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Sensitization potential and potency of terpene hydroperoxides in the cocultured activation test method

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Abstract

Background: Positive patch test reactions to mixtures of oxidized terpenes containing allergenic hydroperoxides are frequently reported. However, human sensitization data for these hydroperoxides are not available.

Objectives: To analyse and evaluate the human sensitization potential and potency of hydroperoxides in vitro by using human cells.

Materials/Methods: Limonene-1-hydroperoxide, limonene-2-hydroperoxide, citronellol-7-hydroperoxide, cumene hydroperoxide, 1-(1-hydroperoxy-1-methylethyl)cyclohexene and mixtures of citronellol hydroperoxides (isomers at positions 6 and 7) and linalool hydroperoxides (isomers at positions 6 and 7) were studied. All compounds were synthesized except for cumene hydroperoxide, which was commercially available. Their potential and potency to activate dendritic cells (DCs) was evaluated by measuring the upregulation of CD86 and CD54 on THP-1 cells upon exposure in the cocultured activation test (COCAT) consisting of HaCaT cells (human keratinocyte cell line) and THP-1 monocytes (as a surrogate for DCs).

Results: Hydroperoxides upregulated CD86 and/or CD54 on cocultured THP-1 cells in a concentration-dependent manner. The results are comparable with their sensitization potency ranking in predictive animal models.

Conclusions: For the first time, the human sensitization potential and potency of several hydroperoxides were determined by the use of human cells and the COCAT method.

KEYWORDS

COCAT, cocultured activation test, fragrances, human skin sensitization potential, hydroperoxides, in vitro, sensitization potency

1 | INTRODUCTION

Allergic contact dermatitis is the clinical manifestation of contact allergy, which is an altered immune status of an individual, induced by a sensitizing substance, that is, a contact allergen. Upon uptake into the living epidermis, a contact allergen needs to become an antigen

and to strongly activate the innate immune system of the skin, providing inflammatory and danger signals from keratinocytes.^{1–3} These signals allow dendritic cells (DCs) to leave the skin and mature in the course of their migration to skin-draining lymph nodes, where they activate specific naive T cells, resulting in expansion of allergen-responsive T cells. At this point, an individual is immunologically

sensitized. Only upon re-exposure to the same or a cross-reacting contact allergen is the elicitation phase triggered, leading to specific T cell activation with clinical symptoms.

Fragrance compounds remain the most commonly diagnosed allergens in consecutive dermatitis patients, although they are typically moderate sensitizers.⁴ The prevalences of contact allergy to fragrances in women are approximately twice as those in men.⁵ Specifically, terpene fragrances are ingredients in up to 60% of cosmetic and household products, and occur naturally in essential oils. Contact allergy to oxidation products of fragrance terpenes such as linalool and limonene is frequently documented,^{6–11} whereas patch test studies for air-oxidized citronellol are absent. Linalool, limonene and citronellol are not contact allergens according to human and animal data.^{12,13}

The sensitizing potency based on the murine local lymph node assay (LLNA) is categorized into extreme (EC3 value of $\leq 0.2\%$), strong ($>0.2\%$ to $\leq 2\%$), and moderate ($>2\%$).¹⁴ The allylic hydroperoxides derived from air-oxidized linalool,¹⁵ limonene,¹⁶ and citronellol,¹⁷ as well as the other air-oxidized compounds, were evaluated in the LLNA, and the results showed that the hydroperoxides are the most potent sensitizers among all oxidation products derived from air oxidation of the parent terpenes. Consequently, limonene-1-hydroperoxide (Lim-1-OOH), limonene-2-hydroperoxide (Lim-2-OOH)¹⁶ and the mixture of linalool hydroperoxides¹⁵ are categorized as strong sensitizers, and the mixture of citronellol hydroperoxides is classified as a moderate sensitizer¹⁷ (Table 2). Furthermore, exposure to hydroperoxides may also occur because of their use as synthetic chemicals. For instance, cumene hydroperoxide (Cum-OOH) is used in the production of acetone and phenol, and as a polymerization initiator.

Overall, the most frequently studied fragrance hydroperoxides are Lim-2-OOH, linalool-6-hydroperoxide (Lin-6-OOH), and linalool-7-hydroperoxide (Lin-7-OOH). The quantification of hydroperoxides in consumer products is challenging, owing to low thermal stability and their lack of chromophores.^{18–21} The link between exposure to the hydroperoxides and allergic contact dermatitis has been well documented in only a few cases. For example, a 7-year-old girl developed severe eyelid dermatitis after using a shampoo that contained linalool and low levels (0.2 $\mu\text{g/g}$) of linalool hydroperoxides.²²

The aim of the present study was to investigate the sensitizing potency of a series of hydroperoxides by using our cocultured activation test (COCAT), which was developed to identify the human skin sensitization hazard and determination of skin sensitization potency in vitro.^{23,24} The COCAT is based on the use of cocultures of HaCaT cells (human keratinocyte cell line) and THP-1 cells (surrogate for antigen-presenting cells). Individually, these cell lines are used to address key steps in the process of sensitization, namely activation of keratinocytes (key event 2) and activation and maturation of DCs (key event 3) of the skin sensitization adverse outcome pathway.²⁵ We found that exposure of the cocultured cells has the potential to increase their response to sensitizing chemicals, enabling the detection of prohaptens and supporting the identification of skin sensitization potency.^{24,26,27} Additionally, 79.5% (31/39) concordance was

found when potency was estimated on the basis of a prediction of four potency subcategories (extreme/strong, moderate, weak, and non-sensitizer) by linear interpolation of the lowest concentration needed to reach positivity for estimated markers (CD54 or CD86) as compared with LLNA EC3 values.²⁸

2 | MATERIALS AND METHODS

2.1 | Chemicals

The following compounds were studied: Lim-1-OOH ([1S,4R]-4-isopropenyl-1-methyl-2-cyclohexene-1-hydroperoxide), Lim-2-OOH ([5R]-5-isopropenyl-2-methyl-2-cyclohexene-1-hydroperoxide), citronellol-7-hydroperoxide (Citr-7-OOH) ([E]-7-hydroperoxy-3,7-dimethyloct-5-ene-1-ol), a mixture of citronellol hydroperoxides (6-hydroperoxy-3,7-dimethyloct-7-ene-1-ol [citronellol-6-hydroperoxide; Citr-6-OOH] and Citr-7-OOH) in a 6:4 ratio (Citr-6/7-OOH), a mixture of linalool hydroperoxides (6-hydroperoxy-3,7-dimethylocta-1,7-diene-3-ol [Lin-6-OOH] and 7-hydroperoxy-3,7-dimethylocta-1,5-diene-3-ol [Lin-7-OOH]) in a 6:4 ratio [Lin-6/7-OOH]), Cum-OOH (Sigma-Aldrich, Saint Quentin Fallavier, France), 1-(1-hydroperoxy-1-methylethyl)cyclohexene (Cyc-hex-OOH), sodium dodecylsulfate (SDS) (Carl Roth, Karlsruhe, Germany), and 2,4-dinitrochlorobenzene (DNCB) (Sigma-Aldrich, Taufkirchen, Germany).

The synthesis of the hydroperoxides was performed in the Dermatochemistry Laboratory (Strasbourg, France). Lim-1-OOH and Lim-2-OOH were synthesized as reported previously in the literature.^{29,30} For the mixture Citr-6/7-OOH, we used exactly the same procedure as described for Lin-6/7-OOH, and obtained, for both mixtures, an isomer ratio of 6:4.³⁰ Cyc-hex-OOH was also synthesized with a method described in the literature.³¹ All synthesized compounds were pure according to the ^1H and ^{13}C nuclear magnetic resonance

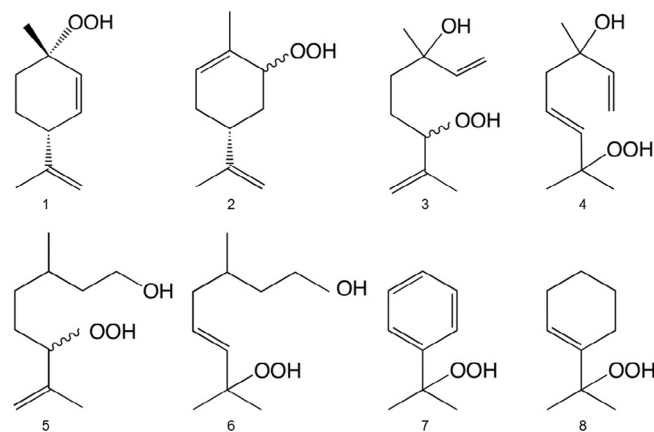


FIGURE 1 Chemical structures of terpene hydroperoxides included in this study: limonene-1-hydroperoxide (1), limonene-2-hydroperoxide (2), linalool-6-hydroperoxide (3), linalool-7-hydroperoxide (4), citronellol-6-hydroperoxide (5), citronellol-7-hydroperoxide (6), cumene hydroperoxide (7), and 1-(1-hydroperoxy-1-methylethyl)cyclohexene (8)

TABLE 1 Concentrations of hydroperoxides used for the experiments

Compound	Solvent (0.2% final concentration)	Maximum concentration (μM) resulting in a 30%-35% reduction in THP-1 cell viability	Total number of tested concentrations
Lim-1-OOH	DMSO	500	9
Lim-2-OOH	DMSO	500	9
Lin-6/7-OOH	DMSO	500	7
Citr-6/7-OOH	DMSO	1000	8
Citr-7-OOH	DMSO	1000	9
Cyc-hex-OOH	DMSO	1000	9
Cum-OOH	DMSO	500	9

Abbreviations: Citr-6/7-OOH, mixture of citronellol-6-hydroperoxide and citronellol-7-hydroperoxide in a 6:4 ratio; Citr-7-OOH, citronellol-7-hydroperoxide; Cum-OOH, cumene hydroperoxide; Cyc-hex-OOH, 1-(1-hydroperoxy-1-methylethyl)cyclohexene; DMSO, dimethylsulfoxide; Lim-1-OOH, limonene-1-hydroperoxide; Lim-2-OOH, limonene-2-hydroperoxide; Lin-6/7-OOH, mixture of linalool-6-hydroperoxide and linalool-7-hydroperoxide in a 6:4 ratio.

characterizations, in agreement with the literature. The chemical structures of the studied hydroperoxides are shown in Figure 1.

2.2 | Cocultured activation test

THP-1 cells (8×10^4 , 96-well plate) were placed on top of confluent HaCaT keratinocytes. The maximal test concentration (up to 4000 μM) for each test chemical was determined on the basis of solubility in the culture medium and/or cell viability. Cocultured cells were exposed to increasing concentrations of the compounds

TABLE 2 Comparison of the skin sensitization potencies of fragrance terpene hydroperoxides and 2,4-dinitrochlorobenzene (DNCB) in vitro (COCAT) and in vivo (LLNA)

Compound	COCAT ECΔ (mM), mean ± SEM ^a	Human sensitization category	LLNA EC3 (mM; % wt/vol)
Lim-1-OOH	0.29 ± 0.01	NA	19; 0.33 ^b
Lim-2-OOH	0.35 ± 0.08	NA	49; 0.83 ^b
Lin-6/7-OOH, ratio 3:5	NA	NA	86; 1.6 ^c
Lin-6/7-OOH, ratio 6:4	0.36 ± 0.04	NA	NA
Citr-6/7-OOH, ratio 6:4	0.29 ± 0.07	NA	100; 2.3 ^d
Citr-7-OOH	0.65 ± 0.17	NA	NA
DNCB	0.014 ± 0.0037	1 ^e	2.96; 0.06 ^e

Abbreviations: Citr-6/7-OOH, mixture of citronellol-6-hydroperoxide and citronellol-7-hydroperoxide; Citr-7-OOH, citronellol-7-hydroperoxide; COCAT, cocultured activation test; Lim-1-OOH, limonene-1-hydroperoxide; Lim-2-OOH, limonene-2-hydroperoxide; Lin-6/7-OOH, mixture of linalool-6-hydroperoxide and linalool-7-hydroperoxide; LLNA, local lymph node assay; NA, not available; SEM, standard error of the mean.

^an = 3, except n = 2 for Citr-6/7-OOH.

^bReference 16.

^cReference 15.

^dReference 17.

^eReference 37.

(7.8–4000 μM) dissolved in a final concentration of 0.2% dimethylsulfoxide (DMSO). Cell viability was then determined, and concentrations inducing not more than a 30% to 35% reduction in THP-1 cell viability were used for the experiments. Overall, seven to nine concentrations were evaluated for each hydroperoxide (Table 1). In each run, 20 μM DNCB and 144 μM SDS were used as positive and negative controls, respectively. After 24 hours of treatment, floating THP-1 cells were harvested, and cell surface expression (geometric mean fluorescence intensities [MFI]) of CD86 and CD54 was analysed on viable THP-1 cells (4,6-diamidino-2-phenylindole [DAPI]/propidium iodide-negative) with flow cytometry (FACSVerse or FACSCalibur; BD Biosciences, Heidelberg, Germany). Briefly, THP-1 cells were stained with fluorescein isothiocyanate (FITC)-labelled anti-CD86 (clone 2331 [FUN-1]) and allophycocyanin (APC)-labelled anti-CD54 (clone HA58) monoclonal antibodies (both mouse IgG₁; BD Pharmingen, Heidelberg, Germany) or corresponding isotype controls. Then, the chemical-induced differences in cell surface levels of CD86 or CD54 (ΔMFI) on viable THP-1 cells (determined with DAPI/propidium iodide exclusion) were calculated for each sample. A ΔMFI of 10.8 for CD86 and a ΔMFI of 300 for CD54 were used as thresholds to identify a sensitizer. These thresholds were established by analysing a set of 21 sensitizers and 12 non-sensitizers in the course of the method development. The lowest effective concentration (ECΔ) (either CD54 or CD86) for each run was used to estimate potency. Mean ECΔ and SE were calculated for each compound.^{24,28}

3 | RESULTS

We investigated human sensitization potential and potency by using the COCAT method. Specifically, Lim-1-OOH, Lim-2-OOH, Citr-7-OOH, Citr-6/7-OOH, Lin-6/7-OOH, Cum-OOH and Cyc-hex-OOH were investigated (Figure 1). Representative flow cytometry histogram plots are shown in Figure 2. All studied hydroperoxides upregulated both markers for activation of DCs (CD86 and CD54) on cocultured THP-1 cells in a concentration-dependent manner. The

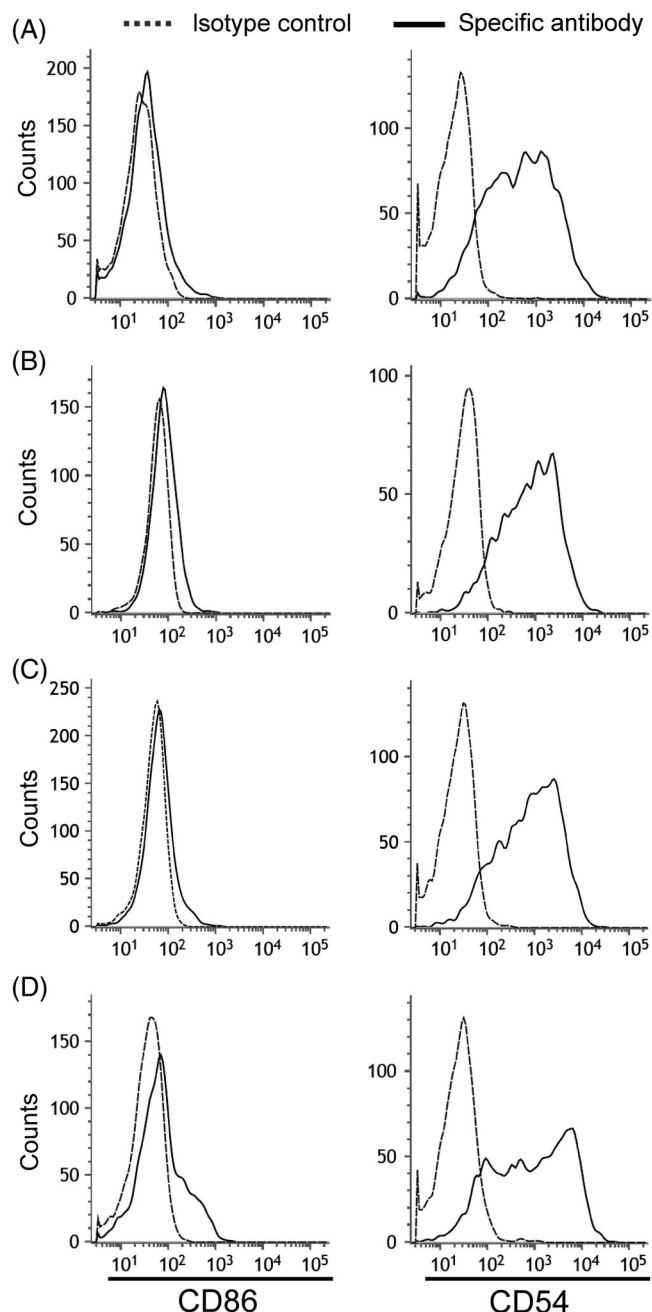


FIGURE 2 Representative flow cytometry plots for the geometric mean fluorescence intensity (MFI) of CD86 and CD54. A–D, Expression of CD86 and CD54 on cocultured THP-1 cells after treatment with solvent (0.2% dimethylsulfoxide) (A), 500 μ M limonene-1-hydroperoxide (B), (C) 750 μ M citronellol-6-hydroperoxide/citronellol-7-hydroperoxide mixture (C), and 20 μ M 2,4-dinitrochlorobenzene (D). Flow cytometry histograms show the number of events vs fluorescein isothiocyanate (FITC) (CD86) and allophycocyanin (APC) (CD54) fluorescence intensities. Solid lines represent histograms for the indicated antibodies (FITC-labelled anti-CD86 or APC-labelled anti-CD54), and dotted lines represent graphs of the corresponding isotype controls (FITC-labelled or APC-labelled IgG₁), respectively

corresponding curves for CD86 and CD54 are shown in Figure 3. Furthermore, all compounds reached the threshold for positivity (CD86, Δ MFI of 10.8; CD54, Δ MFI of 300) for one or two markers in at least

two of three runs. All hydroperoxides were correctly predicted to be sensitizers. The lowest concentration needed to reach a threshold for positivity (ECA) was used to estimate the potency of the molecule.²⁴ Here, we found that the tested hydroperoxides needed similar concentrations to reach positivity; values varied between 0.29 and 0.65 mM. In contrast, for DNCB, which is classified as an extreme sensitizer in the LLNA, at least a 10-fold lower concentration was sufficient to reach positivity (Table 2). In the case of the limonene hydroperoxides, we found that Lim-1-OOH was more potent than Lim-2-OOH, similarly to what was shown by the LLNA data. The values for Lin-6/7-OOH are close to those for Lim-2-OOH. Interestingly, Citr-7-OOH alone was less potent than Citr-6/7-OOH. The concentrations needed for positivity of Cum-OOH and Cyc-hex-OOH were comparable to those for Lim-2-OOH (Table 3).

4 | DISCUSSION

In this study, we analysed the skin sensitization potential and potency of allergenic hydroperoxides *in vitro* by using our COCAT method.^{24,26–28} All hydroperoxides were correctly classified as skin sensitizers on the basis of analysis of activation of DCs in their natural environment of keratinocytes. We found concentration-dependent upregulation of CD86 and CD54 over a wide range of concentrations. The concentration needed for positivity ranged between 0.29 and 0.65 mM. Thus, our potency ranking is comparable to reported *in vivo* data from predictive animal testing (Tables 2 and 3). The highest potency was found for Lim-1-OOH, followed by Lim-2-OOH. Although exploration of the mechanism(s) leading to the upregulation of the two markers CD86 and CD54 was not the focus of this study, it seems reasonable to speculate that the difference might reflect the different capacities of the two molecules to generate radicals. For instance, a higher ability of Lim-1-OOH to generate different kinds of radicals was detected in radical trapping experiments.³² For Lim-1-OOH, both carbon-centred and oxygen-centred radicals were trapped, whereas experiments with Lim-2-OOH retrieved only small amounts of carbon-centred radicals. Instead, the main product detected in experiments testing Lim-2-OOH was carvone. However, carvone is much less sensitizing in animal experiments (LLNA EC₃ value of 0.86 M, 13% wt/vol),³³ and cannot account for the results obtained with the COCAT. This notion is also supported by the ECA value for carvone (0.55 ± 0.11 mM) in the COCAT.²⁸ In addition, published prevalences of positive patch test reactions to oxidized fragrance hydroperoxides³⁴ are in accordance with the potency ranking obtained in this study. Specifically, a human patch test study including 763 eczema patients, all tested with Lim-1-OOH and Lim-2-OOH, showed that 18 individuals responded to Lim-1-OOH and 13 individuals responded to Lim-2-OOH.³⁴

Our results for Lim-OOH and Lin-OOH are in general agreement with a previous study investigating THP-1 cell activation in the absence of keratinocytes, among further in-depth mechanistic aspects.³⁵ The correlation of the compound concentrations showed that we needed much lower concentrations in the presence of

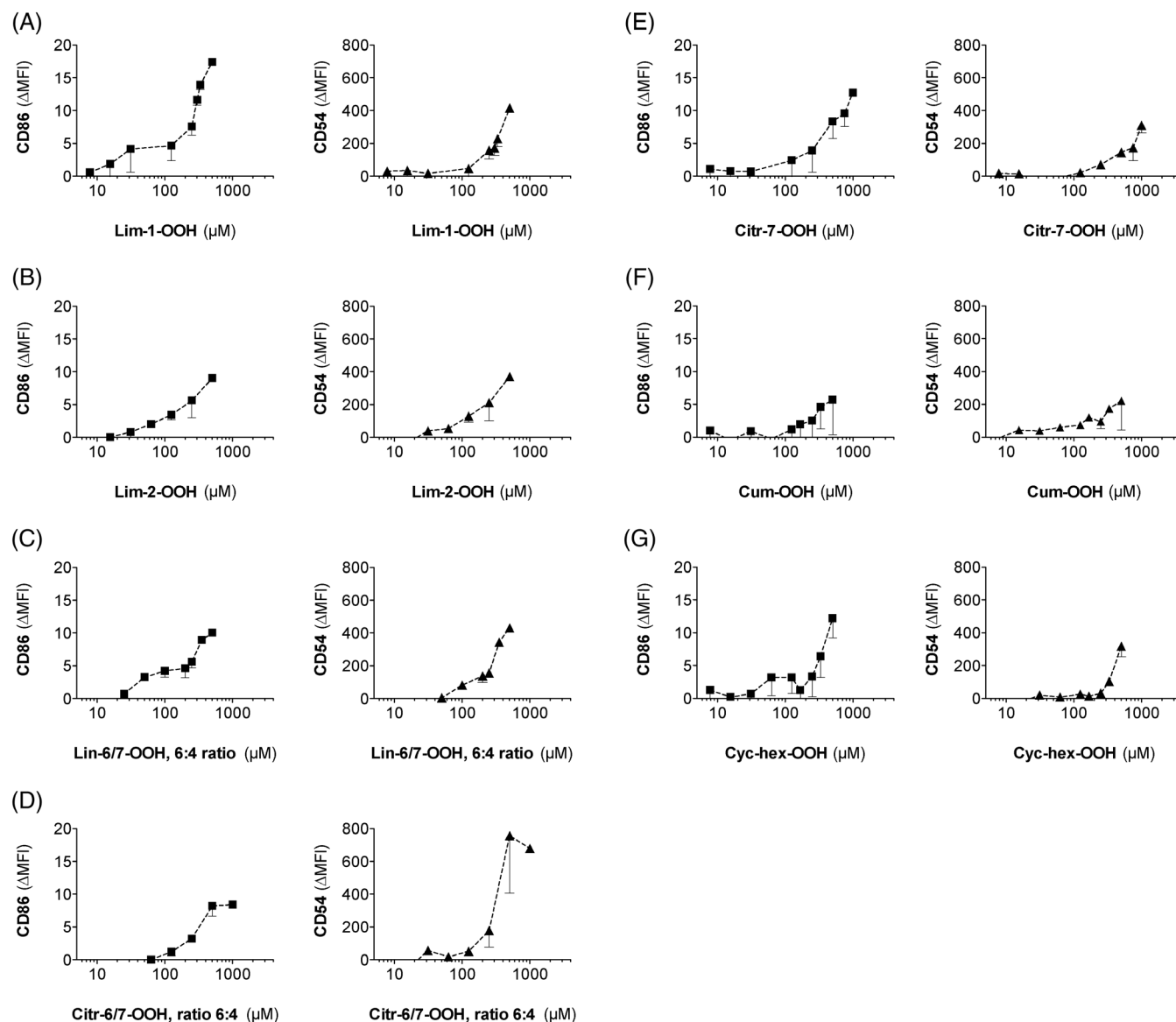


FIGURE 3 Concentration-dependent upregulation of CD86 and CD54 by hydroperoxides. Cocultured HaCaT and THP-1 cells were exposed to limonene-1-hydroperoxide (Lim-1-OOH) (A), limonene-2-hydroperoxide (Lim-2-OOH) (B), a mixture of linalool-6-hydroperoxide and linalool-7-hydroperoxide (Lin-6/7-OOH) (C), a mixture of citronellol-6-hydroperoxide and citronellol-7-hydroperoxide (Citr-6/7-OOH) (D), citronellol-7-hydroperoxide (Citr-7-OOH) (E), cumene hydroperoxide (Cum-OOH) (F) and 1-(1-hydroperoxy-1-methylethyl)cyclohexene (Cyc-hex-OOH) (G) for 24 hours. The expression of CD86 and CD54 was analysed on harvested THP-1 cells with flow cytometry, and given as chemical-induced differences in cell surface levels of CD86 or CD54 (mean $\pm$ standard error of the mean of $n = 3$; Citr-6/7-OOH and Cum-OOH, $n = 2$)

keratinocytes. This suggests that keratinocytes support THP-1 cell activation. Different responsiveness between monoculture and coculture of THP-1 cells has already been observed with a different set of chemicals^{24,28}; as compared with exposing THP-1 cells alone, coculture resulted in up to 3-fold enhanced maximal CD86 and/or CD54 upregulation on THP-1 cells.

Cum-OOH and Cyc-hex-OOH are comparably potent in the COCAT. The comparable potencies of Cum-OOH and Cyc-hex-OOH with that of Lim-2-OOH observed in the COCAT is in good agreement with *in vivo* results also indicating comparable potencies of these molecules.³¹ The lowest potency was detected for Citr-7-OOH.

Citronellol is also one of the highly used fragrances in terms of the relative volume sold.³⁶ With respect to its oxidation products, it was shown that Citr-7-OOH, together with Citr-6-OOH, contributes most to the sensitizing potency of autoxidized citronellol in the LLNA.¹⁷ Comparison of our results for Citr-7-OOH alone with the results for Citr-6/7-OOH indicate that Citr-7-OOH may have influenced the results obtained for the mixture. This is in contrast to results reported for Lin-6/7-OOH and Lin-7-OOH obtained after exposure of THP-1 cells in the absence of HaCaT keratinocytes, where no difference was found for Lin-7-OOH and the Lin-6/7-OOH.³⁵ Besides the different experimental setups, there is no obvious difference in the structure of

TABLE 3 Comparison of the skin sensitization potencies of limonene-2-hydroperoxide (Lim-2-OOH), cumene hydroperoxide (Cum-OOH) and 1-(1-hydroperoxy-1-methylethyl)cyclohexene (Cyc-hex-OOH) in vitro (cocultured activation test [COCAT]) with published in vivo results derived from guinea-pig tests (Freund's complete adjuvant test [FCAT])

Compound	COCAT	Guinea pig-test (FCAT)	
	ECA (mM), mean $\pm$ SEM ^a	Sensitized animals ^b (%) after challenge with 0.3%	Sensitized animals ^b (%) after challenge with 1%
Lim-2-OOH	0.35 $\pm$ 0.08	33.3 ^c	100 ^c
Cum-OOH	0.30 $\pm$ 0.12	13.3 ^d	86.7 ^d
Cyc-hex-OOH	0.38 $\pm$ 0.01	25 ^e	91.7 ^e

Abbreviations: SEM, standard error of the mean.

^an = 3, except n = 2 for Cum-OOH.

^bAt 72 hours after challenge.

^cInduction was performed with 1.5% Lim-2-OOH.³⁸

^dInduction was performed with 1% Cum-OOH.³⁸

^eInduction was performed with 3% Cyc-hex-OOH.³¹

Citr-7-OOH and Lin-7-OOH, but, clearly, more detailed structure-related analyses are necessary to explain the observed differences according to chemical structures.

At present, comparison of our data for the hydroperoxides with results from other alternative methods explicitly evaluating the potency of these compounds is not possible, owing to the lack of data. Furthermore, in the absence of appropriate experimental data, no meaningful results regarding potency can be derived from defined approaches capable of potency prediction, such as DC ITS (at <http://its.douglasconnect.com>). In conclusion, this study is the first to evaluate the human sensitization potential and potency of several hydroperoxides, mainly fragrance hydroperoxides, in vitro with the COCAT method. The results obtained are in line with the results from the literature obtained with in vivo methods. These data, together with our earlier results,^{23,24,26-28} show that the COCAT can help in the assessment of chemicals with as yet limited or absent human sensitization data.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to report.

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