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Anti-inflammatory effects of new catechin derivatives in a hapten-induced mouse contact dermatitis model

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Abstract

Contact dermatitis is a common skin disease, with various treatments available to dermatologists. According to general guidelines, the first line of treatment involves topical steroids; however, this treatment has application-site restrictions in order to avoid adverse cutaneous events. Accordingly, increased demand exists for the development of new treatments. In Japan, the recent use of catechin-containing health foods and their beneficial effects has attracted attention. Indeed, several studies have examined the anticancer, anti-obesity, anti-inflammatory, and antioxidant effects of catechins. In this study, we synthesized planar catechin (PC) from natural (+)-catechin, and further chemically modified it with the intent to clarify the anti-inflammatory and antioxidant effects of new catechin derivatives. Methylate-PC (methyl PC) and acetylate-PC (acetyl PC) were modified to increase lipid solubility. Their antioxidant effects were examined with electron spin resonance by evaluating the ability to remove hydroxyl radicals. *In vitro*, the antioxidant effects were in the order of PC > (+)-catechin > acetyl PC > methyl PC. In addition, we used a 1-fluoro-2,4-dinitrobenzene (DNFB)-induced allergic contact dermatitis model in BALB/c mice. Our results demonstrated that catechin derivatives inhibited ear swelling induced by DNFB, with acetyl PC demonstrating a greater inhibitory effect than PC and methyl PC. Moreover, acetyl PC downregulated the mRNA levels of inflammatory cytokines, including tumor necrosis factor- α , interleukin (IL)-1 β , and IL-4, as well as myeloperoxidase activity, in the ear tissue of DNFB-treated mice. Collectively, our novel findings suggest that catechin derivatives may be a promising new choice for the treatment of contact dermatitis.

Keywords : contact dermatitis, catechin derivative, planar catechin, anti-inflammation, cytokine

1. Introduction

Allergic contact dermatitis is a common clinical skin disease, mainly caused by cosmetics and personal care products, which account for 60%–70% of contact dermatitis cases (Ito et al., 2017). The first line of treatment includes topical steroids (Brasch et al., 2014); however, this treatment is restricted in its application sites to avoid additional adverse cutaneous events, such as atrophy and telangiectasia. Additionally, using external steroids by pregnant women may result in low birth weight infants (Chi CC et al., 2013). Therefore, additional treatment options are warranted. Catechins, which are found in green tea, have garnered increased attention for their beneficial properties. The principal catechins include (+)-catechin, (+)-galliccatechin (GC), (–)-epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epicatechin gallate (ECg), and (–)-epigallocatechin gallate (EGCg).

Particularly, EGCg has various bioactivities, including anticancer, -inflammatory, and -oxidant effects (Hashimoto et al., 2003; Matsuo et al., 1997; Thichanpiang and Wongprasert, 2015). EGCg's anti-inflammatory effects include histamine release inhibition and apoptosis induction in monocytes (Kawai et al., 2005; Maeda-Yamamoto et al., 2012). Given the diverse physiological activities, the gallate group is considered important for bioprocess modulation. EC, which does not have a galloyl moiety, is used to inhibit mouse type IV allergies. Thus, flavanol/flavane structures may be important modulators of EC's inhibitory effects in these allergies (Suzuki et al., 2000). However, few studies have investigated this phenomenon. We focused on the flavanol structure of catechins and synthesized planar catechins (PC) in which the natural (+)-catechin was bridged in the chroman structure by a Fenton reaction using acetone (Fig. 1). The ortho-dihydroxy functionality of the catechol ring is responsible for the radical-scavenging activity of (+)-catechin, which can be modulated by introducing electron-donating or -withdrawing substituents to the catechol ring. Previous studies have reported that PC has radical-scavenging activity that is approximately 5-fold more than that of (+)-catechin (Fukuhara et al., 2002, 2003, 2009; Hakamata et al., 2006). We

synthesized PC from natural (+)-catechin and introduced further chemical modifications to evaluate their properties. Methylate-PC (methyl PC) and acetylate-PC (acetyl PC) were modified to increase their lipid solubility; their anti-inflammatory effects were also examined (Fig. 1). Reactive oxygen species produced by ultraviolet rays or environmental pollutants have been implicated in the etiology of dermatosis (Bickers and Athar, 2006; Honda et al., 2013). In contact dermatitis, reactive oxygen species is associated with immunization reactions such as those involving dendritic cells and keratinocytes. In one study using the strong antioxidant N-acetylcysteine, hexavalent chromium-induced hypersensitivity suppression through cytokine expression inhibition was reported (Lee et al., 2015). Inflammatory cytokines, such as tumor necrosis factor- α (TNF- α) and interleukin (IL)-1 β , are closely associated with contact dermatitis exacerbation. Here, we induced allergic contact hypersensitivity (CHS) by administering haptens to examine the effects of synthesized compounds on contact dermatitis *in vivo*. Moreover, we also investigated TNF- α , IL-1 β , IL-4, and IL-6 mRNA levels in 1-fluoro-2,4-dinitrobenzene (DNFB)-induced mouse ear tissue. We found that the therapeutic effect of catechin derivatives on contact dermatitis may be related to their anti-inflammatory action. Thus, catechin derivative-based treatments may be a promising therapeutic approach for contact dermatitis.

2. Materials and methods

2.1. Synthesis

(+)-Catechin (Sigma-Aldrich Corporation, St. Louis, MO, USA) was purified by recrystallization from EtOAc-Et₂O.

(6aS,12aR)-5,5-dimethyl-5,6a,7,12a-tetrahydroisochromeno[4,3-b]chromene-2,3,8,10-tetraol (PC) was synthesized according to our previously reported procedure (Hakamata et al., 2006). The purity of all compounds was measured using ¹H nuclear magnetic resonance (NMR) and was found to be

>95%. To obtain

(6a*S*,12a*R*)-5,5-dimethyl-5,6a,7,12a-tetrahydroisochromeno[4,3-*b*]chromene-2,3,8,10-tetraol tetraacetate (acetyl PC), we prepared a solution of 330 mg of 1 mmol PC in 5 ml of acetic anhydride, 2 ml of pyridine, and dimethylaminopyridine (12 mg, 0.1 mmol) under argon. The solvent was removed in vacuo, and the residue was dissolved with ethyl acetate and washed with 2 × 10 ml of brine, dried over Na₂SO₄, and evaporated under reduced pressure. The residue was subjected to column chromatography with ethyl acetate/toluene in the ratio of 2:1 as eluent to obtain 260 mg of acetyl PC (white solid; yield, 67%). The ¹H NMR (400 MHz, dimethyl sulfoxide-*d*₆ [DMSO-*d*₆]) characterization data were as follows: δ 1.49 (s, 3H), 1.54 (s, 3H), 2.24 (s, 3H), 2.27 (s, 3H), 2.28 (s, 3H), 2.31 (s, 3H), 2.51 (dd, 1H, *J* = 10.0 Hz, *J* = 15.6 Hz), 2.92 (dd, 1H, *J* = 5.6 Hz, *J* = 15.6 Hz), 4.02 (ddd, 1H, *J* = 5.6 Hz, *J* = 9.2 Hz, *J* = 10.0 Hz), 4.78 (d, 1H, *J* = 9.2 Hz), 6.64 (d, 1H, *J* = 2.4 Hz), 6.73 (d, 1H, *J* = 2.4 Hz), 7.28 (s, 1H), 7.41 (s, 1H); [M+H]⁺ 499, high-resolution mass spectrometry [HRMS, electrospray ionization (ESI)][M]⁺ found 498.1516 calcd for C₂₆H₂₆O₁₀ 498.1526. To obtain

(6a*S*,12a*R*)-2,3,8,10-tetramethoxy-5,5-dimethyl-5,6a,7,12a-tetrahydroisochromeno[4,3-*b*]chromene (methyl PC), we dissolved 330 mg of 1 mmol PC in dry tetrahydrofuran (THF) and added 176 mg of 4.4 mmol NaH at 0°C under argon. Thereafter, we added methyl iodide, and the reaction mixture was heated at reflux for 12 h. The mixture was allowed to cool, following which the solvent was removed in vacuo, and the residue was dissolved with 100 ml of ethyl acetate and washed with 2 × 10 ml of brine, dried over Na₂SO₄, and evaporated under reduced pressure. The residue was subjected to column chromatography with ethyl acetate/toluene in the ratio of 1:1 as eluent to obtain 210 mg of methyl PC (white solid; yield, 42%). The ¹H NMR (400 MHz, DMSO-*d*₆) characterization data were as follows: δ 1.47 (s, 3H), 1.56 (s, 3H), 2.39 (dd, 1H, *J* = 10.8 Hz, *J* = 15.6 Hz), 2.87 (dd, 1H, *J* = 5.6 Hz, *J* = 15.6 Hz), 3.72 (s, 3H), 3.75 (s, 3H), 3.77 (s, 3H), 3.79 (s, 3H),

3.84 (ddd, 1H, $J = 5.6$ Hz, $J = 8.8$ Hz, $J = 10.8$ Hz), 4.54 (d, 1H, d, $J = 8.8$ Hz), 6.17 (d, 1H, $J = 2.4$ Hz), 6.18 (d, 2H, $J = 2.4$ Hz), 6.80 (s, 1H), 7.06 (s, 1H); $[M+H]^+$ 387, HR-MS (ESI) $[M]^+$ found 386.4436 calcd for $C_{22}H_{26}O_6$ 386.4440.

2.2. Hapten-induced contact dermatitis in BALB/c mice

The mouse studies were approved by the Institutional Animal Care and Use Committees of the Showa University. Female BALB/c mice (6–12 weeks old) were purchased from Sankyo Labo Service Corporation, INC. The mice were housed at room temperature (25 ± 1 °C), with a relative humidity of $60\% \pm 10\%$, under a 12-h day and night cycle. The mice had free access to food and water. First, the mice were sensitized with 0.5% DNFB in acetone/olive oil (4/1, vol/vol) on the abdominal skin. After 5 days, the ventral surfaces of the ears were challenged by administration of 20 μ l of 0.3% DNFB to their dorsal and ventral surfaces. The ear swelling was measured for each mouse both before induction of contact dermatitis and at various time periods after the induction. These measurements were taken at a predetermined site using a micrometer. Differences in ear tissue thickness were interpreted as tissue swelling (Miki et al., 2013).

2.3. (+)-Catechin and catechin-derivative treatment groups

A topical steroid (0.05% betamethasone butyrate propionate [BBP]; Torii Pharmaceutical Co., Ltd., Japan) was used as the positive control. (+)-Catechin was purchased from Wako Pure Chemical Industries, Ltd. and mixed in acetone/olive (1/1, vol/vol) with Vaseline (Kozakai Pharmaceutical Co., Ltd.) at 1% (w/w). Catechin-derivative creams were prepared in the same manner. These creams were applied to both ears at 15 min and 18 h after the challenge. The experimental mice were randomly divided into three groups ($n = 12$) as follows: the vehicle group (DNFB-challenge plus Vaseline treatment), the (+)-catechin or catechin-derivative group

(DNFB-challenge plus drug treatment), and the DNFB+BBP group (DNFB-challenge plus 0.05% BBP treatment). The control group included the mice that did not receive the DNFB-challenge. The ear swelling inhibition ratio was calculated by the following formula: $\text{Vehicle-Sample}/\text{Vehicle} \times 100$ (cutoff untreated).

2.4. Histopathology

The mice were euthanized by cervical dislocation at 24 h after the challenge and the tissues were fixed with 10% neutral buffered formalin. Ear tissue sections were prepared by cutting at a position about 5 mm from the tip of the ear. Paraffin-embedded sections were sectioned (3 μm thick) and stained with hematoxylin and eosin. Next, we evaluated the auricular swelling using a microscope. The sections were consigned to the Biopathology Institute Co., Ltd.

2.5. Quantitative RT-PCR

Total RNA was extracted from ear tissues 24 h after the challenge by using TRIzol reagent (Invitrogen). First-strand cDNA synthesis was performed using a High Capacity cDNA Reverse transcription kit (Applied Biosystems). PCR reactions were performed using the Power SYBR Green PCR system (Applied Biosystems) with the Step One™ PCR system (Applied Biosystems). Primers were obtained from Sigma-Aldrich Co. LLC. The primer sequences of IL-4 and IL-6 were determined as previously described (Olivier et al., 2014; Yoon et al., 2015). Other primers were designed using primer Express Software Version 3.0 (Applied Biosystems). Table 1 lists the primer sets used. β -Actin was used as the internal control. All samples were normalized to the values of (+)-catechin or catechin derivative groups. Using the $\Delta\Delta\text{Ct}$ method, the results were expressed as relative fold changes of the threshold cycle (Ct) value relative to the vehicle group.

2.6. Myeloperoxidase (MPO) assay

The ear with induced inflammation was indirectly assayed for neutrophilic infiltration by the MPO assay (Szczepanik et al., 2000; Zemelka-Wiącek et al., 2013). The MPO assay kit was purchased from Abcam Ltd. The ears were removed 24 h post-challenge and 10 mg of the central part of the ear was obtained. The ear was homogenized on ice in 100 μ L of MPO assay buffer, centrifuged at 13,000 $\times$ g for 5 min at 4 $^{\circ}$ C, and the supernatant was collected. Reaction Mix (50 μ L) was added to the tissue sample of 50 μ L (the volume was adjusted to 50 μ L /well using assay buffer). The output at Ex/Em = 475/550 nm was measured on a microplate reader every 3 min for 30 min. One unit of MPO activity was considered the amount of MPO that oxidized the aminophenyl fluorescein substrate to generate 1.0 μ mol of fluorescein per minute at 25 $^{\circ}$ C.

2.7. Measurement of hydroxyl radical-scavenging activity using electron spin resonance (ESR)

Acetonitrile (30%) in water was used as the solvent for aliquots of 1 mmol/l samples. The spin-trapping solution was mixed with 160 μ L of 50 mmol/l phosphate buffer (pH 8.0), 30 μ L of 1 mmol/l diethylenetriaminepentaacetic acid, 30 μ L of 0.5 mmol/l ferrous sulfate solution, 5 μ L of 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), 30 μ L of 1 mmol/l hydrogen peroxide, and 50 μ L of 1 mmol/l sample solution. L-ascorbic acid (Wako Pure Chemical Industries, Ltd.) was used as the positive antioxidant control. The spin-trapping solution was added strictly within 1 min following the introduction of the ferrous sulfate solution. The reaction was conducted at room temperature. The reacted solution was subsequently placed within the ESR cell cavity and the spectrum was measured 40 s after the mixing step (Arakawa et al., 2004). The spectrum was obtained using a JES-FR30 ESR spectrometer (JEOL RESONANCE Inc.). The ESR measurement conditions were as follows: microwave power, 4 mW; modulation frequency, 9,420.0 MHz; magnetic field, 335.250 mT (width, 5.0×1 mT); field modulation, 100 kHz (width, 1.0×0.1 mT); modulation amplitude, 5.0×10 ; time constant, 0.03 s; and sweep time, 1 min. The ESR spectrum of the DMPO-OH spin adduct was also

recorded. The antioxidative activity of each sample was calculated according to the following equation: antioxidative activity (%) = $100 - (\text{signal intensity of DMPO-OH with sample} / \text{signal intensity of DMPO-OH} \times 100)$.

2.8. Statistical analysis

Data are expressed as mean S.E.M. or S.D.. Data were obtained using one-way analysis of variance followed by Tukey's test, where appropriate. Statistical significance was considered as $P < 0.05$.

3. Results

3.1. Hydroxyl radical-scavenging activity of the catechin derivatives

The hydroxyl radical-scavenging activity of catechin derivatives were measured to evaluate the *in vitro* activity of new catechin derivatives. The results of the elimination hydroxyl radical spectrum by hydrogen peroxide are shown in Fig. 2. The spectrum almost disappeared by the addition of ascorbic acid, which is a powerful antioxidant. Using a similar method, we added EGCg, (+)-catechin, PC, methyl PC, and acetyl PC in hydrogen peroxide and calculated the spectrum elimination rate for each compound. PC showed the highest antioxidant ability of the catechin derivatives for hydroxyl radicals. Acetyl PC and (+)-catechin showed similar responses, whereas methyl PC was found to have almost no antioxidant ability. Ascorbic acid, EGCg, and PC had significantly higher antioxidative activity than (+)-catechin ($P < 0.05$). However, other catechin derivatives did not demonstrate a significant difference when compared with (+)-catechin.

3.2. The effect of catechin derivatives on the induction of ear swelling in CHS mice

Our results of applying the treatment ointment sequentially at 15 min and 18 h after

induction are shown in Fig. 3. The ear swelling in mice was dependent on the time after the challenge. In (+)-catechin, we did not observe suppression of the ear swelling. Conversely, the ear swelling in PC-treated mice was significantly reduced compared with that in vehicle mice administered DNFB after 6 h; in methyl PC treated mice, the ear swelling reduced at 3, 6, and 18 h after the challenge. However, methyl PC did not inhibit the ear swelling 24 h after the challenge. For acetyl PC, suppression of the ear swelling was observed at 3 h after the challenge; the ear swelling continued to be suppressed until the 24-h endpoint (Fig. 3, B–E). A comparison between the effects of (+)-catechin, PC, methyl PC, and acetyl PC is shown in Table 2. The inhibition of the ear swelling by acetyl PC treatment was 62% and 50% at 6 h and 24 h, respectively, after the challenge. The results of the hematoxylin and eosin staining showed that the peak of the inflammation occurred 24 h after the challenge (Fig. 4). Acetyl PC inhibited the histopathological changes of acanthosis and dermis. In comparison with the untreated group, ear swelling and edema were found in the vehicle-administered mice group. Histopathology showed suppression of the ear swelling with methyl PC treatment 24 h after the challenge. Therefore, suppression of the ear swelling was found in the groups treated with PC, methyl PC, and acetyl PC compared with the vehicle-treated group.

3.3. Effect of topical application of catechin derivatives on cytokine mRNA expression in inflammatory regions determined by quantitative RT-PCR

Genes encoding for the inflammatory cytokine TNF- α , IL-1 β , IL-4, and IL-6, which are important in the induction of CHS, were measured by quantitative RT-PCR. In mice administered 0.3% DNFB, an increase in the mRNA levels of these four cytokines was observed 24 h after the challenge. Acetyl PC significantly inhibited the mRNA levels of three of the cytokines (TNF- α , IL-1 β , and IL-4) compared with the vehicle-administered group, whereas methyl PC was found to significantly inhibit the mRNA expression TNF- α and IL-1 β . Furthermore, the suppression of IL-1 β

and IL-4 was comparable in level to that induced by BBP, which is currently used for CHS treatment. PC was found to inhibit the expression of IL-6, but it had no effect on the other cytokines. The results showed that 3 h after the challenge, methyl PC inhibits TNF- α and acetyl PC inhibits IL-6. In addition, 9 h after the challenge, neither group showed suppression of the ear swelling.

3.4. Effect of the topical application of catechin derivatives on neutrophilic infiltration measure by the MPO assay

We indirectly measured neutrophil infiltration using the MPO assay to investigate the effect of catechin derivatives on these inflammatory cells (Fig. 6). Neutrophilic mobilization is indispensable to the invasion of T cells, which are the important inflammatory cells of CHS. Accordingly, we hypothesized that we could inhibit CHS if the neutrophil infiltration was decreased. MPO activity was observed 24 h after the challenge. MPO activity significantly increased in the vehicle-administered group treated with DNFB; significant suppression of MPO activity was found with the external application of the steroid medicine BBP. (+)-Catechin, PC, and acetyl PC were also found to significantly decrease the MPO activity whereas the catechin derivative methyl PC behaved similarly to the vehicle.

4. Discussion

This study showed for the first time the *in vivo* effect of catechin derivatives of (+)-catechin. In particular, acetyl PC inhibited DNFB-induced contact dermatitis in BALB/c mice. In addition, acetyl PC inhibited not only the clinical manifestations of dermatitis, such as erythema, edema, and flare, but also the histopathological changes of acanthosis and dermis (Fig. 4). In this study, we assumed that PC would be a lead compound since it was developed in reference to the structure of quercetin. Quercetin is known as a strong flavonoid-based antioxidant; however,

carcinogenicity at large doses has been reported *in vitro*. Although mutagenicity and carcinogenicity related to catechin has not been reported, the antioxidant properties of these compounds were not so high. As shown in Fig. 2, PC had higher antioxidant properties toward the hydroxyl radical than (+)-catechin. These results are similar to those of our previous analysis of the radical-scavenging activity using the galvinoxyl radical (Fukuhara et al., 2009; Hakamata et al., 2006). Furthermore, PC showed antioxidant properties not inferior to those of EGCg, which is known to have high antioxidant properties.

In this study, we synthesized methyl PC and acetyl PC for the purpose of improving their adsorption efficiency by the skin. PC, in which the geometry of (+)-catechin is constrained to be planar, shows strong antioxidative activity compared with (+)-catechin. Both compounds demonstrated difficulty in penetrating the cell membrane because of the hydrophilic property of hydroxyl groups in their catechol structure. Therefore, increasing lipophilicity by protecting the hydroxyl group of PC's catechol structure can improve the membrane permeability of both methyl PC and acetyl PC. In addition, their radical-scavenging activities toward hydroxyl radicals are weak because of the protection of catechol hydroxyl groups that are responsible for their antioxidative properties. Nevertheless, methyl PC and acetyl PC showed anti-inflammatory effects for CHS. Epicatechin-3-*O*-(3''-*O*-methyl)-gallate, included in 'Benifuuki', showed that the anti-allergic effects are higher than those of EGCg by inhibiting the activity of mast cells (Maeda-Yamamoto et al., 2012). Furthermore, Epicatechin-3-*O*-(3''-*O*-methyl)-gallate showed a depression effect stronger than that of EGCg in the application for mouse type IV allergy. Accordingly, this was believed to be associated with the stability of the *O*-methylated derivatives of EGCg (Suzuki et al., 2000; Yoshino et al., 2004). In addition, recent study succeeded in reinforcing the antiviral actions by artificially modifying the long-chain fatty acids in EGCg (Matsumoto et al., 2012). We showed that new catechin derivatives have beneficial effects for relieving dermatitis induced by the application of

DNFB (Figs. 3, 4). The ear swelling in EGCg-treated mice was significantly reduced compared with the vehicle group 24 h after the challenge. The inhibition rate was 19% (data not shown). Our results indicate that PC derivatives might have a high anti-inflammatory effect compared with EGCg. By preliminary research, the CHS model of ear swelling showed that 24 h was the peak of the second antigen invasion by applying DNFB to the mouse. This was possible to be measured by a micrometer (Asherson and Ptak, 1968). In our model, auricular swelling was observed, and significant auricular suppression was found with BBP treatment (Fig. 3B–E). The depression effect in the less than 9 h was high with catechin derivatives, particularly with acetyl PC, whereas auricular inhibition by the steroid medicine BBP was highest at 24 h. Glucocorticoid treatment of oxazolone-sensitized mice 1 h before re-challenge with oxazolone did not substantially affect the early response measured after 2 h, but substantially affected the response measured after 24 h. It has been reported that glucocorticoid treatment inhibits CHS through the suppression of macrophages and neutrophils infiltration but not that of leukocytes or keratinocytes of the first wave (Tuckermann et al., 2007). In contrast, catechin derivatives may serve to inhibit this early leukocytic infiltration that glucocorticoids do not affect. The hapten used to induce CHS causes a local inflammation response after administration within the first several hours (Enk and Katz, 1992). This early immune response shows an adjuvant effect for promoting T cells specific for the following allergen (Dudeck et al., 2011). A creation phase is then started by the hapten with re-exposure, and the IgM activates C5a within 1 h after CHS induction. C5a then binds to the C5a receptor and activates platelet and mast cells. Mast cells release TNF- α and serotonin. These vasoactive mediators stimulate local post capillary venules, thereby inducing the expression of adhesion molecules such as ICAM-1 and VCAM-1 on the luminal surface (Askenase, 2001). Furthermore, in a recent study, DNFB was reported to have direct activity with mast cells *in vitro* (Manabe et al., 2017). It was subsequently suggested that mast cells play an important role in both atopic dermatitis and contact dermatitis

(Dudeck et al., 2011). The catechin derivatives that we examined showed a depression effect in time-course experiments that was different from that of BBP. Collectively, our results suggest that catechin derivatives and BBP inhibit CHS through different mechanisms. To investigate the detailed mechanism underlying the effect of the catechin derivatives, we assumed that inflammatory cytokines were a target and examined the depression effect on CHS. We focused our attention on the differences in the cellular release of cytokines and evaluated the catechin derivatives that showed an effect on this process. Our results showed an increase of each cytokine assumed to be through the up-regulation of IL-1 β in the beginning. IL-1 β is a cytokine that increases first after haptenic contact (Enk and Katz, 1992). IL-1 β released from the Langerhans cells induces keratinocytes to release TNF- α and IL-6. Meanwhile, T-cell activation specific to the antigen clinically results in contact dermatitis, and it induces mediator release by infiltrating T cells specific to the hapten (Dudeck et al., 2011; Grabbe and Schwarz, 1998). Methyl PC and acetyl PC significantly inhibited TNF- α and IL-1 β 24 h after the challenge. Furthermore, acetyl PC significantly inhibited not only these cytokines but also IL-4. From antigen stimulation, naive CD4⁺ T cells differentiate into lasting cells of different functions (Th1 and Th2). Th1 cells produce IFN- γ , whereas Th2 cells produce IL-4 in humoral immunity (Iwakura et al., 2008). Several studies have reported the suppression of CHS in a cytokine-knockout mouse (He et al., 2009; Traidl et al., 1999). In addition, we found that catechin derivative compounds inhibited at least three of these cytokines. Acetyl PC showed an anti-inflammatory effect by inhibiting cytokine IL-1 β , which was increased during the early response. After compound application, no major changes were observed in the Langerhans cells (data not shown).

The human skin is exposed to various allergens, including ultraviolet rays and metals present in cosmetics (Avadhani et al., 2017; Tan et al., 2014; Warshaw et al., 2009). Allergens induce reactive oxygen species production, which further worsens contact dermatitis. We hypothesized that

if the production of reactive oxygen species decreased, contact dermatitis would be inhibited.

However, our results showed that hydroxyl radical inhibition was not necessary, as methyl PC and acetyl PC inhibited CHS without having an effect on hydroxyl radical. Methyl PC and acetyl PC demonstrated lower antioxidant properties compared with PC. Thus, methyl PC and acetyl PC may inhibit inflammation through the inhibition of the NF- κ B, and MAP kinase. This may serve a central role in inhibiting the activation of the early phase. It was reported in *O*-methylated catechins (Byun et al., 2011; Maeda-Yamamoto et al., 2004). Thus, it is necessary to perform further examination. In recent years, a 67 kDa laminin receptor (67LR) of the EGCg receptor on the cell membrane has been reported. EGCg has been found to exert various physiological functions through this receptor. Structure of the gallate group appears to be important in binding EGCg to this receptor. The receptor for non-gallate type catechins has not yet been identified, but a similar receptor may be identified at some point. As for PC seemed to work different from methyl PC and acetyl PC. PC inhibited the infiltration of neutrophils (Weber et al., 2015), which are important cells for the induction period of contact dermatitis, and this was shown in the MPO assay results (Fig. 6). It has been reported that EC, a diastereomer of (+)-catechin, inhibits neutrophils through reactive oxygen species suppression (Flemmig et al., 2014; Marinovic et al., 2015). Therefore, PC, with its associated antioxidant properties, may inhibit inflammation by removing the reactive oxygen species produced by neutrophils. In contrast, only methyl PC, which does not have antioxidant properties, did not inhibit neutrophils. Sensitizers, such as DNFB and oxazolone, induce an immune response through reactive oxygen species. In contrast, nickel (Ni²⁺) directly binds to human Toll-like receptor 4 on dendritic cells without reactive oxygen species and causes CHS (Honda et al., 2013). Therefore, future studies are required to investigate the effects of catechin derivatives on CHS caused without reactive oxygen species. PC may still have promising treatment effects for various diseases; further studies are warranted. In summary, our study of catechin derivatives suggests that they have the potential as

promising new therapeutic agents for the prevention and treatment of contact dermatitis, as a viable alternative to steroid treatments.

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Figure captions

Fig. 1. Chemical structures of tea catechin and catechin derivatives.

Fig. 2. Hydroxyl radical-scavenging activity of catechin derivatives by ESR. (A) Hydroxyl radical of hydrogen peroxide. (B–G) Spectrum that added each compound to hydrogen peroxide. (H) Antioxidative activity (%) of catechin derivatives. Mean $\pm$ S.E.M., ANOVA, and Tukey's test ($n = 7-14$). * $P < 0.05$ compared with (+)-catechin. ESR, electron spin resonance; S.E.M., standard error of the mean; ANOVA, analysis of variance.

Fig. 3. Effect of catechin derivatives on the time-dependent ear swelling response of mice following the challenge. The mice were sensitized for 5 days using 0.5% DNFB and thereafter challenged. The ear thickness was measured before the challenge and at 15 min, 3 h, 6 h, 9 h, 18 h, and 24 h after the

challenge. (+)-Catechin, PC, methyl PC, acetyl PC, and BBP were topically applied to challenged mice after 15 min and 18 h. (A) Administration schedule. (B–E) Effect of catechin derivatives on ear swelling. Mean $\pm$ S.D., ANOVA, and Tukey's test ($n = 12$). * $P < 0.05$ compared with the vehicle group (DNFB plus Vaseline treatment); ** $P < 0.001$ compared with the vehicle group; # $P < 0.05$ compared with BBP; ## $P < 0.001$ compared with BBP. DNFB, 1-fluoro-2,4-dinitrobenzene; PC, planar catechin; methyl PC, (6aS,12aR)-2,3,8,10-tetramethoxy-5,5-dimethyl-5,6a,7,12a-tetrahydroisochromeno[4,3-b]chromene; acetyl PC, (6aS,12aR)-5,5-dimethyl-5,6a,7,12a-tetrahydroisochromeno[4,3-b]chromene-2,3,8,10-tetraacetate; BBP, betamethasone butyrate propionate; S.D., standard deviation; ANOVA, analysis of variance.

Fig. 4. Histopathological analysis of effect of catechin derivatives on DNFB-induced contact dermatitis after 24 h. (A) Untreated control ear. (B) DNFB + Vaseline-treated ear. (C–F) DNFB + (+)-catechin- or catechin derivative-treated ear. (G) DNFB + BBP-treated ear. Each sample was stained by three mice ears. A representative image is shown ($n = 1$). Scale bar = 100 μ m. DNFB, 1-fluoro-2,4-dinitrobenzene; BBP, betamethasone butyrate propionate.

Fig. 5. Effect of catechin derivatives on mRNA levels of cytokines in ear tissues of mice. Ear tissues were taken 3, 9, and 24 h following the last treatment by DNFB and quantified using RT–PCR. Mean $\pm$ S.E.M., ANOVA, and Tukey's test ($n = 3–6$). * $P < 0.05$ compared with the vehicle group (DNFB plus Vaseline treatment); ** $P < 0.001$ compared with the vehicle group. mRNA, messenger ribonucleic acid; DNFB, 1-fluoro-2,4-dinitrobenzene; RT–PCR, reverse transcription polymerase chain reaction; S.E.M., standard error of the mean; ANOVA, analysis of variance.

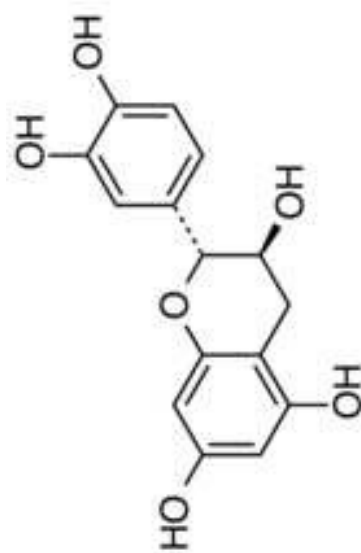
Fig. 6. Effect of catechin derivatives on MPO activity in ear tissues of mice. Ear tissues were taken 24 h following the last treatment by DNFB and quantified by MPO assay. Mean $\pm$ S.E.M., ANOVA, and Tukey's test ($n = 5-6$). * $P < 0.05$ compared with the vehicle group (DNFB plus Vaseline treatment). MPO, myeloperoxidase; DNFB, 1-fluoro-2,4-dinitrobenzene; S.E.M., standard error of the mean; ANOVA, analysis of variance.

Table 1. Primer sequences used in this study.

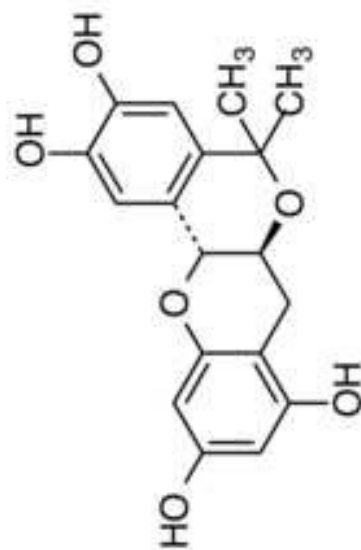
Gene	Forward primer (5' – 3')	Reverse primer (5' – 3')
TNF- α	CAGCCGATGGGTGTGTACCTT	GGCAGCCTTGTCCCTTGA
IL-1 β	TTGACGGACCCCAAAAGATG	TGGACAGCCCAGGTCAAAG
IL-4	CTTCCAAGGTGCTTCGCATA	AAGCCCGAAAGAGTCTCTGC
IL-6	GCCCACCAAGAACGATAGTCA	CAAGAAGGCAACTGGATGGAA
β -actin	CCTGTATGCCTCTGGTCGTA	CCATATAATGCTCGAAGTCT

Table 2. Inhibition of the ear swelling ratio.

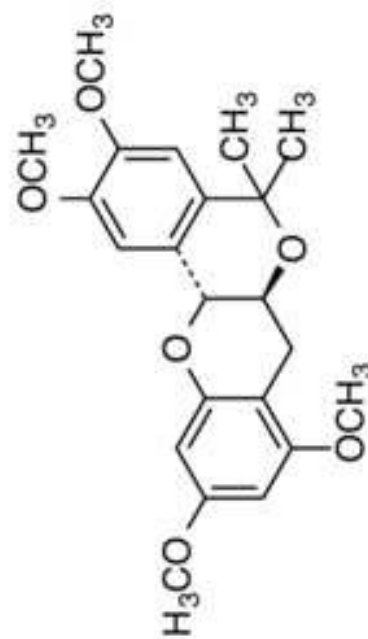
[%]	3 h	6 h	9 h	18 h	24 h
(+)-Catechin	-4	0	6	3	8
PC	16	52	67	55	39
Methyl PC	64	43	42	31	18
Acetyl PC	66	62	84	59	50
BBP	28	37	52	69	71



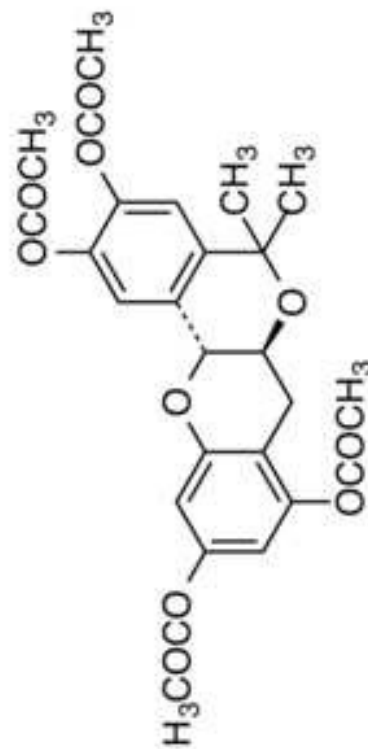
(+)-Catechin (C)



Planar catechin (PC)



Methylate-PC (Methyl PC)



Acetate-PC (Acetyl PC)

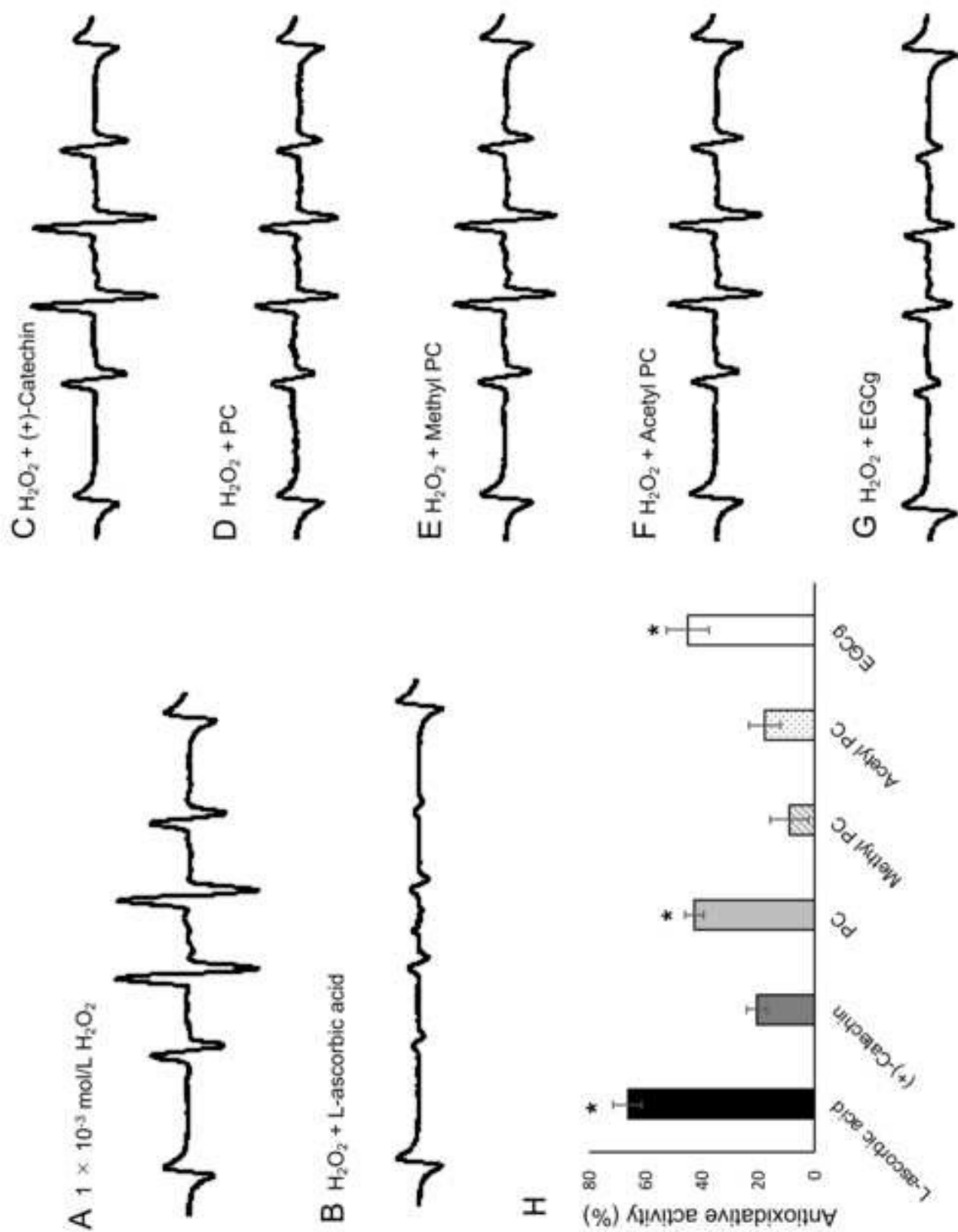


Figure 2

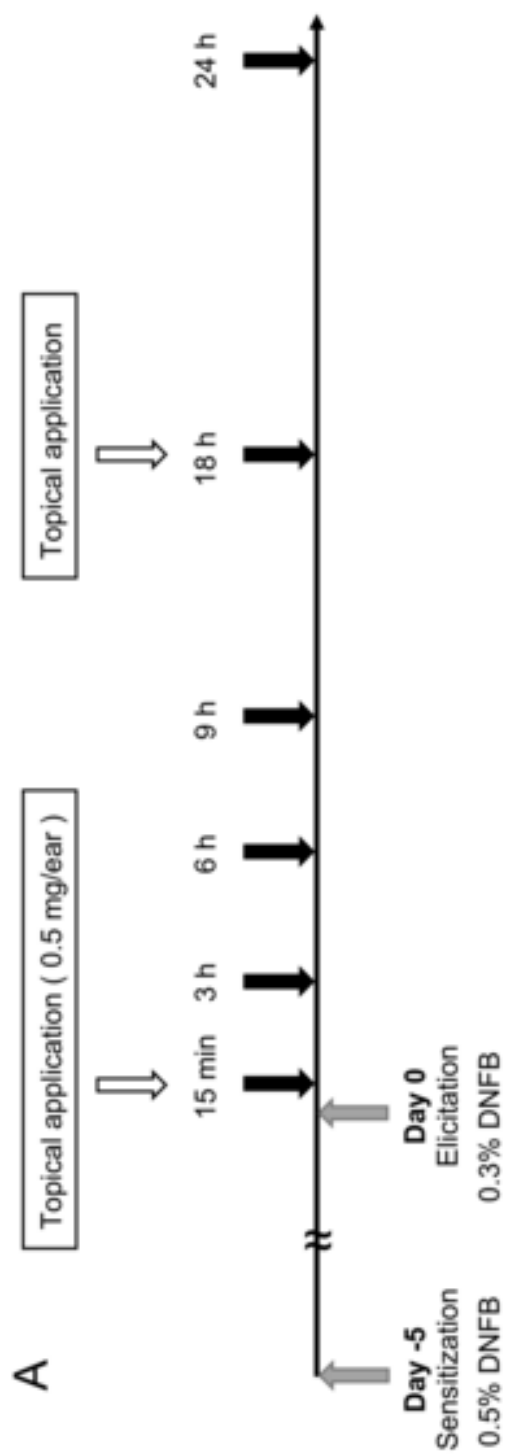


Figure 3A

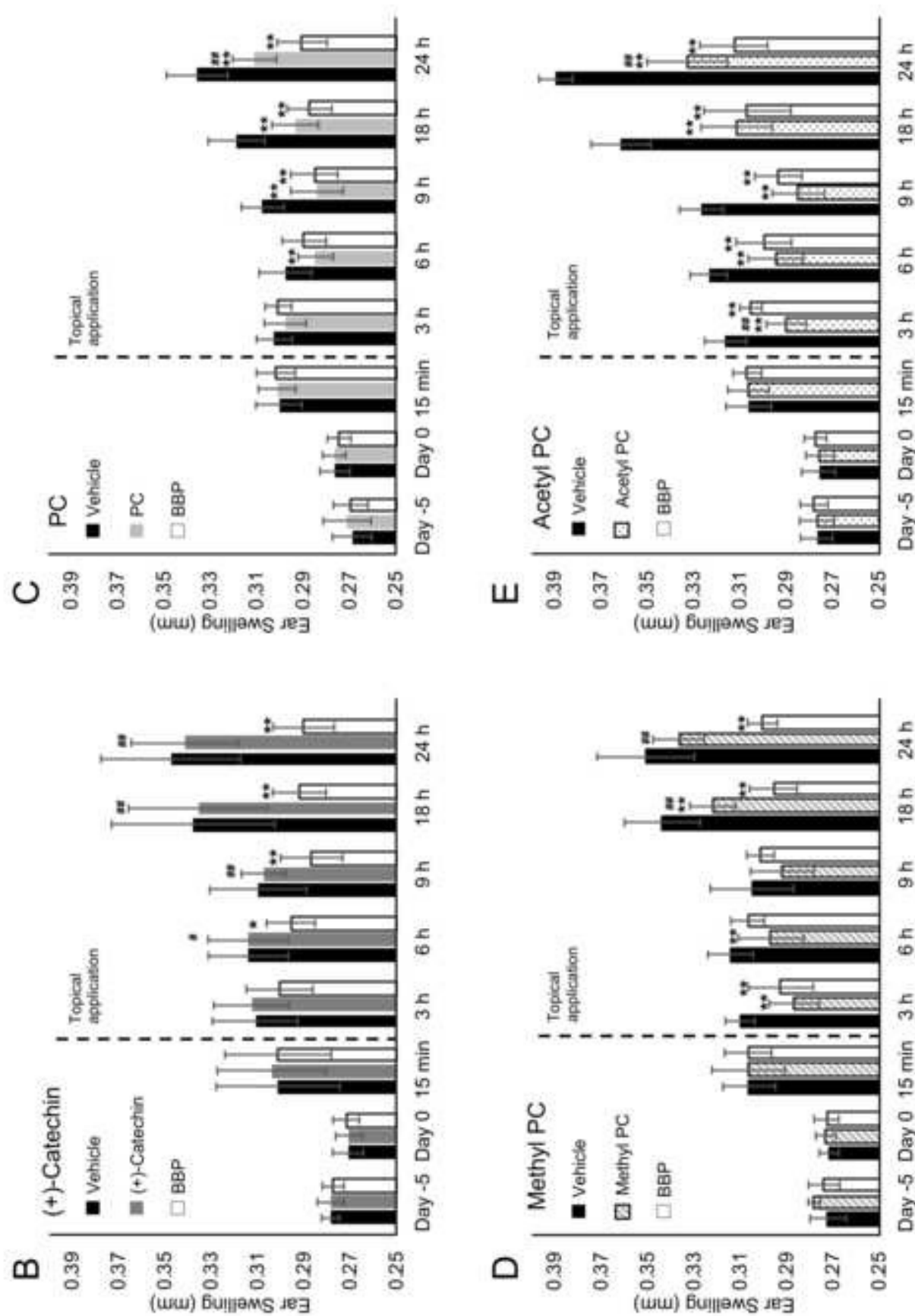


Figure 3B-E

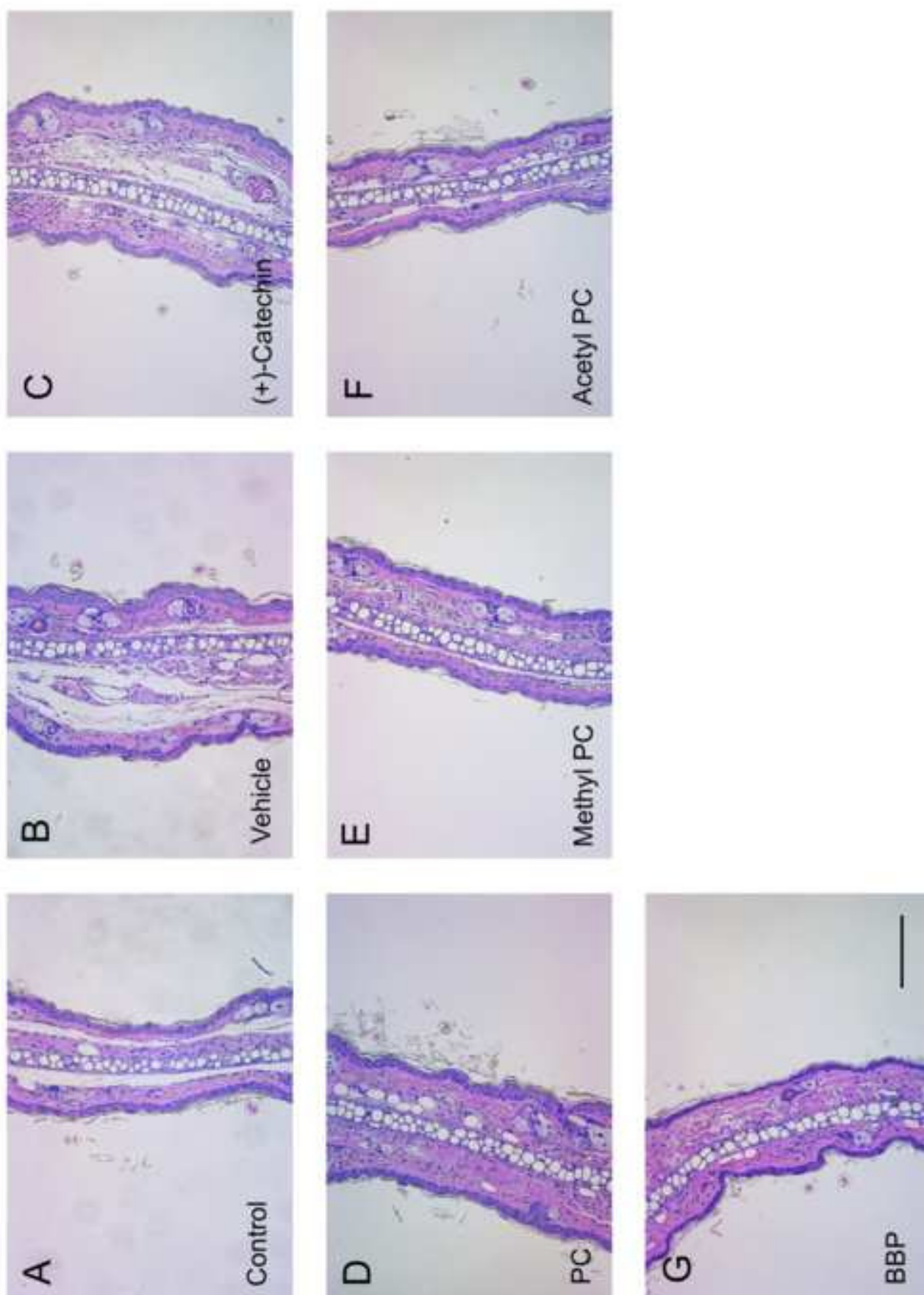


Figure 4

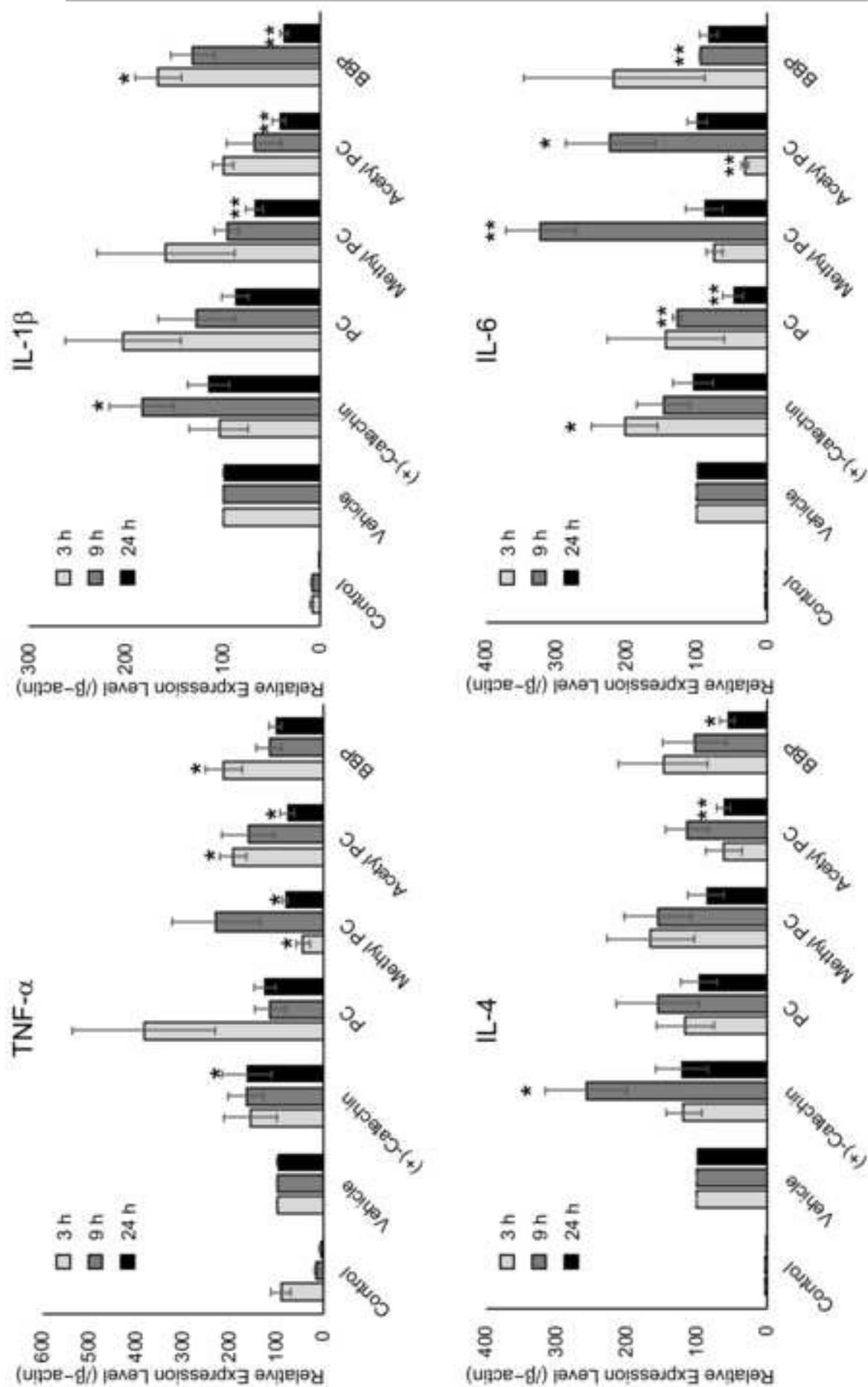


Figure 5

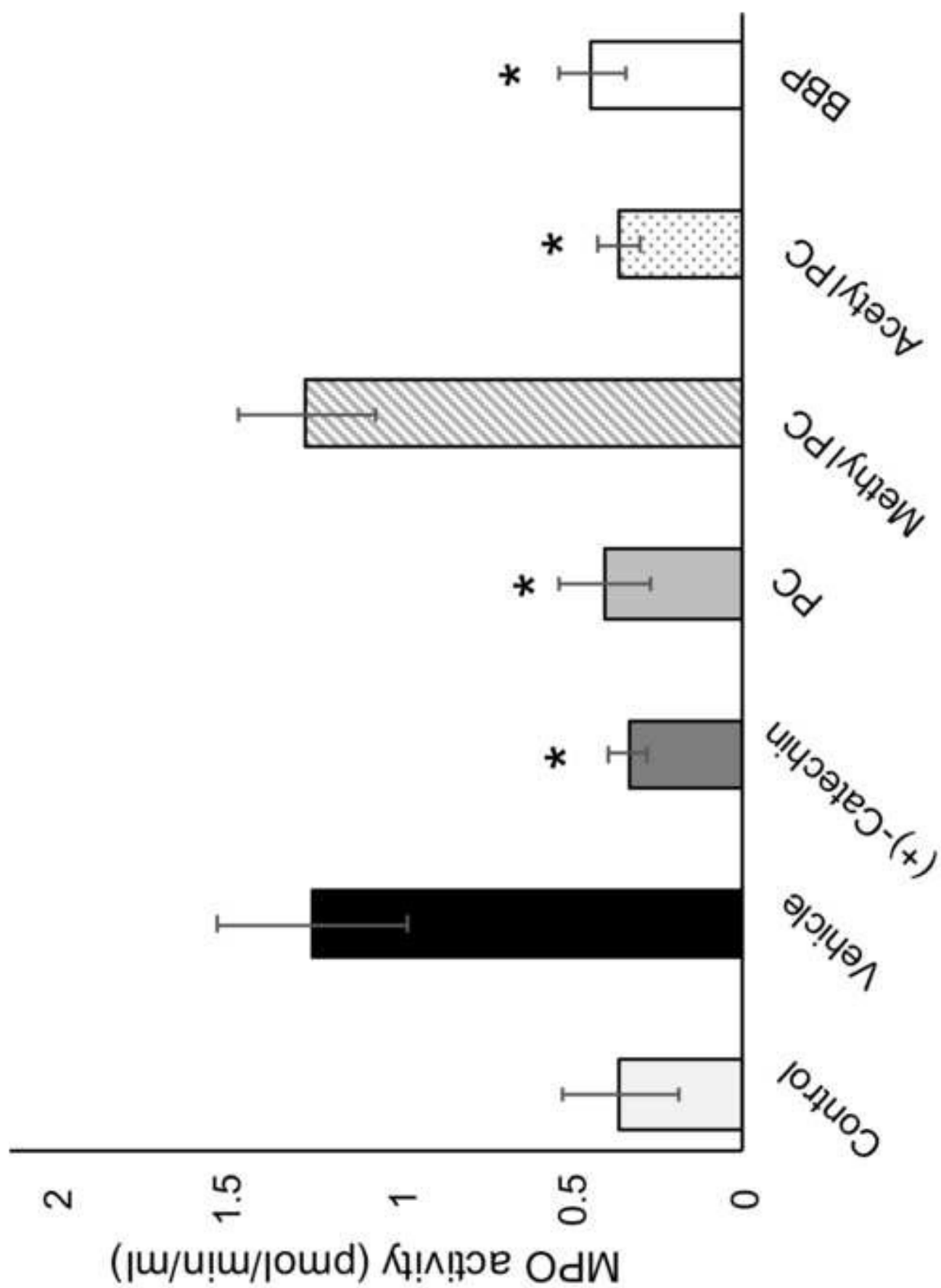


Figure 6