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The Effects of Essential Oils on Skin Homeostasis and Inflammation Using Human Keratinocyte HaCaT cells

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Abstract

Some essential oils used in aromatherapy are known to have antibacterial, anti-inflammatory, and/or antioxidant effects although it has not been scientifically validated. In this study, we screened essential oils with anti-inflammatory effects using an *in-vitro* inflammation model. Inflammatory cytokines were added to the human epidermal keratinocytes HaCaT to induce the expression of inflammatory and thymus and activation-regulated chemokine (CCL17/TARC). As a result, 7 of the 46 kinds of essential oils, including German chamomile and patchouli, were found to suppress IFN-γ- and TNF-α-induced TARC. Moreover, both essential oils were shown to suppress TARC induction, disregarding the countries of origin and product manufacturers. We also performed RT-PCR to examine the inflammation- and skin homeostasis-related gene expression fluctuation. The expressions of inflammatory chemokines and the genes responsible for the skin barrier function, such as filaggrin, fluctuated when inflammatory cytokines were applied to HaCaT cells. Then, we examined the effect of patchouli on the expression of several genes in HaCaT cells. As a result, patchouli was found to suppress the inflammatory chemokine expression induction to some extent and modulate MLN64 and MMP2 expressions. From the above, it was strongly suggested that some essential oils may have effects to suppress skin inflammation.

Keywords

Atopic dermatitis
HaCaT
TARC
Chemokine
Patchouli

1. Introduction

According to the Atopic Dermatitis (AD) Treatment Guidelines 2018 [1], AD is a condition characterized by the recurrence of exacerbation and remission, with pruritic eczema as the primary lesion. Many patients have atopic predisposing factors, such as a family history, a medical history of conditions such as bronchial asthma, allergic rhinitis, conjunctivitis, one or more types of AD, or a propensity to produce IgE antibodies. AD is a chronic inflammatory skin disease, and the prevalence of AD in developed countries has increased two to three times since the 1970s. It is estimated that approximately 15% to 20% of children and 1% to 3% of adults worldwide are affected [2].

Topical corticosteroids have become the most commonly prescribed treatment for AD since their introduction in the early 1950s. These agents have strong anti-inflammatory properties and are considered the mainstay therapy for improving the inflammatory state of the skin. However, they can sometimes cause localized cutaneous side effects [3]. Prolonged use may lead to skin thinning, weakening of the anti-inflammatory effect, and exacerbation after discontinuation, among other serious side effects [4]. Consequently, some patients have a strong aversion to topical steroids, and there is a demand for drugs with fewer side effects [5].

AD can significantly reduce a patient's quality of life, leading to research on inflammatory substances involved in AD to identify the causes and treatment targets [6]. Among these substances, thymus and activation-regulated chemokine (CCL17/TARC, hereafter TARC) produced by epidermal cells in AD has garnered attention in recent years. TARC is a chemokine with leukocyte chemotactic properties, and when overproduced, it attracts Th2 cells to the site of inflammation. This, in turn, is believed to stimulate the production of IgE antibodies and activate eosinophils, leading to allergic inflammatory reactions [7]. TARC levels in the serum are used as a prominent biomarker in AD because they are considered more sensitive indicators of the disease's pathophysiology compared to total serum IgE levels, specific IgE antibody levels, peripheral blood eosinophil counts, and serum lactate dehydrogenase [8]. It is well-known that a decrease in TARC levels is associated with a reduction in the inflammatory state, bringing it closer to remission.

Therefore, this study aimed to express TARC in an *in vitro* cell culture system and attempted to screen TARC inhibitors. In this study, immortalized human epidermal keratinocytes, HaCaT cells, were used. It is known that HaCaT cells induce TARC production when exposed to cytokines that promote inflammation, such as tumor necrosis factor-α (TNF- α) and interferon-γ (IFN- γ) [9], making them a suitable model for AD inhibitor screening. This model is being used for the discovery of AD-induced inhibitors [10,11].

Essential oils are relatively low-molecular-weight compounds that contain a high concentration of volatile terpenes, such as monoterpenes and sesquiterpenes, each with its own unique aroma [12]. In addition, essential oils exhibit characteristics such as antibacterial, anti-inflammatory, and antioxidant properties, and they are commonly used in aromatherapy [12]. Thus, this study aimed to explore essential oils that inhibit TARC production using a skin inflammation cell model based on HaCaT cells (**Figure 1**).

In previous studies, the expression changes of immune system genes (such as chemokines and interleukins) were extensively studied in a skin inflammation cell model, where HaCaT cells were exposed to IFN-γ/TNF-α to induce TARC. However, detailed verification of the expression changes of genes outside the immune system has not been conducted. Recent studies have reported that in human skin, genes related to the homeostasis of the nervous, immune, and endocrine systems are closely interconnected and expressed similarly to the whole human body [13,14]. Thus, this study aimed to verify the mRNA expression changes of 28 genes that are believed to have an impact on maintaining homeostasis in epidermal keratinocytes using RT-PCR. Notable

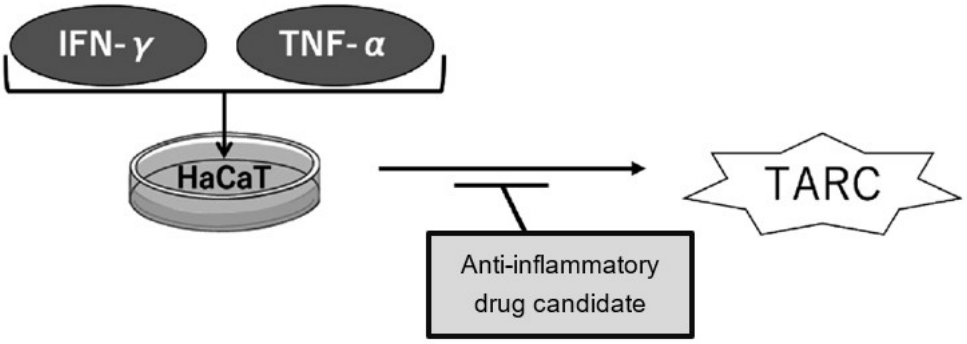

Figure 1. Skin inflammation cell model using human epidermal keratinocytes (HaCaT). When IFN-γ and TNF-α were added to HaCaT cells (final concentration of 10 ng/mL), the inflammatory chemokine TARC was induced. This model was used to examine whether the induction of TARC can be inhibited by adding candidate anti-inflammatory agents (various essential oils).

examples include CCL5/RANTES (a chemokine similar to TARC), various interleukins, glucocorticoid receptor α/β, 11β-hydroxysteroid dehydrogenase type 1/2 (11β-HSD1/2, an endocrine protein), nerve system protein substance P, and PAR-2. Attention was also given to filaggrin, which is associated with skin barrier function and is considered relevant to AD. Furthermore, this study aimed to investigate whether essential oils that inhibit TARC production in the skin inflammation cell model, where IFN-γ/TNF-α were applied, affect the expression of other genes or contribute to homeostasis.

Moreover, this study attempted to utilize liposomes, which are currently gaining attention as drug transport carriers, to ensure anti-inflammatory drug candidates are effectively taken up in the skin inflammation model cells. Liposomes are artificial vesicles consisting of lipid bilayers similar to cell membranes [15,16], making them susceptible to endocytosis. Therefore, by encapsulating essential oils in liposomes, it was considered that essential oils could be efficiently delivered to the skin inflammation cell model [16]. In the future, the aim is to establish a delivery system into skin cells using liposomes that do not cause toxicity when applied to the skin. In summary, this study employed a skin inflammation cell model to select essential oils with anti-inflammatory properties. Additionally, it investigated how effective essential oils that inhibit TARC induction affect the expression of genes related to homeostasis. Furthermore, it attempted to encapsulate effective essential oils in liposomes. The ultimate goal is to contribute to maintaining skin homeostasis and alleviating and treating AD symptoms.

2. Materials and methods

2.1. Materials

As experimental materials, 30 essential oils were obtained from Tree of Life Co., Ltd. and 16 from Ease Co., Ltd (**Table 1**). These essential oils were screened as candidate anti-inflammatory agents that could inhibit TARC production. The products from Tree of Life Co., Ltd. used in this study were those commonly employed in the qualification exams of the Japanese Aromatherapy Environmental Association, and they are widely used by many aromatherapists, making them the focus of this study. In addition, the remaining essential oils, apart from these 30 types, were primarily obtained from Ease Co., Ltd., which also handles a wide variety of other essential oils. Patchouli was obtained from Tree of Life, Ease, Aroma Station Shape, and Inscent Co. Online Shop, all of which were produced in Indonesia. German chamomile was obtained from Tree of Life (from Egypt), Green Gables Hashimoto (from Egypt), Seimi Laboratory (from Hungary), Mieux Sélection (from Egypt), and Un Petite Réve

Table 1. Essential oils used in this study

No.	Essential oil name	Species name	Production origin
Essential oils from Tree of Life Co., Ltd.			
1	Benzoin	*Styrax tonkinensis*	Laos
2	Bergamot	*Citrus bergamia*	Italy
3	Bitter orange	*Citrus aurantium*	Tunisia
4	Black pepper	*Piper nigrum*	Sri Lanka
5	Clary	*Salvia sclarea*	Hungary
6	Damask rose	*Rosa damascena*	Bulgaria
7	Damask rose	*Rosa damascena*	Morocco
8	Eucalyptus	*Eucalyptus globulus*	China
9	Frankincense	*Boswellia carteri*	Somalia
10	Grapefruit	*Citrus paradisi*	Argentina
11	Geranium	*Pelargonium graveolens*	Egypt
12	German chamomile	*Matricaria chamomilla*	Egypt
13	Indian sandalwood	*Santalum album*	India
14	Jasmine	*Jasminum officinale*	Egypt
15	Juniper berry	*Juniperus communis*	Albania
16	Lavender	*Lavandula officinalis*	France
17	Lemon	*Citrus limon*	Italy
18	Lemon balm	*Melissa officinalis*	France
19	Lemongrass	*Cymbopogon flexuosus*	Nepal
20	Marjoram	*Origanum majorana*	Egypt
21	Mediterranean cypress	*Cupressus sempervirens*	Spain
22	Myrrh	*Commiphota myrrha*	Somalia
23	Patchouli	*Pogostemon cablin*	Indonesia
24	Peppermint	*Mentha piperita*	France
25	Roman chamomile	*Anthemis nobilis*	Italy
26	Rosemary	*Rosmarinus officinalis*	Morocco
27	Sweet orange	*Citrus sinensis*	Brazil
28	Tea tree	*Melaleuca alternifolia*	Australia
29	Vetiver	*Vetiveria zizanoides*	Indonesia
30	Ylang-ylang	*Cananga odorata*	Madagascar
Essential oils from Ease Co., Ltd.			
1	Basil	*Ocimum basilicum*	Egypt
2	Bergamot	*Citrus bergamia*	Italy
3	Bitter orange	*Citrus aurantium*	India
4	Bitter orange	*Citrus aurantium*	Italy
5	Grapefruit	*Citrus paradisi*	Argentina
6	Grapefruit	*Citrus paradisi*	Spain
7	Helichrysum, Immortelle	*Helichrysum italicum*	Albania
8	Hyssop	*Hyssopus officinalis*	Spain
9	Lavandin	*Lavandula × intermedia*	France
10	Lavandin Grosso	*Lavandula × intermedia Emeric ex Loisel. Clone Grosso*	France
11	Lime	*Citrus aurantifolia*	Brazil
12	Mandarin	*Citrus reticulata*	Brazil
13	Sage	*Salvia officinalis*	Spain
14	Spearmint	*Mentha spicata*	India
15	Sweet orange	*Citrus sinensis*	Australia
16	White thyme	*Thymus vulgaris*	Spain

(from Nepal). These different sources of patchouli and German chamomile were used to verify whether they have the same effects despite differences in their production country or manufacturer. All these essential oils were kept refrigerated and used within six months of purchase.

All the essential oils used in this study were dissolved in ethanol (99.5%, Kanto Chemical Co., Ltd.), prepared at dilution factors of 300, 1,000, 3,000, 10,000, and 30,000, and added to the cultured cells in a 1/100 volume (v/v). The final dilution factors added to the cultured cells were 3.0×10^4, 1.0×10^5, 3.0×10^5, 1.0×10^6, and 3.0×10^6.

2.2. Cultured cells

Human adult low calcium high temperature (HaCaT) keratinocyte cell line (CLS Cell Lines Service GmbH) was cultured in D-MEM medium containing 10% fetal bovine serum (FBS; Biowest), penicillin-streptomycin mixed solution (Nacalai Tesque Inc.), L-glutamine, sodium pyruvate, and 4.5 g/L glucose (Nacalai Tesque Inc.) under the conditions of 37°C and 5% CO_2. Cell passaging was conducted from the semi-confluent state to the confluent state.

2.3. WST assay

HaCaT cells in the proliferation phase were added to a 96-well culture plate (Nippon Genetics Co., Ltd.) at a concentration of 5.0×10^4 cells/mL, with 100 µL in each well, and cultured for 24 hours in a CO_2 incubator. To determine the appropriate solvent, various concentrations of DMSO (10%, 20%, 30%, 40%, 50%) or ethanol (5%, 25%, 50%, 100%) were added to the cultured cells in a 1/100 volume ratio (v/v%), resulting in final concentrations of 0.1%, 0.2%, 0.3%, 0.4%, and 0.5% for DMSO, and 0.05%, 0.25%, 0.5%, and 1% for ethanol. After one hour, each well received 1 µL of 1 µg/mL human IFN-γ (PeproTech Inc.) and 1 µg/mL human TNF-α (Miltenyi Biotech Co., Ltd.) at a final concentration of 10 ng/mL each. After 24 hours, 10 µL of Cell Counting Kit-8 (CCK-8; Dojindo Chemical Laboratory Co., Ltd.) was added to each well, and the cultures were incubated for an additional hour in the CO_2 incubator. Subsequently, the absorbance at 480 nm was measured using a 96-well plate reader (Multiskan FC, Thermo Fisher Scientific). Concurrently, a TARC enzyme-linked immunosorbent assay (ELISA) was performed to verify the impact of solvents on the TARC values (refer to Section 2.4 for details on the TARC ELISA method). These assays were conducted to determine the solvent conditions that were irrelevant to cell proliferation and had minimal impact on TARC values.

Next, essential oils were dissolved in the selected solvents, and experiments were conducted to investigate their effects on cell proliferation. HaCaT cells were added to a 96-well culture plate with 100 µL in each well, and they were cultured for 24 hours in the CO_2 incubator. Six wells received 1 µL of 99.5% ethanol (vehicle), 1 µg/mL of human IFN-γ, and 1 µg/mL of human TNF-α, resulting in a final concentration of 10 ng/mL for each [EtOH (+) + Cyto (+)]. Three wells received ethanol only, without the addition of cytokines [EtOH (+) + Cyto (-)], serving as controls. In contrast, each essential oil was added to the cells in sets of 3 wells, with final concentrations of 3.0×10^4, 1.0×10^5, 3.0×10^5, 1.0×10^6, and 3.0×10^6 fold dilutions. After an hour, 1 µL of human IFN-γ and human TNF-α were added to each well, with a final concentration of 10 ng/mL each. After 24 hours, 10 µL of CCK-8 was added in each well as previously described, and the WST assay was performed to measure absorbance at 480 nm.

2.4. TARC ELISA

Simultaneously with cell seeding for the WST assay, HaCaT cells were added to another 96-well plate at a concentration of 4×10^5 cells/mL, with 200 µL in each well, and cultured for 24 hours in the CO_2 incubator. The medium was then removed, and 200 µL of FBS-free medium was added to exchange the medium. To determine suitable solvent conditions, the final

concentrations of DMSO (0.1%, 0.2%, 0.3%, 0.4%, 0.5%) and ethanol (0.05%, 0.25%, 0.5%, 1%) were added similarly to the WST assay. One hour later, 2 μL of 1 μg/mL human IFN-γ and 2 μL of 1 μg/mL human TNF-α were added to each well, with a final concentration of 10 ng/mL each. After 24 hours, TARC ELISA was performed following the manufacturer's protocol (Human CCL17/TARC DuoSet ELISA, R&D Systems, Inc.). Nunc Maxisorp™ ELISA plates (Thermo Fisher Scientific) were used, and a blocking agent of 1% BSA dissolved in PBS was applied. All other steps followed the manufacturer's protocol for ELISA. This experiment verified the solvent concentrations that did not impact TARC production.

Next, essential oils were dissolved in the previously determined solvents and added to HaCaT cells to examine their effects on TARC production. Two controls were prepared as in the WST assay, with 2 μL of ethanol and 2 μL of cytokine solutions used. In contrast, 2 μL of each essential oil was added to the cells in sets of three wells, with final concentrations of 3.0×10^4, 1.0×10^5, 3.0×10^5, 1.0×10^6, and 3.0×10^6 fold dilutions. An hour later, 2 μL of 1 μg/mL human IFN-γ and 2 μL of 1 μg/mL human TNF-α were added to each well, with a final concentration of 10 ng/mL for each cytokine). After 24 hours, TARC ELISA was performed as described above.

2.5. RT-PCR

First, HaCaT cells were cultured to semi-confluent in 100 φ Petri dishes, and the culture medium was replaced with a D-MEM FBS-free medium to halt cell proliferation. Subsequently, patchouli was diluted 3×10^5 times, and the vehicle ethanol solution was added at a 1/100 volume ratio, resulting in a final dilution of 3×10^5). After an hour, 1 μg/mL IFN-γ and 1 μg/mL TNF-α were added at a final concentration of 10 ng/mL each. Cells were harvested at 0, 1, 2, 3, 6, and 12 hours, and RNA was purified using the RNeasy® Mini Kit (Qiagen), following the manufacturer's protocol. The purified RNA was diluted 25 times in 10 mM Tris-HCl buffer (pH 8.0), and the optical densities at 260 nm, 280 nm, and 320 nm of the collected RNA were measured. Subsequently, the RNA was treated with RNA Clean & Concentrator™-5 (Zymo Research Co., Ltd.) to remove genomic DNA. The RNA was then purified again using the RNeasy® Mini Kit, dissolved again in 100 mM Tris-HCl Buffer (pH 8.0), and the concentration was measured based on the OD_{260} value.

cDNA was synthesized according to the manufacturer's protocol using the Prime Script® RT-PCR Kit (Takara Bio Inc.). cDNA was used as a template, and PCR amplification was carried out for each RNA using GoTaq® Master Mixes (Promega Corp.). Primers (Eurofine Genomics, Inc.) are shown in **Table 2**, and GAPDH was used as the housekeeping gene for control. DNA was denatured at 94°C for 1 minute, followed by PCR conditions consisting of 30 cycles: denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute, and ended with a final extension at 72°C for 4 minutes (except for LEKTI, which run for 32 cycles, Filaggrin for 34 cycles, and GRβ for 40 cycles). To verify the amplified PCR products, electrophoresis was conducted using a 1.5% agarose gel. The gel was stained with ethidium bromide, and images were captured using a gel documentation system (WSE-5200 Printgraph 2M, Atto Co., Ltd.).

2.6. Liposome preparation

Phosphatidylcholine (PC) and cholesterol (Cho) were prepared at a concentration of 10 mg/mL in chloroform. 52.5 μL of PC, 26.3 μL of Cho, and 320 μL of chloroform were added to a round bottom glass test tube, and the chloroform was evaporated while gently rotating the test tube and blowing nitrogen to create a dry film at the bottom of the test tube. Subsequently, 1 mL of PBS was added along the wall of the test tube, and the mixture was allowed to swell. The tubes were kept at 55°C for 1 hour. In the meantime, the extruder for the Complete NanoSizer Extrusion Kit with a 200 nm filter (Funakoshi Co., Ltd.) was

set up on a syringe and placed on a hot plate kept at 55°C. The solution that swelled with PBS was then transferred to the syringe, and liposomes were prepared by moving the syringe left and right 11 times. Three types of liposomes were prepared: liposomes without the addition of essential oil, liposomes prepared with the addition of 1 μL essential oil during dry film preparation, and liposomes prepared with the addition of essential oil during swelling with PBS. Each of these liposomes was prepared at a 3-fold dilution in PBS. In addition, an ethanol solution (with essential oil EtOH) was prepared by adding 1 μL of essential oil to 1 mL of ethanol without making liposomes, and this solution was further diluted 3-fold with ethanol. Liposomes or ethanol dilutions of 1 μL each were added for the WST assay and 2 μL for the TARC ELISA, and TARC induction was performed with cytokines as in the protocol described above to verify the inhibition rate of cell proliferation and TARC production. In both cases, the final dilution of essential oil added to cultured cells

Table 2. List of primers for amplifying various genes

Gene	Coding strand	Template strand	bp
GAPDH	5’-GCCAAGGTCATCCATGACAACTT TGG	5’-CGACGCCTGCTTCACCACCT	318
CCL17/TARC	5’-ACTGCTCCAGGGATGCCATCGTTT TT	5’-ACAAGGGGATGGGATCTCCCTCA CTG	270
CCL5/RANTES	5’-CCGTGCCCACATCAAGGAGTATT T	5’-CCAGCCTGGGGAAGGTTTTTGTA A	308
CCL22/MDC	5’-ATGGCTCGCCTACAGACCTGCAC TC	5’-CACGGCAGCAGACGCTGTCTTCC A	80
IL-8	5’-ATGACTTCCAAGCTGGCCGTGGC T	5’-TCTCAGCCCTCTTCAAAAACTTCT C	292
IL-1α	5’-ATGGCCAAAGTTCCAGACATGTT T	5’-GTGACTGCCCAAGATGAAGACCA A	600
IL-1β	5’-ATGATGGCTTATTACAGTGGCAA T	5’-TTCACCATGCAATTTGTGTCTTCC	753
IL-2	5’-CTGCTGGATTTACAGATGATTTTG	5’-TTCAGATCCCTTTAGTTCCAGAAC	655
IL-4	5’-CTGCTTCCCCCTCTGTTCTT	5’-CTGTGAAGGAAGCCAACCAG	378
IL-6	5’-CTTCTCCACAAGCGCCTTC	5’-CTCCTCATTGAATC	137
IL-10	5’-GCAACCTGCCTAACATGCTTCG	5’-GAAGATGTCAAACTCACTCATGG C	388
IL-13	5’-AGGGAGCTCATTGAGGAGCTGGT C	5’-GAGCAGGTCCTTTACAAACTGGG C	144
IL-13RA1	5’-GGAGCCAGCTCAAATTGTAG	5’-ACACGGGAAGTTAAAGGC	509
IL-22	5’-GCAGGCTTGACAAGTCCAACT	5’-GCCTCCTTAGCCAGCATGAA	187
IL-22RA1	5’-CTGTCCGAGATCACCTACTTAGG	5’-GCACATTTGGGTCAGATGTTCTGT C	500
IL-31	5’-TCGAGGAATTACAGTCCCTCTC	5’-TGTCGAGGTGCTCTATGATCTC	213
GRα	5’-CCTAAGGACGGTCTGAAGAGC	5’-ATTATCCAGCACTTCATAGACAC AAAT	478
GRβ	5’-GGCAATACCAGGTTTCAGGAACT TACA	5’-ATTATCCAGCACTTCATAGACAC AAAT	1,002
11β-HSD1	5’-GAATTCAGACCAGAGATGCTC	5’-AACTGAGGAAGTTGACTTCCA	380
11β-HSD2	5’-ACCGTATTGGAGTTGAACAGC	5’-TCACTGACTCTGTCTTGAAGC	477
StAR	5’-CATCCAGCAAGGAGAGGAAG	5’-CGTGAGTTTGGTCTTTGAGG	496
MLN64	5’-GCTCAGGAGCGGGAGTACATCCG C	5’-AGCTCGCTGATGCGCTGTCGCAG G	626
MMP2	5’-GTGCTGAAGGACACACTAAAGAA GA	5’-TTGCCATCCTTCTCAAAGTTGTAG G	580
PAR-2	5’-TGGATGAGTTTTCTGCATCTGTCC	5’-CGTGATGTTCAGGGCAGGAATG	491
Substance P	5’-GACAGCGACCAGATCAAGGAGG AA	5’-GCATTGCACTCCTTTCATAAGCCA	208
Semaphorin 3A	5’-ACTCACTGTTCAGACTTA	5’-AGAGACTTCATGCAGCTC	435
Filaggrin	5’-TGATGCAGTCTCCCTCTGTG	5’-TGTTTCTCTTGGGCTCTTGG	338
LEKTI	5’-AAGAATGAAGATCAGGAAATGTG CCATGA	5’-ATTCTTTGCCTGATTTTGATATTG ACTGC	863
Tryptase (α)	5’-TGCAGCAAGCGGGTATCGT	5’-AGTCTGGATGATGTAGAACTGT	278

Abbreviation: bp, Base pairs; GADPH, Glyceraldehyde 3-phosphate dehydrogenase; CCL17/TARC, CC chemokine ligand 17 / thymus and activation-regulated chemokine; CCL5/RANTES, CC chemokine ligand 5 / regulated on activation, normal T cell expressed and secreted; CCL22/MDC, CC chemokine ligand 22 / macrophage-derived chemokine; IL, interleukin; IL-13RA1, interleukin 13 receptor subunit alpha 1; GR, glucocorticoid receptor; HSD, hydroxysteroid dehydrogenase; StAR, steroidogenic acute regulatory protein; MLN64, metastatic lymph node 64; MMP2, matrix metalloproteinase 2; PAR-2, protease-activated receptor-2; LEKTI, lymphoepithelial Kazal-type-related inhibitor.

was 1.0×10^5 or 3.0×10^5 times. For the WST assay control, 1 μL of PBS without essential oil was added to cultured cells, whereas for the TARC ELISA control, 2 μL of PBS was added to cultured cells.

2.7. Statistical analysis

Statistical analysis was performed using the analysis of variance (ANOVA) test, and $P < 0.05$ was judged as a significant difference.

3. Results

3.1. Determination of essential oil dilution solvent

Essential oils are highly hydrophobic, and when diluted with water or aqueous solvents, they tend to separate. Therefore, an experiment was conducted to determine the dilution solvent for essential oils that has minimal impact on the skin inflammation cell model used in this study. A comparison was made between DMSO and ethanol, aiming to verify the concentration conditions that do not inhibit cell growth (cytotoxicity) while not affecting TARC production. As shown in **Figure 2**, both 0.5% DMSO and 1% ethanol, the highest concentrations, showed strong inhibition of cell proliferation. It was found that even 0.1% DMSO was very strongly inhibitory to TARC induction. While repeated validation showed that this result could vary depending on the cell's condition, using DMSO as a vehicle was deemed impractical. On the other hand, 1% ethanol had a slight effect on the induction of TARC production but did not show significant differences

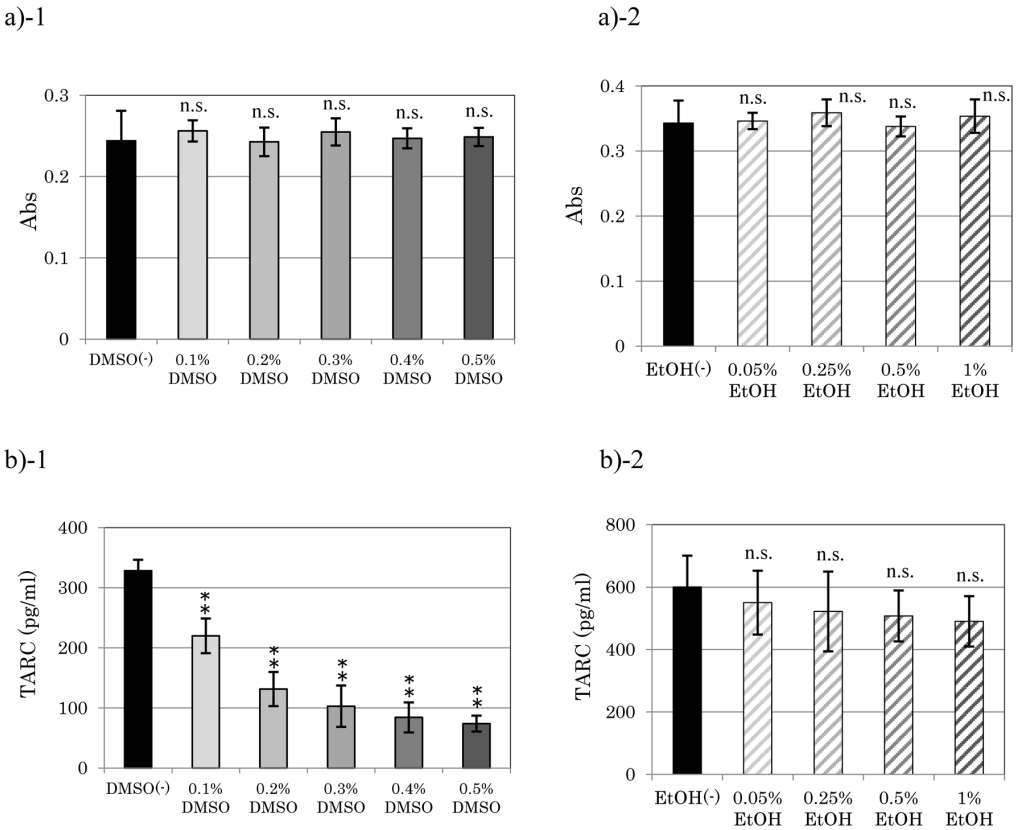

Figure 2. Validation of essential oil dilution solvents. **a)-1** shows cell proliferation when DMSO is added, and **b)-1** shows TARC levels. **a)-2** shows cell proliferation when ethanol (EtOH) is added, and **b)-2** shows TARC levels. HaCaT cells were seeded in a 96-well culture plate, and after 24 hours, the solvents were added (final concentrations of 0.1%–0.5% for DMSO and 0.05%–1% for ethanol). After 1 hour, human IFN-γ and human TNF-α were added. After another 24 hours, 10 μL of Cell Counting Kit-8 was added to the plates for cell proliferation assays, and a WST assay was performed. TARC ELISA was conducted following the manufacturer's protocol. The data were obtained from six replicate measurements, and error bars represent the standard deviation. Statistical analysis was performed using ANOVA and Dunnett test, with * indicating *P*-value < 0.05 and ** indicating *P*-value < 0.01.

compared to the control ($P > 0.05$). Additionally, it was observed that some essential oils were less likely to dissolve uniformly in ethanol diluted with water. Therefore, undiluted ethanol was used as the vehicle, and 1% v/v ethanol was added to the cell culture medium.

3.2. Inhibition of TARC production by essential oils

Next, experiments were conducted in which essential oils were diluted with ethanol and added to the skin inflammation cell model. As shown in **Figures 3 a)-1** and **a)-2**, the addition of the cytokines IFN-γ and TNF-α resulted in a nearly 50% inhibition of cell proliferation compared to the control group without essential oil addition. In other words, the cytokines themselves exhibited some degree of cytotoxicity. Furthermore, when these cytokines were absent, the TARC production level was shown to be below 20 pg/mL in most assays (**Figures 3b)-1** and **3b)-2**). The direct control for essential oils was prepared with the addition of ethanol and cytokines [EtOH (+) + Cyto (+)].

The TARC production inhibitory effects of each essential oil were determined by the following method. Among the tested essential oils, some showed a concentration-response curve with cell proliferation inhibition in the range of final dilutions from 3.0×10^{6} to 3.0×10^{4}, while others showed minimal proliferation inhibition. Therefore, among the essential oil concentrations that showed more than 50% inhibition of TARC production compared to the control [EtOH (+) + Cyto (+)] at concentrations below IC_{10} (10% inhibition of cell proliferation), those with a significant difference ($P < 0.05$) were considered valid. As a

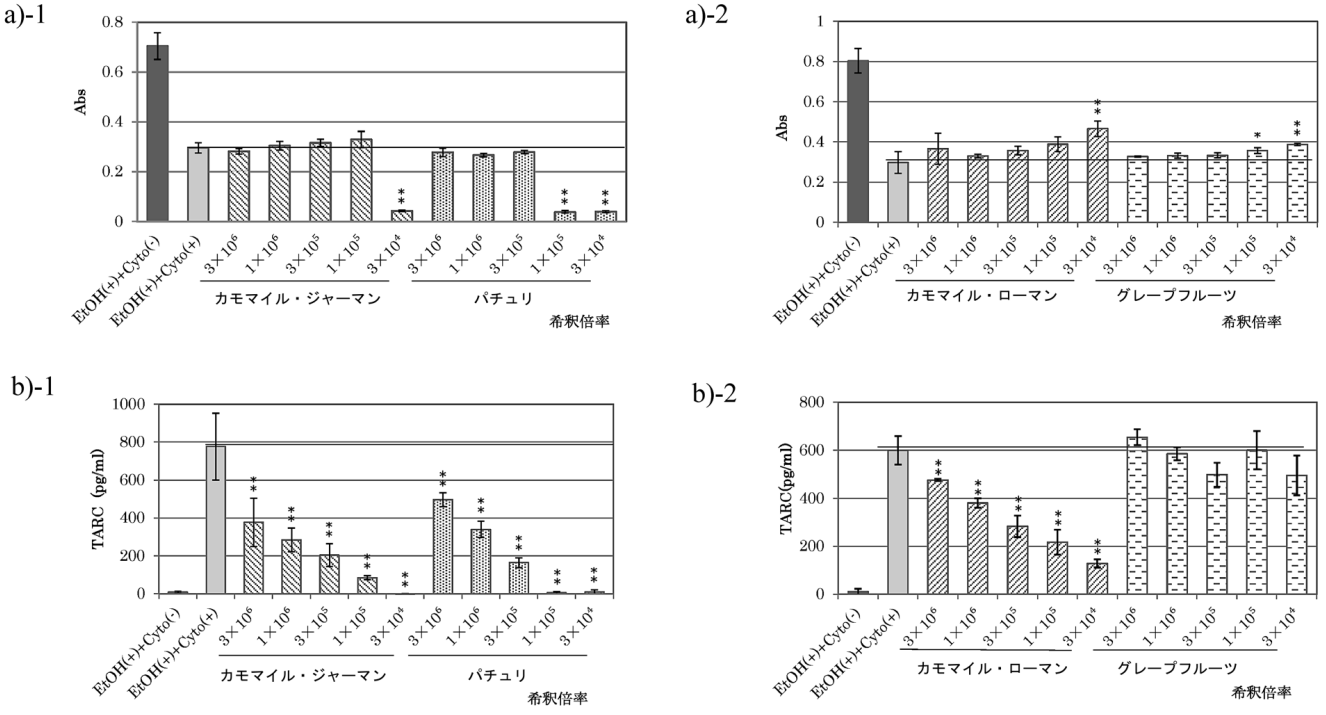

Figure 3. Impact on cell proliferation and TARC production by essential oil addition. **a)-1** shows cell proliferation and **b)-1** shows TARC values for German chamomile and patchouli, **a)-2** shows cell proliferation and **b)-2** shows TARC values for Roman chamomile and grapefruit. HaCaT cells were seeded into a 96-well culture plate and various essential oils were added 24 hours later. One hour after the addition of human IFN-γ and human TNF-α, Cell Counting Kit-8 was added to the plates for cell proliferation assays. TARC ELISA was performed following the protocol provided with the kit. Two controls were set up for this experiment, as follows: EtOH (+) + Cyto (-) received vehicle addition but no cytokines, while EtOH (+) + Cyto (+) had vehicle addition along with cytokines. EtOH (+) + Cyto (+) served as the direct control for essential oil-added samples. The controls were measured in sextuplicate, while the others the measured in triplicate. Error bars indicate standard deviations. Statistical analysis was conducted using ANOVA and the Dunnett test, with * indicating a P-value < 0.05 and ** indicating a P-value < 0.01.

result, seven essential oils – German chamomile, patchouli, Roman chamomile, black pepper, vetiver, sage, and white thyme – were determined to be effective (**Table 3**). These essential oils showed significant TARC production inhibition and exhibited minimal differences between assay repetitions, with German chamomile, patchouli, and Roman chamomile being the most pronounced among them (**Figure 3**). **Figure 3 b)-2** also includes grapefruit as a reference, which did not exhibit TARC production inhibition.

Among these, for German chamomile and patchouli, essential oils were obtained from multiple manufacturers, and their activities were compared across different products with varying countries of origin and producers (data not shown). In the case of German chamomile, all products showed no more than 10% inhibition of cell proliferation at a 1.0×10^5 dilution, and the inhibition of TARC production at this concentration exceeded 50% with statistical significance. On the other hand, all products of patchouli showed no more than 10% inhibition of cell proliferation at a 3.0×10^5 dilution, and the inhibition of TARC production at this concentration exceeded 50% with statistical significance.

3.3. Comparison of gene expression related to skin homeostasis

The cytokines IFN-γ and TNF-α were added to HaCaT cells, and the expression of genes that may be involved in skin homeostasis, including those of the immune system, were examined by RT-PCR. The effect of patchouli, one of the selected essential oils, on gene expression was also examined.

First, regarding the impact of IFN-γ and TNF-α addition on the expression of various genes, three chemokines – CCL17/TARC, CCL5/RANTES, and CCL22/MDC – showed time-dependent induction, while 11β-HSD2 and filaggrin were found to decrease (**Figure 4**). As for IL-8, classified as a chemokine, no induction or inhibition was observed in response to IFN-γ and TNF-α. Among the 28 genes examined for expression, seven other genes were found to be expressed in HaCaT cells: interleukin 13 receptor-alpha 1 (IL13RA1), glucocorticoid receptor-α (GRα), glucocorticoid receptor-β (GRβ), metastatic lymph node 64 (MLN64), matrix metalloproteinase 2 (MMP2), protease-activated

Table 3. The inhibition rates of the selected essential oils on cell proliferation and TARC values

Essential oils	Final dilutions	Cell proliferation inhibition rate	TARC values inhibition rates
German chamomile	3×10^5	<0%	74%
	1×10^5	<0%	89%
Patchouli	1×10^6	9%	56%
	3×10^5	6%	79%
Roman chamomile	3×10^5	<0%	53%
	1×10^5	<0%	64%
Black pepper	1×10^6	6%	37%
	3×10^5	2%	84%
Vetiver	3×10^5	2%	58%
	1×10^5	4%	68%
Sage	1×10^6	6%	62%
	3×10^5	8%	73%
White thyme	1×10^6	8%	65%
	3×10^5	9%	63%

Note: All the inhibition rates of TARC values shown here had significant differences compared to the control [EtOH (+) + Cyto (+)], with $P < 0.01$ for all.

receptor-2 (PAR-2), and lymphoepithelial Kazal-type-related inhibitor (LEKTI). Data regarding gene expression of these 7 genes were not shown.

Hence, the induction and inhibition of mRNA expression in HaCaT cells for these genes were validated by adding vehicle (99.5% ethanol) or patchouli diluted with ethanol (**Figure 5**). The results showed a slight inhibition in CCL17/TARC production and a clear inhibition in CCL5/RANTES and CCL22/MDC production. No significant impact of patchouli addition was observed for IL-8. MLN64 and MMP2, for which the vehicle had minimal changes, were inhibited by patchouli addition. There was no effect of cytokine addition on GRα, and patchouli addition did not influence it either (data not shown). In addition, the expression of other genes (IL13RA1, GRβ, PAR-2, and LEKTI) could not be verified due to either low expression levels or poor reproducibility (data not shown). The trends in the gene expression reported in **Figures 4** and **5** were consistent with similar independent experiments.

3.4. Comparison of essential oil activity by liposome encapsulation

To facilitate the penetration and introduction of active ingredients such as essential oils into skin cells, it is considered safer to encapsulate the essential oils in liposomes and add them to the cells, as opposed to using ethanol as the vehicle, considering potential skin irritation. Therefore, the effects of liposome encapsulation were examined. The final dilutions of patchouli were set at 1.0×10^5 and 3.0×10^5, which are believed to be the borderline concentrations showing cytotoxicity and inhibition of TARC production. During the preparation of liposomes, experiments were conducted with two different timings of adding the essential oil: before swelling and after swelling (**Figure 6**). As a result, in most cases, there was no significant difference in cell proliferation under the experimental conditions performed here when compared to the control with PBS addition, whether liposomes or low-concentration patchouli were added (**Figure 6a)**, lanes 1–5). However, significant differences were observed between the addition of liposomes and high-concentration patchouli samples (**Figure 6a)**, lanes 1 and 6–9). Unexpectedly, the addition of patchouli led to a significant increase in cell proliferation rather than inhibition (**Figure 6a)**, lane 9). Therefore, in this experiment, patchouli's cytotoxicity was weaker than shown in **Figure 3**.

Regarding TARC production, in all cases, it significantly decreased compared to the control (**Figure 6b)**, lanes 1 and 2–9). Firstly, when patchouli was added with ethanol as the vehicle, as expected, a significant inhibition of TARC production was observed compared to the control (**Figure 6b)**, lanes 1 and 5, 9). However, even when liposomes were added alone, there was a significant decrease compared to the control (**Figure 6b)**, lanes 1 and 2, 6; asterisks in the figure are not mentioned). Furthermore, when patchouli at a dilution of 3.0×10^5 was encapsulated in liposomes and added, there was no difference compared to the case without patchouli (**Figure 6b)**, lanes 2 and 3, 4; 'n.s.' in the figure are not mentioned). However, when 1.0×10^5 dilution of patchouli encapsulated in liposomes, there was a significant reduction in TARC production compared to the case without patchouli (**Figure 6b)**, lanes 6 and 8). Nonetheless, at both concentrations, the inhibitory effect of patchouli encapsulated in liposomes was significantly smaller than when patchouli was added with ethanol as the vehicle (**Figure 6b)**, lanes 3, 4 and 5, 7–9).

4. Discussion

In this study, the induction of TARC production that occurs during the exacerbation of AD was mimicked simply using an *in vitro* cell culture system of skin inflammation cells. Essential oils that could inhibit TARC production were selected through TARC ELISA. As a result, seven essential oils - German chamomile, patchouli, Roman chamomile, black pepper, vetiver,

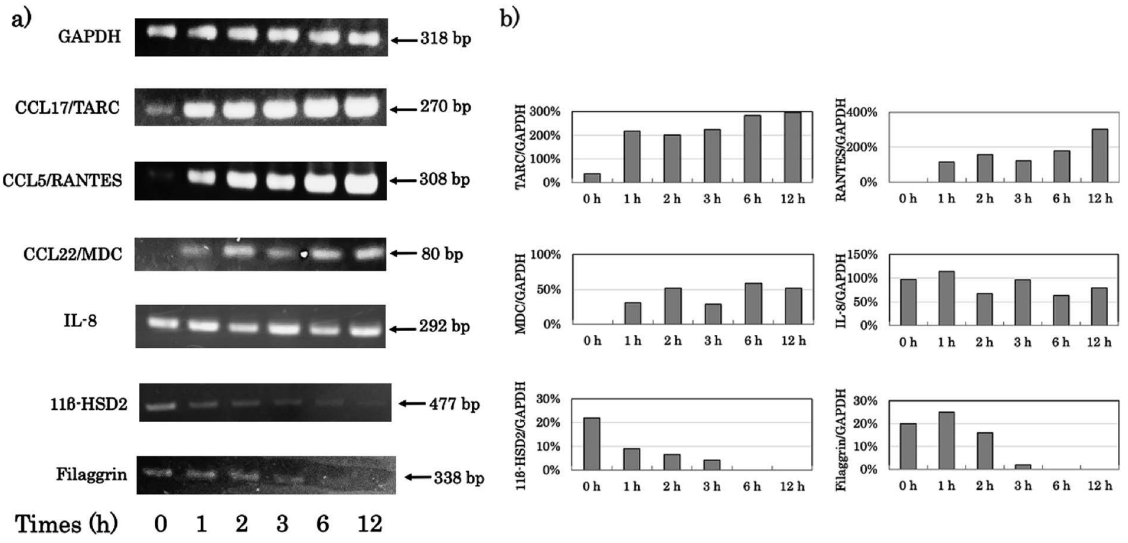

Figure 4. Validation of mRNA expression upon cytokine addition. After the addition of IFN-γ and TNF-α, cells were cultured for 0, 1, 2, 6, and 12 hours, and then cells were collected and RNA was purified. The purified RNA was used as a template to synthesize cDNA, and RT-PCR was performed using specific primers. Subsequently, 10 μL of PCR products were loaded onto a 1.5% agarose gel, electrophoresis was carried out. Panel **a)** shows the electrophoresis image, while panel **b)** illustrates the relative changes in the expression of each gene normalized to the band of GADPH, which is set at 100% at each time point.

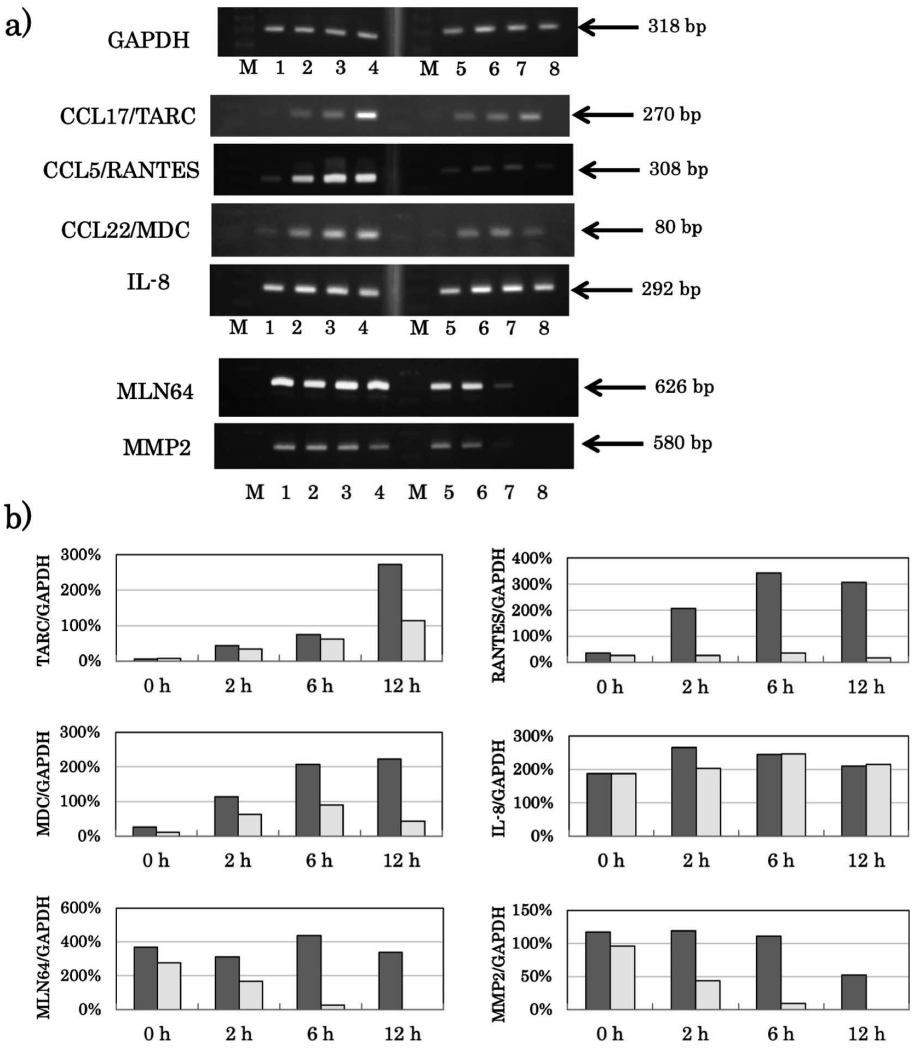

Figure 5. Verification of mRNA expression by RT-PCR. Panel **a)** shows the electrophoresis graph, where lanes 1 to 4 represent the addition of ethanol as a vehicle to the cells, while lanes 5 to 8 represent the addition of the ethanol-diluted patchouli essential oil. The cells were cultured for 0, 2, 6, and 12 hours, and RNA was purified from the collected cells, followed by RT-PCR using the RNA as a template. The resulting products were loaded onto a 1.5% agarose gel, and electrophoresis was performed. 'M' represents the All Purpose Hi-Lo DNA marker (Bionexus^{TM}) added at 3 μL. Panel **b)** illustrates the relative change in the expression of each gene, with the bands of GADPH at each time point normalized to 100%. The black bars represent the addition of ethanol (vehicle) and the white bars represent the addition of ethanol-diluted essential oil.

sage, and white thyme – demonstrated the ability to inhibit TARC production. Among these, German chamomile and patchouli were tested using products obtained from multiple manufacturers and consistently showed inhibition of TARC production at non-cytotoxic concentrations. This inhibition activity is presumed to be due to components commonly found in German chamomile and patchouli essential oils and is not unique to a specific product. However, this study did not investigate which specific components within these essential oils are responsible for the inhibition of TARC production. Previous studies have shown that German chamomile contains anti-inflammatory compounds such as α-bisabolol and chamazulene [17,18], while

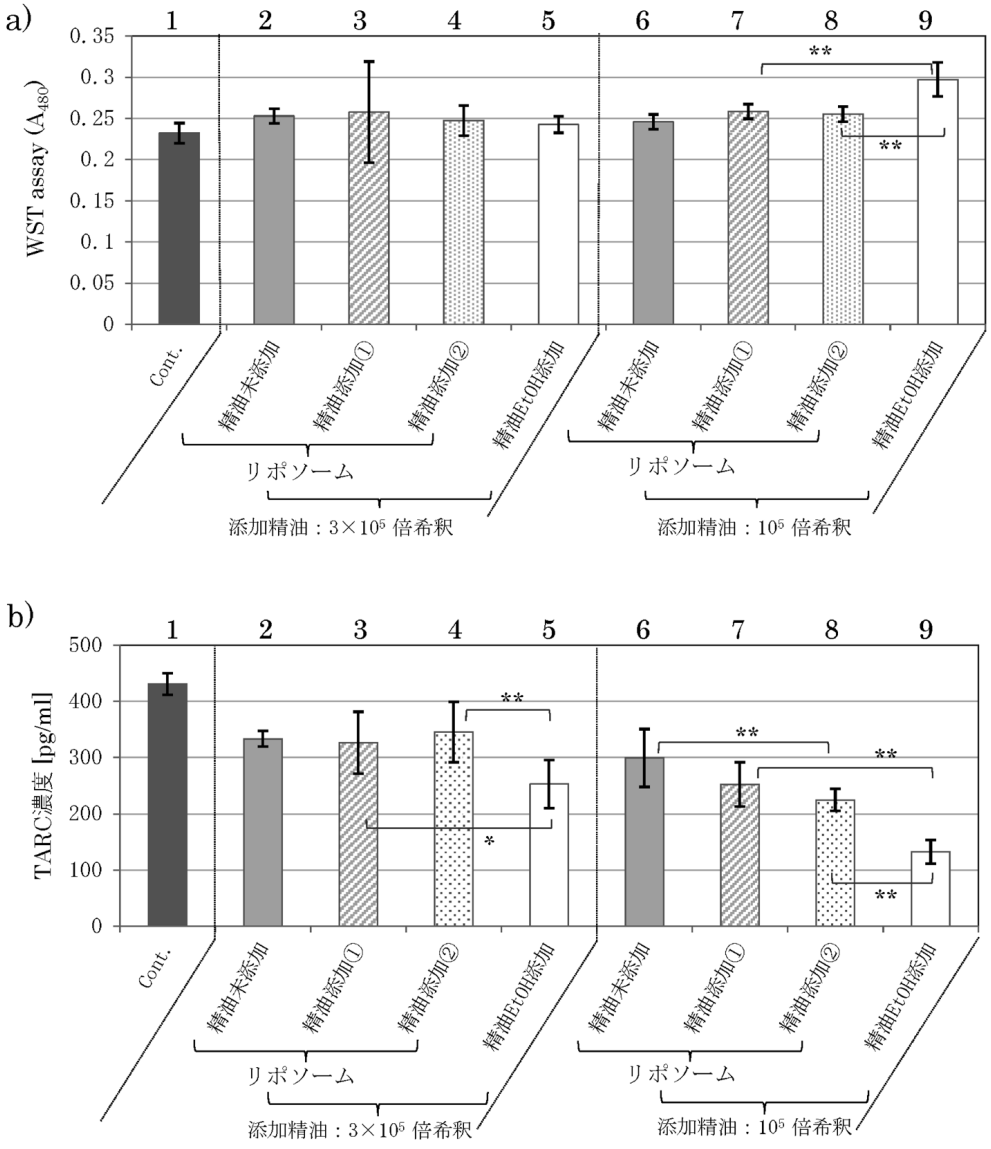

Figure 6. Cell proliferation and the effect on TARC production due to liposome encapsulation. HaCaT cells were seeded in a 96-well culture plate as in the experiment in **Figure 3**. In the control sample, PBS was added as the vehicle (Lane 1). In the 'no essential oil added' samples, only the prepared liposomes were added (Lanes 2 and 6). In the 'essential oil added' and 'essential added and swell with PBS' samples, liposomes containing essential oil were added (Lanes 3, 4, 7, and 8). However, in the 'essential oil EtOH added' samples, the essential oil (patchouli) was dissolved in ethanol and added, similar to the experiment in **Figure 3** (Lanes 5 and 9). WST assay and TARC ELISA were performed as in **Figure 3**. The control was tested in sextuplicates, while the other samples were tested in triplicates, and the error bars represent the standard deviation. ANOVA and Tukey test were conducted, and * indicated *P*-value < 0.05, and ** indicated *P*-value < 0.01. (Significance difference tests were performed for the combinations 2 and 3, 2 and 4, 3 and 5, 4 and 5, 6 and 7, 6 and 8, 7 and 9, and 8 and 9. Only those with significant differences are marked with * or **.)

patchouli contains patchouli alcohol and β-patchoulene, which have excellent anti-inflammatory effects [19,20]. Therefore, there is a possibility that these components contributed to the anti-inflammatory effect in this model. While this is a speculative conclusion, it is important to consider the possibility that the inhibition activity may not be solely due to a single component in each essential oil but rather a synergistic effect of multiple components. Considering the concept of aromatherapy, the focus should not only be on the therapeutic effects of individual components but also the effects of mixtures.

Subsequently, the changes in the expression of genes involved in maintaining the homeostasis of the skin were examined in the skin inflammation cell model of this study. As a result, the addition of IFN-γ and TNF-α induced the production of not only TARC but also chemokines such as CCL5/RANTES and CCL22/MDC, which is consistent with previous research findings [21]. However, no similar induction was observed for IL-8, a chemokine whose production was induced in other studies [21]. According to previous literature, IL-8 mRNA expression in cells upon cytokine addition is relatively low, but some baseline expression was already observed in the HaCaT cells used in this study, indicating a potential abnormality in the cells. Notably, the expression of the filaggrin, an essential gene for the skin's barrier function, was reduced in this *in vitro* skin inflammation cell model. This phenomenon was not observed in HaCaT cells' filaggrin gene expression upon Th cytokine stimulation [22], but reports are suggesting that Th cell cytokines can reduce filaggrin gene expression [23], suggesting a need for further investigation into the expression of genes related to barrier function. In addition, this model system showed a decrease in the gene encoding 11β-HSD2, an enzyme involved in the inactivation of glucocorticoids. This suggests that changes in genes related to the endocrine system can occur in this skin inflammation cell model. For genes with minimal expression changes, it is considered that using more sensitive real-time PCR might provide further insights.

Patchouli, the most stable and prominent inhibitor of TARC production, was also used to examine its effects on the expression of genes involved in skin homeostasis. Patchouli strongly inhibited the production of CCL5/RANTES and CCL22/MDC at the mRNA level, even more than TARC, suggesting that this essential oil has a role in suppressing chemokine production. Of particular interest was its strong suppression of the gene expression of MLN64 and MMP2, which both are involved in glucocorticoid synthesis and cholesterol uptake into mitochondria [24]. This indicated that patchouli suppresses the expression of these proteins in the skin inflammation cell model and affects the gene expression related to the endocrine system in the skin. Moreover, MMP2 is an enzyme that degrades collagen and elastin in the skin, contributing to skin aging [25]. Hence, patchouli may contribute not only to the suppression of inflammation but also to the prevention of skin aging.

In this study, the possibility of cellular uptake of essential oils by using liposomes instead of ethanol as the vehicle was considered, with the actual application of essential oils to the skin in mind. However, liposomes did not show more efficient inhibition of TARC induction than ethanol. It might be easier to introduce essential oils into cells by directly dissolving them in a solvent. Nevertheless, this experiment was preliminary, and it was not possible to examine the detailed localization of patchouli when encapsulated in liposomes or taken up by cells. Therefore, in the future, it will be necessary to investigate the step-by-step process of patchouli encapsulation into liposomes and cellular uptake.

This study demonstrated that several essential oils can inhibit the chemokine TARC induction, a factor in the exacerbation of AD. Patchouli, which was extensively examined as a representative example, was shown to influence the gene expression in the skin inflammation cell model. Although liposomes were not shown to inhibit inflammation better than ethanol in this study, considering the irritative nature of solvents, the introduction of liposomes remains an attractive avenue. It is necessary to devise a more stable and

efficient method than the one used in this study. Based on these findings, conducting similar research on other promising essential oils may contribute to improving the quality of life for AD patients suffering from itching. Furthermore, many related genes that were not investigated in this study remain to be explored. Thus, further investigations into the impact of essential oils on the expression of various genes may shed light on their effects and mechanisms.

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Disclosure statement

The authors declare no conflict of interest.

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