixture F-12 (DMEM/F12, 1:1). The mixture was supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 2.5 µg/mL NaHCO₃ (HyClone, Logan, UT, USA), 0.5 µg/mL hydrocortisone (Sigma, St. Louis, MO), 1% GlutaMax (Gibco, Grand Island, NY, USA), 1% antibiotic-antimycotic (Gibco, Grand Island, NY, USA), 5 mg/mL human insulin, 20 ng/mL recombinant human epidermal growth factor protein (Bio-Techne, USA), and 0.5 µM ALK5 inhibitor (Miltenyi Biotec, Germany). The culture was maintained at 37 °C in a 5% CO₂-humidified incubator for 14 days to facilitate the growth of limbal epithelial cell colonies. After 14 days, the 3T3 feeder layers were detached using 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) (Gibco, Grand Island, NY, USA) at 37 °C for 1 min. The limbal epithelial cell colonies were then dissociated with 0.25% trypsin/EDTA at 37 °C for 5 min to yield single limbal epithelial cells. Subsequently, the cells

from the 2nd passage were seeded onto dishes coated with 10 µg/mL collagen type I (Bio-Techne, USA) at a density of 5×10^5 cells and bathed in 3T3-J2-conditioned medium for further experiments.

Cell viability assay

The cytotoxicity of curcuminoids and their analogs on primary human corneal limbal epithelial cells was assessed using PrestoBlue™ cell viability reagent (Invitrogen, Waltham, MA, USA). Cells were plated in 96-well plates at a density of 5×10^3 cells/well in serum-free media and were incubated at 37 °C with 5% CO₂ for 24 h. Curcuminoids (curcumin, DC, and BDC) and curcuminoid analogs (THC, THDC, and THBDC) were introduced at final concentrations of 0, 1, 10, and 100 µM. Cyclosporin A at 0.05% was diluted 1:500 in DMEM/F12. The cells, in the presence of curcuminoids, curcuminoid analogs, and cyclosporin A, were further incubated for 24 h at 37 °C with 5% CO₂. Subsequently, they were treated with 10 µL of PrestoBlue™ reagent for 30 min. Cell viability was quantified by measuring fluorescence intensity using a microplate reader at wavelengths of 560 nm and 590 nm (Thermo Scientific, Waltham, MA, USA). The control group of primary human corneal limbal epithelial cells (untreated with any reagent) was set as the benchmark for 100% viability.

Hyperosmoticity assay

Cells were seeded into 6-well plates at a density of 5×10^5 cells/well and incubated for 24 h. Following this, cells were exposed to 90 mM sodium chloride (NaCl) to induce a hyperosmoticity of 450 mOsm for 6 h. Post NaCl exposure, cells were treated with either cyclosporin A at a dilution of 1:500 or with curcuminoids and their analogs at final concentrations of 0, 1, 10, and 100 µM. Subsequently, they were incubated for an additional 24 h at 37 °C in an atmosphere containing 5% CO₂.

RNA extraction and real-time PCR

After the 24-h incubation, cells were harvested by centrifugation for RNA extraction. Total RNA was isolated using the TRIzol® reagent (Invitrogen, Waltham, MA, USA). The RNA concentration and quality were ascertained using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) with measurements taken at 260 nm. The cDNA synthesis was achieved using the RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA), adhering to the manufacturer's guidelines. Quantitative reverse transcription PCR was executed using the Power Up™ SYBR™ Green Master Mix in accordance with the provided instructions. The PCR amplification was carried out on the QuantStudio™ 6 Flex Real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA)

under the stipulated conditions: 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The expression levels of target genes were normalized against the endogenous glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels and then compared to a normalized calibrator. The primers used in this study, which include tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-17 A, matrix metalloproteinase-9 (MMP-9), intercellular adhesion molecule-1 (ICAM-1), and GAPDH, are itemized in Table S1.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism version 9. Differences in means were assessed for statistical significance using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison tests. *P*-values < 0.05 were deemed significant.

Results

Cell viability assay

The viability of primary human corneal limbal epithelial cells, when treated with curcuminoids and their analogs at varying concentrations (1, 10, and 100 µM) for 24 h, showed no significant difference in comparison to the control group (*P* > 0.05) (Fig. 3). As a result, curcuminoids and curcuminoid analogs at concentrations of 100 µM or less were employed in subsequent experiments. Additionally, certain curcuminoid analogs displayed significantly higher cell viability in primary human corneal limbal epithelial cells compared to those treated with cyclosporin A at a 1:500 ratio, as detailed in Table 1. The mean cell viability, complete with standard deviation and a 95% confidence interval for all agents, is illustrated in Table S2.

Proinflammatory cytokine mRNA expression levels

Upon 6-h exposure to 450 mOsm hyperosmoticity (90 mM NaCl), the mRNA expression levels of proinflammatory cytokines surged in hyperosmotic-induced cells. A statistically significant difference (*P* < 0.0001) was observed in mRNA expression levels of all the proinflammatory cytokines (TNF-α, IL-1β, IL-6, IL-17 A, MMP-9, and ICAM-1) between cells treated with and without 90 mM NaCl (Figs. 4, 5, 6, 7, 8 and 9).

Following the 6-h exposure to 90 mM NaCl, the cells were subsequently treated with curcuminoids and their analogs at varying concentrations (1, 10, and 100 µM) over 24 h. The data revealed that both curcuminoids and their analogs suppressed the mRNA expression levels of proinflammatory cytokines (TNF-α, IL-1β, IL-6, IL-17 A, MMP-9, and ICAM-1) instigated by hyperosmoticity, in comparison to the hyperosmotic-induced cells (Figs. 4, 5, 6, 7, 8 and 9).

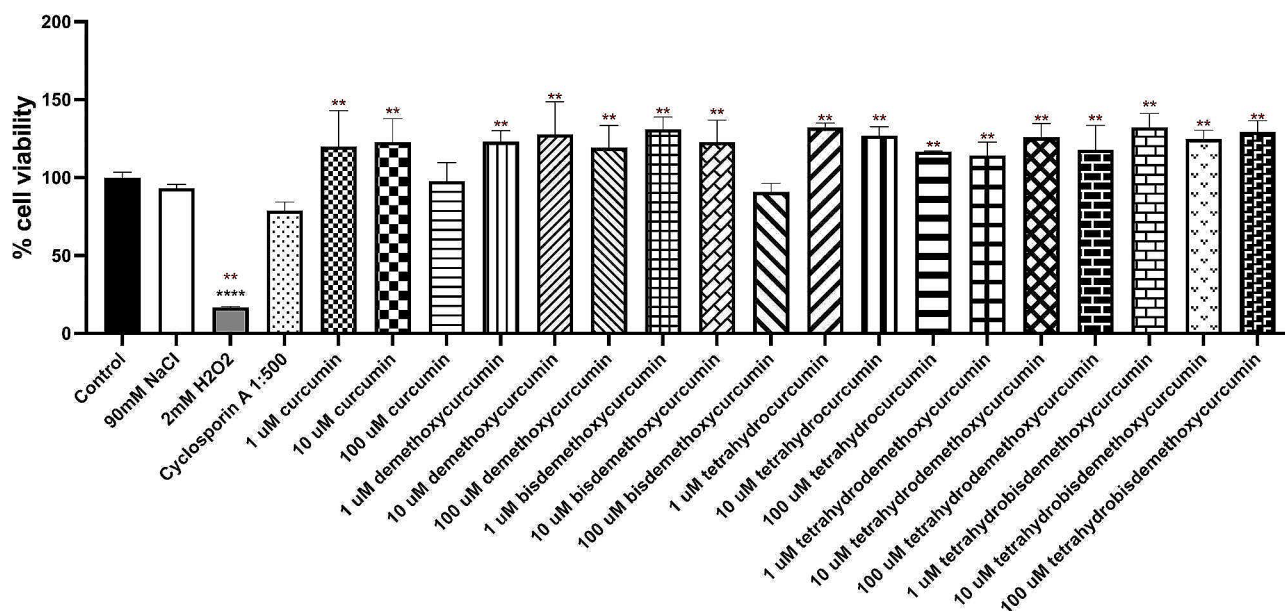


Fig. 3 Effect of curcuminoids and curcuminoid analogs on primary human corneal limbal epithelial cells at concentrations of 1, 10, and 100 μM over 24 h. Cell viability was determined using PrestoBlue™ cell viability reagent. ** $P < 0.0001$, compared with the control group; and * $P < 0.05$, compared with the cyclosporin A 1:500 group

Furthermore, the concentrations of all proinflammatory cytokines treated with 1 μM curcumin, 10 μM curcumin, 100 μM curcumin, 100 μM BDC, 100 μM THC, 1 μM THDC, and 100 μM THDC were not significantly different from those treated with cyclosporin A at a 1:500 ratio. However, certain proinflammatory cytokine levels from cells treated with 1 μM DC, 10 μM DC, 100 μM DC, 1 μM BDC, 10 μM BDC, 1 μM THC, 10 μM THC, 10 μM THDC, 1 μM THBDC, 10 μM THBDC, and 100 μM THBDC were significantly different compared to levels treated by cyclosporin A at a 1:500 ratio (Figs. 4, 5, 6, 7, 8 and 9). The average mRNA expression level of proinflammatory cytokines in each group is presented in Tables S3–S8.

Discussion

In this study, curcumin, DC, BDC, THC, THDC, and THBDC at concentrations of 1, 10, and 100 μM demonstrated no influence on cell viability. All curcuminoids and their analogs at these concentrations significantly reduced TNF- α , IL-1 β , IL-6, IL-17 A, MMP-9, and ICAM-1 mRNA expression in primary human corneal limbal epithelial cells when stimulated by 90 mM NaCl. This was in comparison to the cytokine mRNA expressions of cells stimulated by 90 mM NaCl without the application of any curcuminoid or its analog. Furthermore, the levels of all proinflammatory cytokines from hyperosmotic cells treated with various concentrations of curcuminoids and their analogs were insignificantly different from those treated with cyclosporin A at a 1:500 ratio.

In previous studies, curcumin and THC, at concentrations ranging from 0.01 to 100 $\mu\text{g}/\text{mL}$, exhibited over 70% cell viability, which decreased with increasing concentrations [5]. Conversely, Chen et al. reported that curcumin concentrations below 30 μM had no effect on cell viability. However, a concentration of 50 μM significantly diminished cell viability [15]. In our study, all curcuminoids and their analogs at 1, 10, and 100 μM retained a cell viability of over 90%. In contrast, cyclosporin A at 1:500 had a lower cell viability (78.97%) than most of the curcuminoids and their analogs, with the exception of 100 μM curcumin and 100 μM BDC. These results confirm that curcuminoid analogs, up to 100 μM , are safer for primary human corneal limbal epithelial cells than cyclosporin A at 1:500.

Stimulating primary human corneal limbal epithelial cells in a hyperosmolarity environment (achieved by adding NaCl) is known to trigger the release of numerous inflammatory cytokines [15]. Our study determined that 90 mM NaCl places these cells in an inflammatory state, resulting in elevated expressions of TNF- α , IL-1 β , IL-6, IL-17 A, MMP-9, and ICAM-1 mRNA compared to cells not subjected to a hyperosmolarity environment.

Inflammation plays a pivotal role in the perpetuating cycle of DED. TNF- α , IL-1 β , IL-17, and MMP-9 are inflammatory cytokines stimulated by tear hyperosmolarity. These cytokines attract more inflammatory cells to the ocular surface, causing damage to the surface epithelial and goblet cells. This damage exacerbates tear hyperosmolarity, initiating the DED cycle. Both adaptive and innate defense mechanisms are implicated in

Table 1 Cell viability of cyclosporin A 0.05% 1:500 compared with each analog

Tukey's multiple comparisons test	Mean difference	95% CI of difference	Adjusted P-value
2 mM H ₂ O ₂ vs. cyclosporin A 1:500	-62.17	-95.46 to -28.87	< 0.0001
cyclosporin A 1:500 vs. 1 μm curcumin	-41.09	-74.38 to -7.79	0.0040
cyclosporin A 1:500 vs. 10 μm curcumin	-43.91	-77.20 to -10.62	0.0015
cyclosporin A 1:500 vs. 100 μm curcumin	-18.87	-52.16 to 14.43	0.8378
cyclosporin A 1:500 vs. 1 μm DC	-44.20	-77.50 to -10.91	0.0013
cyclosporin A 1:500 vs. 10 μm DC	-48.85	-82.15 to -15.56	0.0002
cyclosporin A 1:500 vs. 100 μm DC	-40.33	-73.62 to -7.04	0.0052
cyclosporin A 1:500 vs. 1 μm BDC	-52.03	-85.32 to -18.73	< 0.0001
cyclosporin A 1:500 vs. 10 μm BDC	-43.93	-77.22 to -10.63	0.0015
cyclosporin A 1:500 vs. 100 μm BDC	-11.98	-45.28 to 21.31	0.9983
cyclosporin A 1:500 vs. 1 μm THC	-53.21	-86.51 to -19.92	< 0.0001
cyclosporin A 1:500 vs. 10 μm THC	-48.04	-81.33 to -14.75	0.0003
cyclosporin A 1:500 vs. 100 μm THC	-37.84	-71.13 to -4.54	0.0120
cyclosporin A 1:500 vs. 1 μm THDC	-35.35	-68.64 to -2.05	0.0268
cyclosporin A 1:500 vs. 10 μm THDC	-47.01	-80.30 to -13.71	0.0005
cyclosporin A 1:500 vs. 100 μm THDC	-39.00	-72.30 to -5.71	0.0081
cyclosporin A 1:500 vs. 1 μm THBDC	-53.32	-86.61 to -20.02	< 0.0001
cyclosporin A 1:500 vs. 10 μm THBDC	-45.86	-79.16 to -12.57	0.0007
cyclosporin A 1:500 vs. 100 μm THBDC	-50.61	-83.90 to -17.31	0.0001

CI=confidence interval, DC=demethoxycurcumin, BDC=bisdemethoxycurcumin, THC=tetrahydrocurcumin, THDC=tetrahydrodemethoxycurcumin, and THBDC=tetrahydrobisdemethoxycurcumin

DED pathology. The innate defense mechanism involves stimulation of pattern recognition receptors, which is associated with increased IL-1, TNF- α , and IL-6 on the ocular surface [16]. A meta-analysis confirmed the presence of IL-17 A, IL-6, IL-1 β , and TNF- α in the tears of patients with DED [17]. ICAM-1, an adhesion molecule, binds to inflammatory cells expressing integrin leukocyte functional antigen 1 (LFA-1). This binding is essential for recruiting inflammatory cells to the ocular surface in the DED cycle [16]. To disrupt this cycle, reducing inflammatory cytokines and ICAM-1 has proven effective in

treatments such as topical corticosteroids, cyclosporin, and LFA-1 antagonists. However, topical corticosteroids come with side effects, including ocular hypertension, cataracts, and opportunistic infections [8]. Topical cyclosporin can cause ocular pain, redness, and eyelid swelling [18]. Additionally, topical LFA-1 may lead to site irritation, reactions, and dysgeusia [19]. Consequently, there is an ongoing search for novel topical drugs.

The anti-inflammatory activity of curcumin and THC has been established both in vitro and in vivo in numerous studies [5, 15, 20–26]. Curcumin was reported to reduce TNF- α , IL-1 β , and MMP-9 in cultured rat corneal epithelial cells [20]. Furthermore, a study that examined the anti-inflammatory action of curcumin in cultured human corneal epithelial cells found that 5 μM curcumin could suppress IL-1 β , IL-6, and TNF- α proteins [15]. THC has been shown to decrease TNF- α and IL-1 β in mouse splenocytes compared to curcumin [5]. Moreover, THC significantly reduced IL-17 A in allergic asthmatic mice, whereas curcumin did not show a similar effect [23]. THC also reduced TNF- α , IL-1 β , and IL-6 in mice [21], IL-1 β and MMP-9 in astrocytes and the hypoxic cerebrum of mice [24], IL-1 β , IL-6, and TNF- α in the articular cartilage of estrogen-deficient rats [25], and TNF- α in high-fat diet obese mice [26].

According to our study, all concentrations of yellow curcuminoid (curcumin, DC, and BDC) significantly reduced the expression of TNF- α , IL-1 β , IL-6, IL-17 A, MMP-9, and ICAM-1 mRNA under hyperosmotic conditions compared to untreated conditions. All curcuminoid analogs, including THC, THDC, and THBDC, which are colorless, also markedly suppressed TNF- α , IL-1 β , IL-6, IL-17 A, MMP-9, and ICAM-1 mRNA expression. Additionally, 1 μM curcumin, 10 μM curcumin, 100 μM curcumin, 100 μM BDC, 100 μM THC, 1 μM THDC, and 100 μM THDC reduced all inflammatory cytokine and ICAM-1 expression levels, which were not significantly different from those inhibited by cyclosporin A 1:500. This study is the first to report the anti-inflammatory effects of DC, BDC, THC, THDC, and THBDC in cultured human corneal limbal epithelial cells compared to the anti-inflammatory effects of cyclosporin A 1:500, an approved treatment for DED.

However, curcuminoids have a yellow color, exhibit low solubility, permeability, and absorption, and are rapidly metabolized with swift elimination from the body [27]. Preparing curcuminoid substances in liquid or eye drop form is challenging. Several formulation-enhancing drug delivery systems, such as nanoparticles, liposomes, micelles, and phospholipid complexes [27, 28], have been employed to maximize the benefits of curcuminoids in medicine. Another approach to addressing the pharmacokinetic challenges of curcuminoids involves using analogs [6], such as THC, THDC, THBDC,

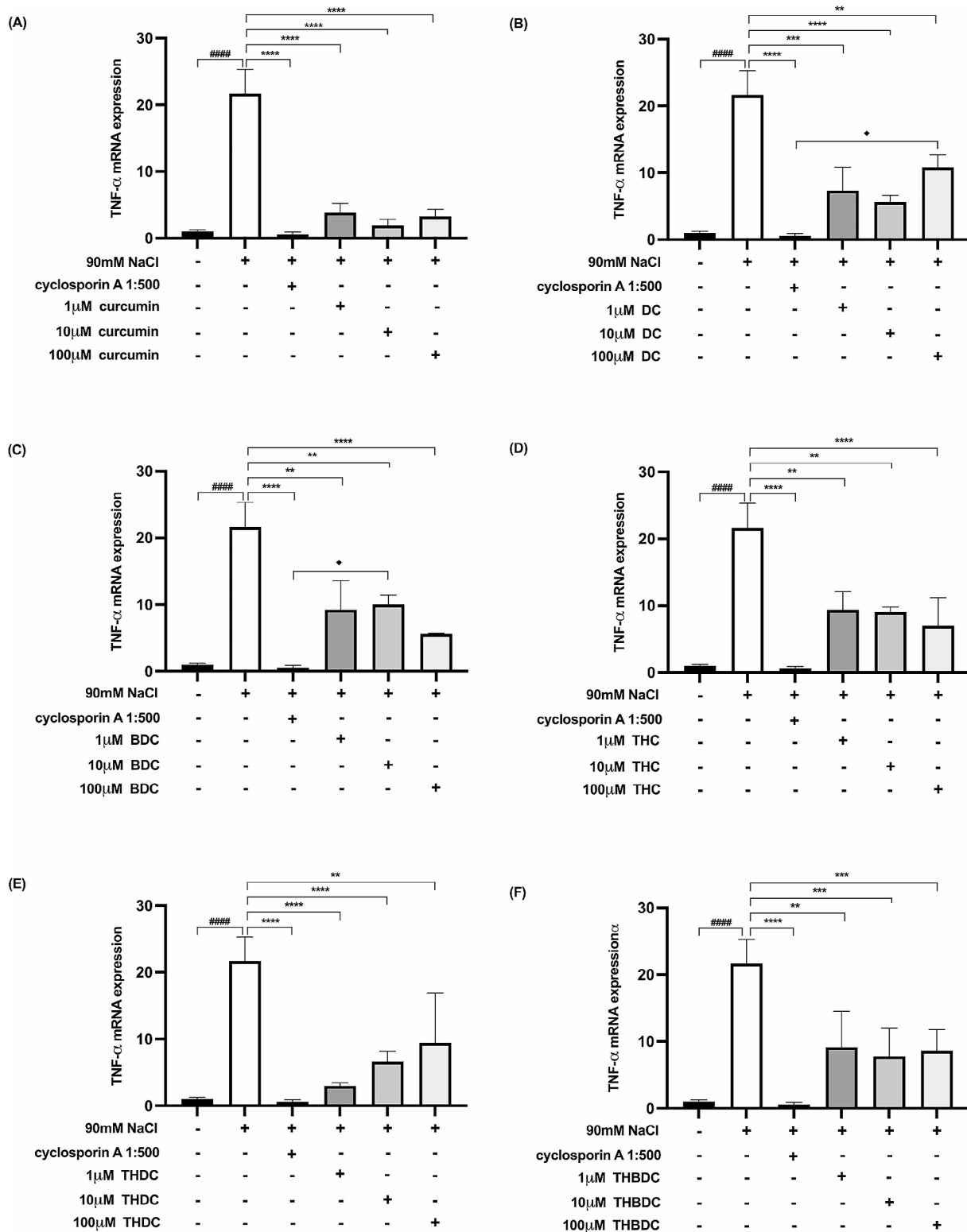


Fig. 4 Effect of curcuminoids and curcuminoid analogs on TNF- α mRNA expressions in hyperosmotic-induced human corneal limbal epithelial cells. After 90mM NaCl exposure for 6 h, TNF- α mRNA expression levels were inhibited by (A) curcumin, (B) demethoxycurcumin (DC), (C) bisdemethoxycurcumin (BDC), (D) tetrahydrocurcumin (THC), (E) tetrahydrodemethoxycurcumin (THDC), and (F) tetrahydrobisdemethoxycurcumin (THBDC). mRNA expression levels were assessed using quantitative RT-PCR. #### $P < 0.0001$ compared to control. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ compared to NaCl alone. ♦ $P < 0.05$ compared to NaCl+cyclosporin A

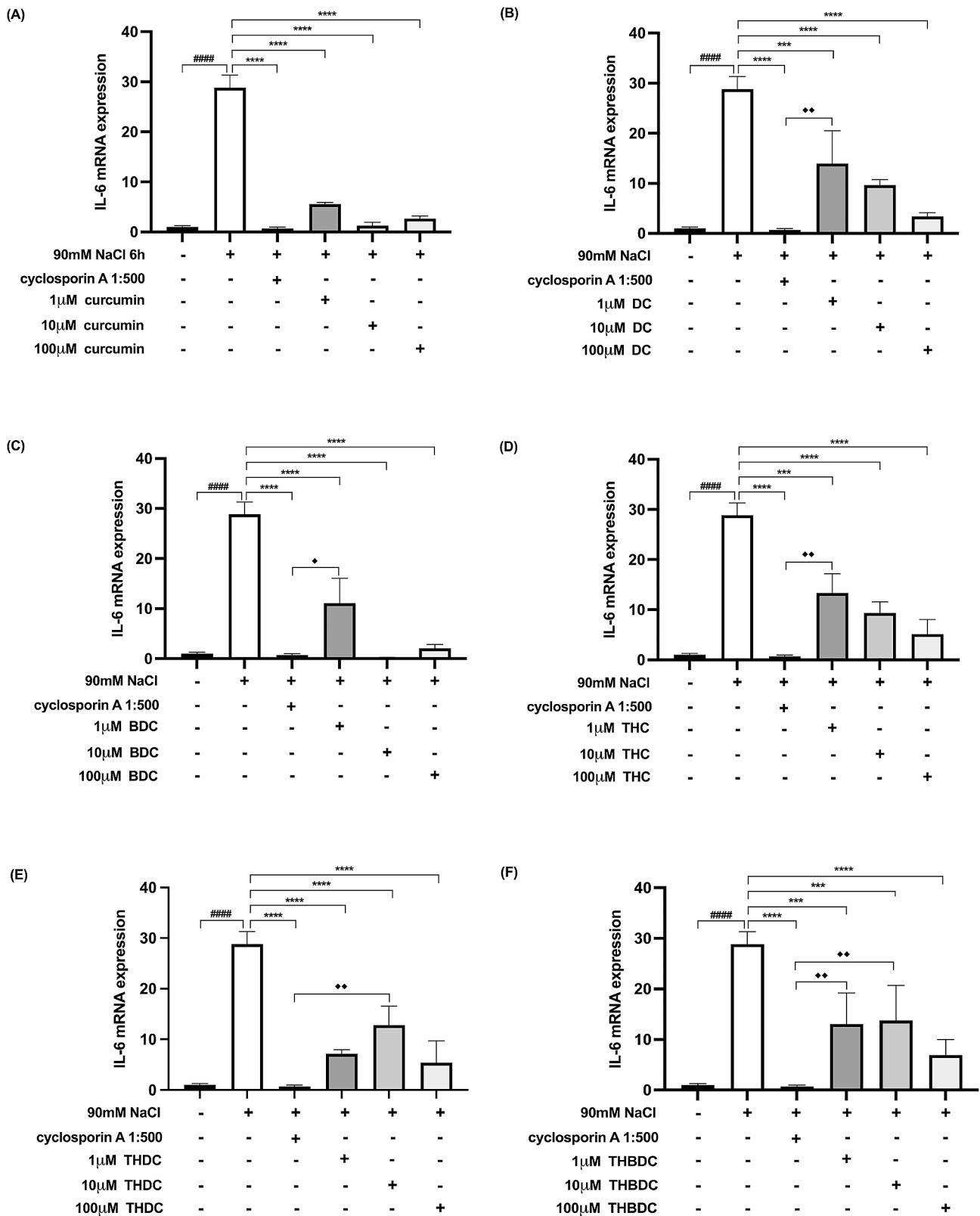


Fig. 5 Effect of curcuminoids and curcuminoid analogs on IL-6 mRNA expressions in hyperosmotic-induced human corneal limbal epithelial cells. After 90mM NaCl exposure for 6 h, IL-6 mRNA expression levels were inhibited by (A) curcumin, (B) demethoxycurcumin (DC), (C) bisdemethoxycurcumin (BDC), (D) tetrahydrocurcumin (THC), (E) tetrahydrodemethoxycurcumin (THDC), and (F) tetrahydrobisdemethoxycurcumin (THBDC). mRNA expression levels were assessed using quantitative RT-PCR. ####*P*<0.0001 compared to control. ****P*<0.001, *****P*<0.0001 compared to NaCl alone. ♦*P*<0.05, ♦♦*P*<0.01 compared to NaCl+cyclosporin A

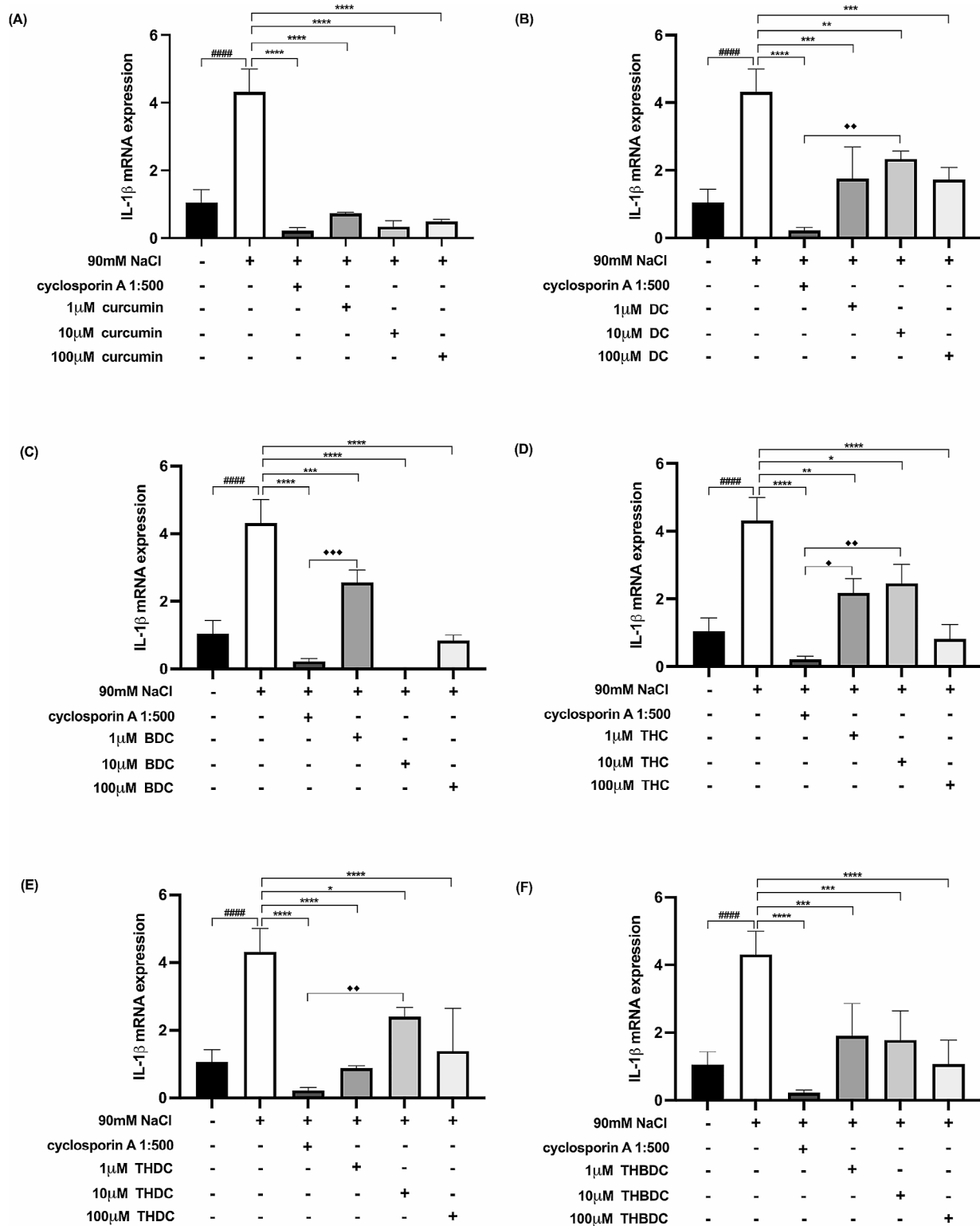


Fig. 6 Effect of curcuminoids and curcuminoid analogs on IL-1β mRNA expressions in hyperosmotic-induced human corneal limbal epithelial cells. After 90mM NaCl exposure for 6 h, IL-1β mRNA expression levels were inhibited by (A) curcumin, (B) demethoxycurcumin (DC), (C) bisdemethoxycurcumin (BDC), (D) tetrahydrocurcumin (THC), (E) tetrahydrodemethoxycurcumin (THDC), and (F) tetrahydrobisdemethoxycurcumin (THBDC). mRNA expression levels were assessed using quantitative RT-PCR. ####*P* < 0.0001 compared to control. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 compared to NaCl alone. ♦*P* < 0.05, ♦♦*P* < 0.01 compared to NaCl + cyclosporin A

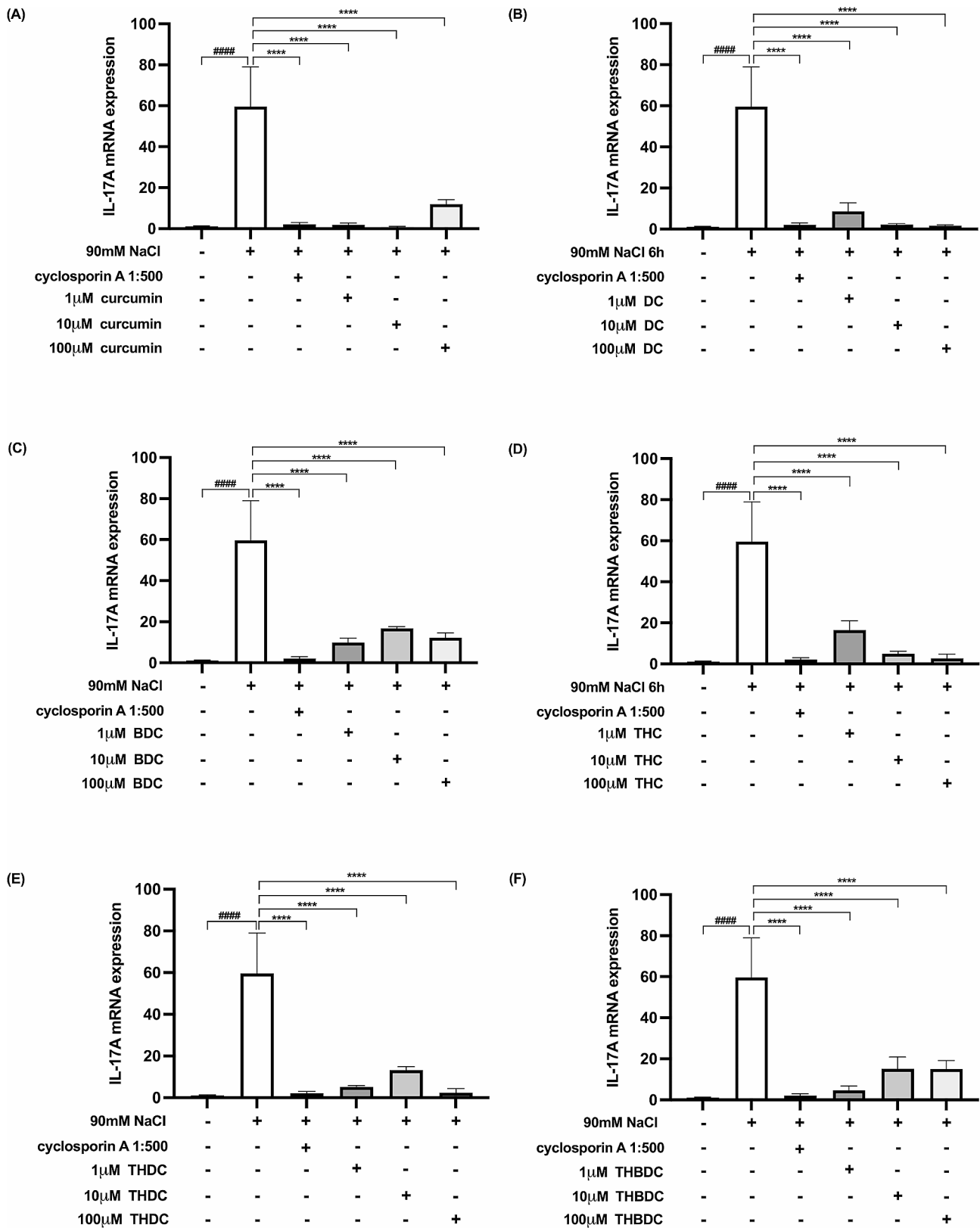


Fig. 7 Effect of curcuminoids and curcuminoid analogs on IL-17 A mRNA expressions in hyperosmotic-induced human corneal limbal epithelial cells. After 90mM NaCl exposure for 6 h, IL-17 A mRNA expression levels were inhibited by (A) curcumin, (B) demethoxycurcumin (DC), (C) bisdemethoxycurcumin (BDC), (D) tetrahydrocurcumin (THC), (E) tetrahydrodemethoxycurcumin (THDC), and (F) tetrahydrobisdemethoxycurcumin (THBDC). mRNA expression levels were assessed using quantitative RT-PCR. #### $P < 0.0001$ compared to control. **** $P < 0.0001$ compared to NaCl alone

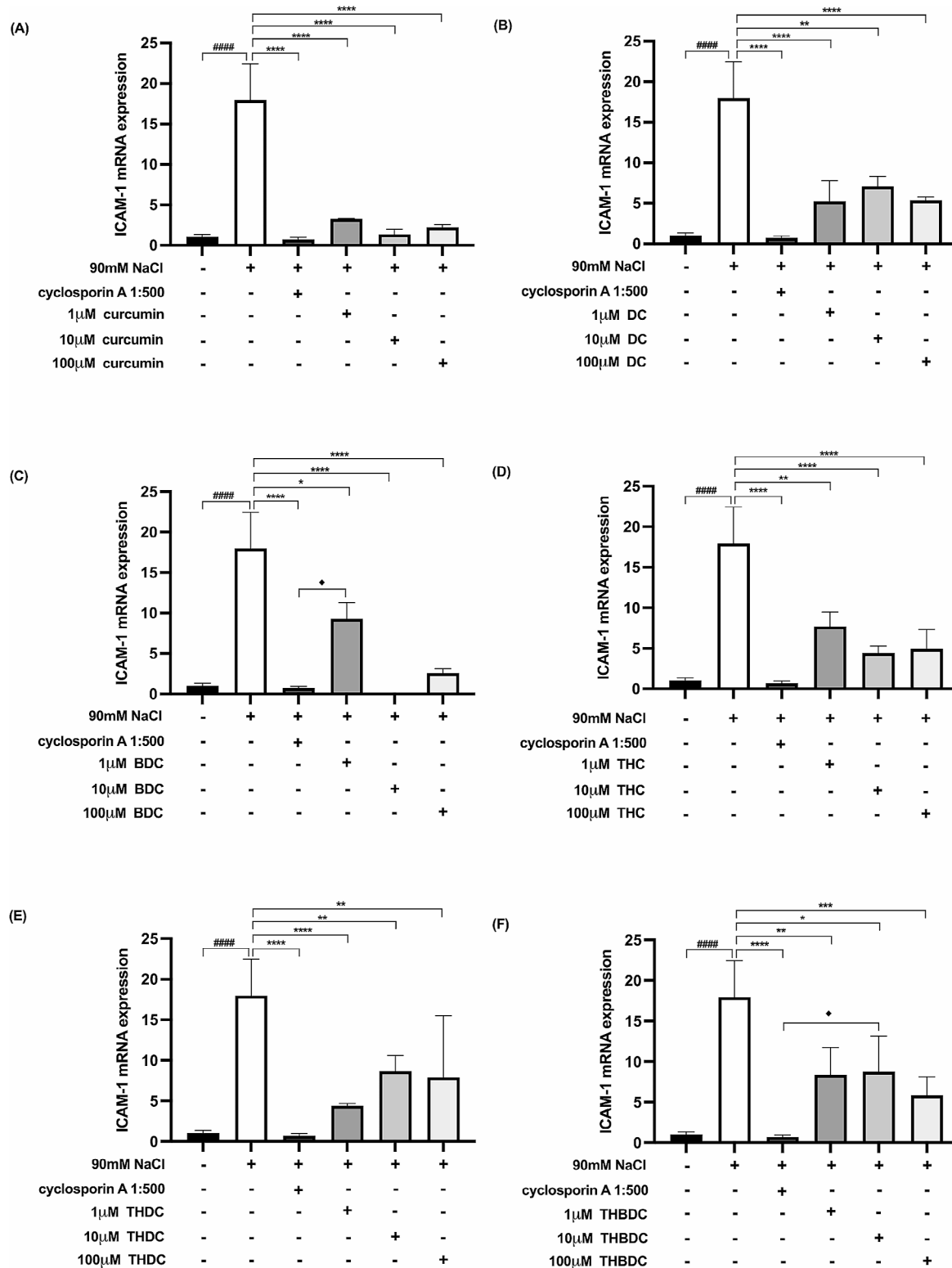


Fig. 8 Effect of curcuminoids and curcuminoid analogs on ICAM-1 mRNA expressions in hyperosmotic-induced human corneal limbal epithelial cells. After 90mM NaCl exposure for 6 h, ICAM-1 mRNA expression levels were inhibited by (A) curcumin, (B) demethoxycurcumin (DC), (C) bisdemethoxycurcumin (BDC), (D) tetrahydrocurcumin (THC), (E) tetrahydrodemethoxycurcumin (THDC), and (F) tetrahydrobisdemethoxycurcumin (THBDC). mRNA expression levels were assessed using quantitative RT-PCR. #### $P < 0.0001$ compared to control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ compared to NaCl alone. * $P < 0.05$ compared to NaCl+cyclosporin A

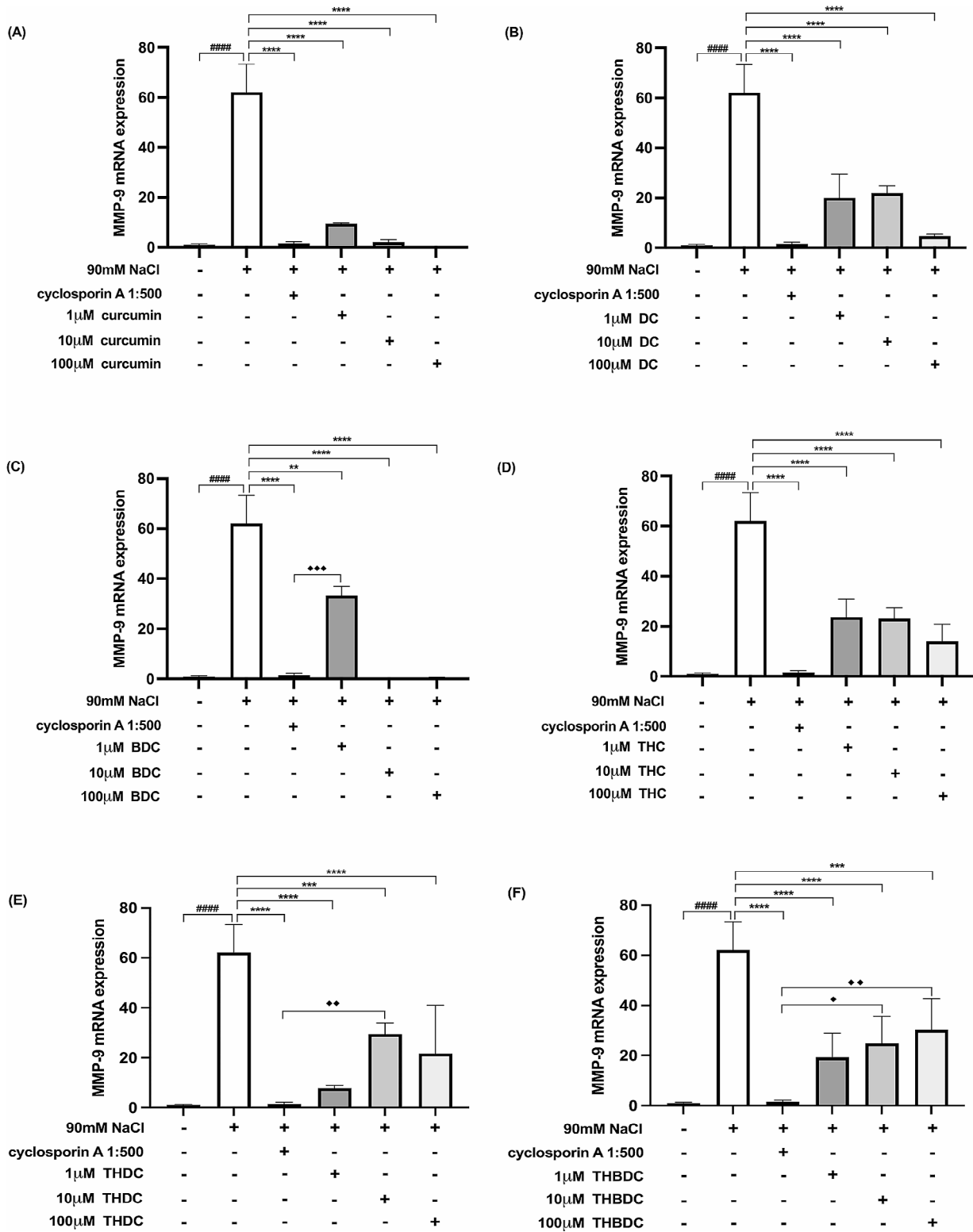


Fig. 9 Effect of curcuminoids and curcuminoid analogs on MMP-9 mRNA expressions in hyperosmotic-induced human corneal limbal epithelial cells. After 90mM NaCl exposure for 6 h, ICAM-1 mRNA expression levels were inhibited by (A) curcumin, (B) demethoxycurcumin (DC), (C) bisdemethoxycurcumin (BDC), (D) tetrahydrocurcumin (THC), (E) tetrahydrodemethoxycurcumin (THDC), and (F) tetrahydrobisdemethoxycurcumin (THBDC). mRNA expression levels were assessed using quantitative RT-PCR. #### $P < 0.0001$ compared to control. *** $P < 0.01$, ** $P < 0.001$, **** $P < 0.0001$ compared to NaCl alone. ♦ $P < 0.05$, ♦♦ $P < 0.01$, ♦♦♦ $P < 0.001$ compared to NaCl + cyclosporin A

dihydrocurcumin, hexahydrocurcumin, octahydrocurcumin, hexahydrodemethoxycurcumin, octahydrodemethoxycurcumin, hexahydrobisdemethoxycurcumin, and octahydrobisdemethoxycurcumin, through structural motif modifications [5]. Compared to curcumin, THC is more stable, hydrophilic, soluble at physiological pH, possesses a longer half-life in plasma at 37 °C, and is more absorbable in the intestines with sustained stability in plasma [29, 30]. In this study, aside from curcumin and BDC, which are yellow, 100 µM THC and 1 and 100 µM THDC, which are colorless, effectively suppressed inflammatory mRNA with higher cell viability than cyclosporin A 1:500. However, 100 µM THC reduced TNF- α , MMP-9, and ICAM-1 mRNA expression slightly more than 100 µM THDC.

Due to the anti-inflammatory effect of 1 µM curcumin, 10 µM curcumin, 100 µM curcumin, 100 µM BDC, 100 µM THC, 1 µM THDC, and 100 µM THDC, which were similar to cyclosporin A 1:500, these agents could be used as a treatment of DED. Most of these agents had better cell viability than cyclosporin A 1:500 except 100 µM curcumin and 100 µM BDC. As a result, 1 µM curcumin, 10 µM curcumin, 100 µM THC, 1 µM THDC, and 100 µM THDC needs to be studied further as a treatment for DED.

This study acknowledges certain limitations. We did not investigate the solubility, stability, and absorbability of curcumin analogs. Future bioavailability studies are recommended before their application in eye drop form for patients.

In conclusion, 1 and 10 µM curcumin, 100 µM THC, and 1 µM and 100 µM THDC can suppress TNF- α , IL-1 β , IL-17 A, IL-6, MMP-9, and ICAM-1 mRNA expression in hyperosmotic stage-induced primary human corneal limbal epithelial cells as effectively as cyclosporin A 1:500 but with higher cell viability. Moreover, 100 µM curcumin and 100 µM BDC can suppress those inflammatory cytokines; however, the resulting cell viability is not significantly different from cyclosporin A 1:500. Aside from yellow curcumin and BDC, colorless 100 µM THC and 1 and 100 µM THDC have potential as novel eye drop treatments.

Abbreviations

BDC	Bisdemethoxycurcumin
THC	Tetrahydrocurcumin
THDC	Tetrahydrodemethoxycurcumin
THBDC	Tetrahydrobisdemethoxycurcumin
ANOVA	Analysis of Variance
TNF	Tumor Necrosis Factor
MMP	Matrix Metalloproteinase
ICAM	Intercellular Adhesion Molecule
DED	Dry Eye Disease
TLC	Thin Layer Chromatography
DMEM	Dulbecco's Modified Eagle Medium
PBS	Phosphate-Buffered Saline
EDTA	Ethylenediaminetetraacetic Acid
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
LFA	Lymphocyte Function-associated Antigen

Supplementary Information

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Supplementary Material 1

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Author contributions

NK, UR and AS: conception and design; NK, LU and NS: Data analysis and interpretation and manuscript drafting; AmS, NS, WC and ApS: Data acquisition. All authors wrote manuscript, reviewed and approved the final version of the manuscript.

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Data availability

The datasets generated/analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

All methods utilized in this investigation, including the *Curcuma longa* plant, were performed in accordance with the relevant institutional, national, and international guidelines and legislation.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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