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# Anti-inflammatory effect of essential oil extracted from *Pinus densiflora* (Sieb. et Zucc.) wood on RBL-2H3 cells

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## Abstract

The aim of this study is to identify the active compounds of the essential oil extracted from the *Pinus densiflora* (Sieb. et Zucc.) wood using the hydrodistillation method and evaluate their anti-inflammatory activity. The chemical composition of the oil was identified by GC–MS analysis, and its anti-inflammatory activity was assessed by investigating its effect on the expression of interleukin-4 (IL-4), interleukin-13 (IL-13), and  $\beta$ -hexosaminidase in lipopolysaccharide (LPS)-stimulated RBL-2H3 cells. Treatment of the LPS-stimulated RBL-2H3 cells with the oil and its fractions down-regulated the production of pro-inflammatory cytokines such as IL-4 and IL-13 and further attenuated the secretion of  $\beta$ -hexosaminidase out of the cells to a significant level. Among the five obtained fractions, fraction E exhibited the best anti-inflammatory activity, and its main constituent, longifolene, was considered as the active compound. Moreover, the inhibitory effect of longifolene on the expression levels of IL-4 and IL-13 and the  $\beta$ -hexosaminidase secretion was similar to that of the *P. densiflora* wood oil, indicating longifolene as the active constituent of the *P. densiflora* wood oil with immunosuppressive effects on inflammation.

**Keywords:** Allergy, Essential oil, Inflammation, Terpene, *Pinus densiflora*, Longifolene

## Introduction

Allergic diseases are mainly caused by hypersensitive response (allergy) to external materials, including bronchial asthma, allergic rhinitis, and atopic dermatitis, and they are one of the most common chronic diseases worldwide [1]. The prevalence of allergic diseases is rapidly increasing because of the rapid development of modern society and the change in personal lifestyle. The most common pharmacological therapies for allergic diseases include inhibitors of chemical mediators that are involved in the allergic reaction or anti-inflammatory drugs that suppress the tissues' inflammation [2]. However, these pharmacological therapies can only improve symptoms when the drug is administered continuously,

and they may also cause side effects. Therefore, further research is still needed, especially on natural resources, for a longer-term therapeutic effect, while limiting undesired side issues.

*Pinus densiflora* (Sieb. et Zucc.) occupies one-quarter of the total forest area in the Republic of Korea, with a high percentage of single species [3]. All pine parts have been used, including leaves, cones, pollen, and bark [4–7]. Pine needles have been used for the treatment of adult diseases such as neuralgia, diabetes mellitus, hypertension, and skin disease in herbal or folk medicine [8]. In addition, pine leaf extracts have been reported to be useful for treating oxidative damage [9].

Essential oils are secondary plant metabolites, which are volatile aromatic substances that consist of bioactive compounds synthesized as self-defense agents against external environmental threats. On the basis of earlier studies, essential oils have antimicrobial effects and may increase memory and immunity, and they can also relieve

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stress and tension [10, 11]. Therefore, in recent years, the exploration of functional materials derived from natural sources and the in-depth investigation of the various bioactivities of essential oils have attracted increasing interest. In particular, essential oils from pine leaves and branches have been reported to have antioxidant and anti-aging activity [12], and it has been demonstrated that the pine leaf essential oil has antimicrobial effects against Gram-positive and Gram-negative bacteria, as well as anticancer activity against the YD-8 human oral squamous cell carcinoma [13, 14]. However, the existing research is limited to the efficacy of the essential oils extracted from pine leaves and branches.

Although pine wood is being extensively used for furniture, flooring, and interior materials, there is limited research and scientific evidence on its benefits to the human body. Previous studies have revealed that pine wood inhibited the expression of inflammatory cytokines (COX-2, TNF- $\alpha$ , IL-1 $\beta$ , and IL-13) in peripheral blood mononuclear cells of a mice model that caused inflammation [15]. To confirm that the anti-inflammatory effect was due to the volatile compounds produced by pine wood, the corresponding essential oil was extracted and evaluated [16]. Although it has been proven that the IL-4 and IL-13 expression and the  $\beta$ -hexosaminidase secretion were effectively inhibited, the active constituents of the pine wood essential oil with effective anti-inflammatory activity were not identified. Therefore, this study aims to elucidate the active compounds of the pine wood essential oil and evaluate their anti-inflammatory effect.

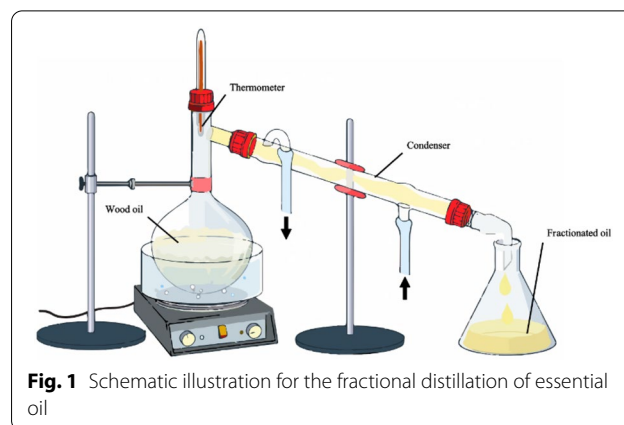
## Materials and methods

### Materials

The wood of *Pinus densiflora* Siebold & Zuccarini was collected from Gapyeong, Gyeonggi province of Korea, in December 2014. The wood was milled, and the obtained sawdust was used for further studies. (+)-longifolene (Cat #. 5395 S, with  $\geq 95\%$  purity) was purchased from Extrasynthese (Rue Jacquard, Genay, France).

### Extraction of the essential oil

The obtained sawdust of the *P. densiflora* wood was hydrodistilled at atmospheric pressure using a Clevenger-type apparatus. A 10-L round-bottom flask containing 1.0 kg of sawdust was placed in a digital heating mantle (MS-DM 608, MTOPS®, Yangju, Korea), and 6.0 L of distilled water was poured in the flask, which was then connected to the Clevenger-type apparatus. The sawdust was extracted for 17 h. The collected essential oil was dried over anhydrous sodium sulfate (SAMCHUN, 98.5%) and filtered through a 0.45- $\mu$ m membrane disk filter. The essential oil was then transferred to dark vials and stored at 4 °C for further



**Fig. 1** Schematic illustration for the fractional distillation of essential oil

**Table 1** Fractionation conditions of the essential oil of *P. densiflora* wood

| Oil fraction | Temperature range |
|--------------|-------------------|
| A            | 23–65 °C          |
| B            | 65–70 °C          |
| C            | 70–75 °C          |
| D            | 75–80 °C          |
| E            | Residues          |

analysis. The yield of the essential oils was calculated using the equation: essential oil yield (%(w/w)) =  $\frac{[\text{mass of essential oil obtained (g)}]}{[\text{mass of over dry matter (g)}]} \times 100$ .

### Fractional distillation of the essential oil

The essential oil was fractionated by fractional distillation, which was based on the difference in the boiling point values of the substances under atmospheric pressure conditions (Fig. 1).

The oil fractions (A, B, C, D, and E) obtained depending on the temperature conditions are presented in Table 1.

### Cell culture

The rat basophilic leukemia cell line RBL-2H3 was purchased from the Korea Cell line bank (Seoul, Korea). The RBL-2H3 cells (CRL-2256™; American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco, Life Technologies, Carlsbad, CA, USA), 1% penicillin–streptomycin (Gibco), and 0.4  $\mu$ L/mL of plasmocin (pH 7.4; Invivogen, San Diego, CA, USA) at 37 °C in a 5% CO<sub>2</sub> incubator (Panasonic, MCO-19AIC).

### GC–MS analysis

For the identification of the volatile components, the oil samples were analyzed by GC–MS (Trace 1300/ISQ-LT, ThermoScientific, USA) equipped with a DB-5MS capillary column (30 m × 0.25 mm, 0.25 µm; ThermoScientific, USA). The injector temperature was 250 °C, and helium was used as the carrier gas with a flow rate of 1 mL/min. The oven temperature was set at 40 °C for 3 min, followed by an increase to 200 °C with a heating rate of 3 °C/min. Then, the temperature was raised by 15 °C/min to 340 °C, where it was maintained for 10 min. The FID and mass spectra of the detected compounds were recorded. The flame ionization detector (FID) temperature was maintained at 280 °C with a helium flow rate of 40 mL/min. The temperatures of the interface of the mass spectrometer and the ion source were maintained at 280 °C and 250 °C, respectively. The mass spectra were acquired at a rate of 0.2 scans/s with an EI-scan range of 35–360 *m/z*. The analyzed components of the essential oils were selected based on the peaks with the highest spectral matching when the signal-to-noise (S/N) ratio reached  $\geq 100$  in total-ion chromatography using the NIST library search program (version 11; National Institute of Standards and Technology, USA). The identification of the Kovats retention index (KI) of the individual compounds was based on the comparison of their relative retention times with an *n*-alkanes mixture (C<sub>8</sub>–C<sub>20</sub>, Sigma-Aldrich) in the DB-5 column.

### Cytotoxic activity

The 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was applied to investigate the cytotoxicity of the pine wood essential oil and its fractions (A–E) on RBL-2H3 cells. Briefly, the oil fractions and single compounds were prepared in dimethyl sulfoxide (DMSO). A stock solution of the ingredients to be tested was prepared by diluting 8 µg/mL ingredients in 1 mL DMSO. In particular, the RBL-2H3 cells were seeded into a 96-well plate at  $8 \times 10^3$  cells/well and incubated in an incubator at 37 °C and 5% CO<sub>2</sub> for 24 h. The cells were then treated with pine wood oil and fractions A–E at a concentration of 0.08–8 µg/mL in an incubator (37 °C) for 24 h. After removing the MTT reagent and washing with Dulbecco's phosphate-buffered saline (DPBS; Gibco), 100 µL of DMSO (Sigma) was added to each well and then stored at room temperature for 10 min. A spectrophotometer (Epoch, model Take 3; BioTek Inc., Winooski, VT, USA) was used to measure the absorbance of the cells treated with wood oils at 540 nm. The percentage of the viable cells was calculated based on the following equation: (% viable cells) = [(absorbance of

the treated cells – absorbance of the blank)/(absorbance of the control – absorbance of the blank)] × 100.

### Evaluation of the IL-4 and IL-13 cytokine levels

The RBL-2H3 cells were grown on six-well plates ( $3 \times 10^5$  cell/well) for 24 h. The cells were treated with 1 µg/mL LPS (Sigma-Aldrich) for 1 h and then washed twice with DPBS (Gibco). The cells were treated with dexamethasone and wood oils for 24 h. Total RNA was extracted from RBL-2H3 cells using the TRIzol reagent (Invitrogen, Life Technologies) following the manufacturer's instructions. The RNA concentration was measured using a microplate spectrophotometer (Epoch, model Take 3; BioTek Inc., Winooski, VT, USA) at 260 nm. Total RNA was reverse transcribed into first-strand complementary DNA (cDNA) using M-MLV reverse transcriptase (Invitrogen, Life Technologies) and random primers (9-mer; Takara Bio Inc., Shiga, Japan). Each cDNA sample was amplified with 2× SYBR® Premix Ex Taq™ (Takara Bio Inc.) and 3 pmol of each primer. The amplification was performed using a 7300 real-time polymerase chain reaction (PCR) system (Applied Biosystems, Foster, CA, USA) with the following parameters: (i) denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, (ii) annealing at 60 °C for 30 s, (iii) extension at 72 °C for 30 s, and (iv) final elongation at 72 °C for 10 min. The sequences of the oligonucleotide primers are presented in Table 2. The relative expression IL-4 and IL-13 levels in each sample (normalized to that of 18S) were determined using the RQ software (version 1.3, Applied Biosystems).

### β-Hexosaminidase secretion assay

The RBL-2H3 cells were grown on six-well plates with a density of  $2 \times 10^5$  cells for 24 h. The cells were then treated with 2,4-dinitrophenyl (DNP)-specific IgE (800 ng/mL, Sigma, USA) overnight. To remove the excess IgE excess to stimulation, the cells were washed twice with the Tyrodes' assay buffer [119 mM NaCl, 4.74 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.19 mM MgSO<sub>4</sub>, 10 mM

**Table 2** Oligonucleotide primer sequences used for quantitative real-time polymerase chain reaction

| Gene  | Primer sequence (5'-3')  |
|-------|--|
| IL-4  | F: TGA TGT ACC TCC GTG CTT GA<br>R: AGG ACA TGG AAG TGC AGG AC |
| IL-13 | F: CTG GAA TCC CTG ACC AAC AT<br>R: CCA TAG CGG AAA AGT TGC TT |
| 18S   | F: CTC AAC ACG GGA AAC CTC AC<br>R: CGC TCC ACC AAC TAA GAA CG |

F forward, R reverse

4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), 5 mM glucose, and 0.1% (w/v) BSA, pH 7.3]. The cells were then stimulated with DNP-BSA (Thermo Fisher Scientific), suspended in 500  $\mu$ L extracellular buffer with 0.1% BSA and incubated at 37 °C for 1 h. After incubation, 50  $\mu$ L of the supernatant was incubated with 200  $\mu$ L of *p*-nitrophenyl-*N*-acetyl- $\beta$ -D-glucosaminide (1 mM, Sigma-Aldrich) in 0.05 M citrate buffer (pH 4.5) at 37 °C for 1 h. The enzyme reaction was terminated by adding 500  $\mu$ L of sodium carbonate buffer (0.05 M; pH 10.0), and the optical density of each reaction was recorded at 405 nm.

#### Enzyme-linked immunosorbent assay (ELISA)

The RBL-2H3 cells were seeded into a 96-well plate at  $8 \times 10^3$  cells/well and incubated at 37 °C in 5% CO<sub>2</sub> incubator for 24 h. The cells were stimulated with 1  $\mu$ g/mL LPS for 1 h, and then treated with wood oil (0.08–8  $\mu$ g/mL) and longifolene (0.008–0.8  $\mu$ g/mL) in an incubator for 24 h. After incubation, the cells were washed twice with DPBS. The production of IL-4 and IL-13 in cell culture supernatants was quantified by commercial ELISA kits (Invitrogen), according to the manufacturer's instructions. The cytokine concentrations were determined using standards provided within each kit.

#### Statistical analysis

All samples were analyzed in triplicate, and the results are presented as mean  $\pm$  SD values. The statistical significance between groups was analyzed using the R x64 4.0.3 program, and *p* values lower than 0.05 or 0.01 were considered statistically significant.

## Results and discussion

#### Yield and chemical composition

The yield of the essential oil extracted from *P. densiflora* wood was 0.75% (g/g). Moreover, the yield of the pine wood oil was higher than that of the essential oil extracted from the leaves of the Pinus species [17, 18]. *P. densiflora* wood oil was divided into five fractions (A–E) using fractional distillation. The yields of the fractions are presented in Table 3.

The chemical composition of the essential oil was identified by GC–MS. As observed in Table 4, the oil consisted mainly of monoterpenes and sesquiterpenes either in their oxygenated or hydrocarbon forms, and the major individual compounds were  $\alpha$ -pinene (49.64%),  $\beta$ -pinene (11.87%), sabinene (12.97%), and longifolene (10.51%).

Fractional distillation of pine wood oil was applied based on the boiling point difference, and five fractions were obtained (A–E; Table 1). On the basis of the data of Table 4, fractions A, B, and C consisted mainly of  $\alpha$ -pinene,  $\beta$ -pinene, and sabinene, and  $\alpha$ -pinene was

**Table 3** Yields of the fractions (A–E) isolated from *P. densiflora* wood oil

| Oil        | Weight of fraction (g) | Yield (%) |
|------------|------------------------|-----------|
| Fraction A | 13.73                  | 43.9      |
| Fraction B | 6.12                   | 19.6      |
| Fraction C | 5.18                   | 16.5      |
| Fraction D | 1.76                   | 5.6       |
| Fraction E | 2.79                   | 8.9       |

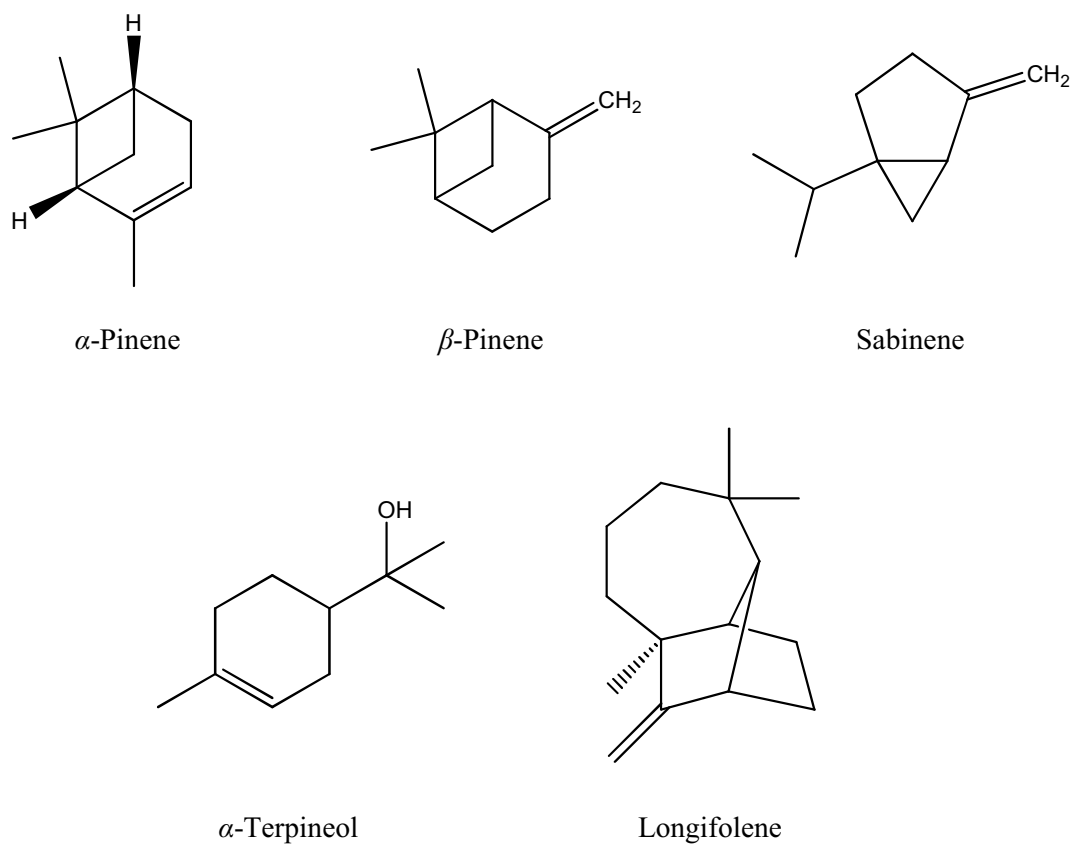
the main component, with GC–MS areas of 68.51%, 59.82%, and 44.59% for A, B, and C, respectively. It was also observed that the contents of monoterpene hydrocarbons in fractions A, B, and C were higher than those of sesquiterpenes. By contrast, the major constituents of fraction D were longifolene (19.71%),  $\alpha$ -terpineol (19.18%), and sabinene (13.53%), and in fraction E, longifolene (63.71%) was the predominant component. Moreover, fraction E consisted mainly of sesquiterpene hydrocarbons, and no monoterpenes were detected. The structure of the main components according to the fractions is shown in Fig. 2.

In pine cone oil, sesquiterpenes such as germacrene D,  $\beta$ -caryophyllene,  $\delta$ -cadinene, and longifolene accounted for a high proportion [19]. Germacrene D, a sesquiterpene type, was the main compound in needle oil, and  $\alpha$ -pinene and  $\beta$ -phellandrene were also identified in pine needle oils [20]. Moreover, germacrene D is one of the main ingredients of pine needle and corn essential oils, and  $\alpha$ -pinene is the main constituent of the pine wood essential oil.

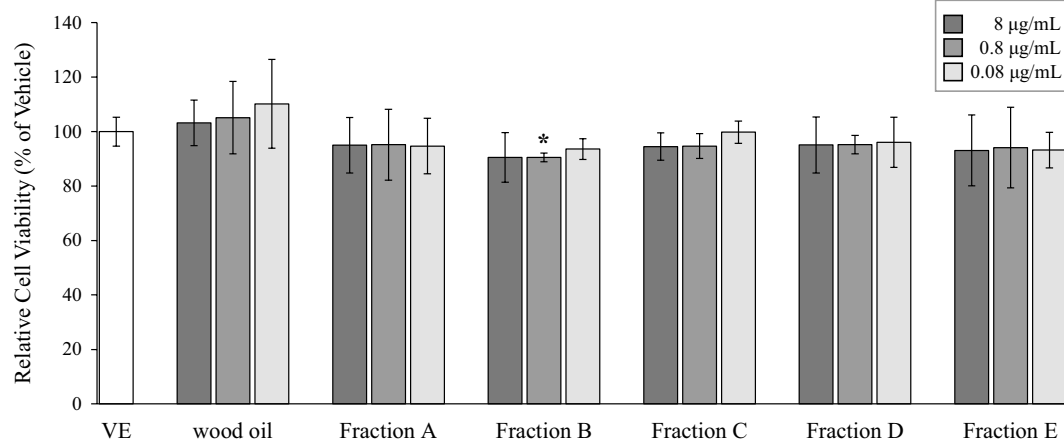
#### Cell viability

The MTT assay was used to evaluate the cytotoxicity of the wood essential oil and its fractions against RBL-2H3 cells. On the basis of the results presented in Fig. 3, the cell viability was higher than 80% at oil concentrations of 0.08–8  $\mu$ g/mL, and almost no cytotoxicity was observed at concentrations lower than 8  $\mu$ g/mL in RBL-2H3. Thus, in order to evaluate the anti-inflammatory effect of the essential oil, the RBL-2H3 cells were treated using a concentration range of 0.08–8  $\mu$ g/mL.

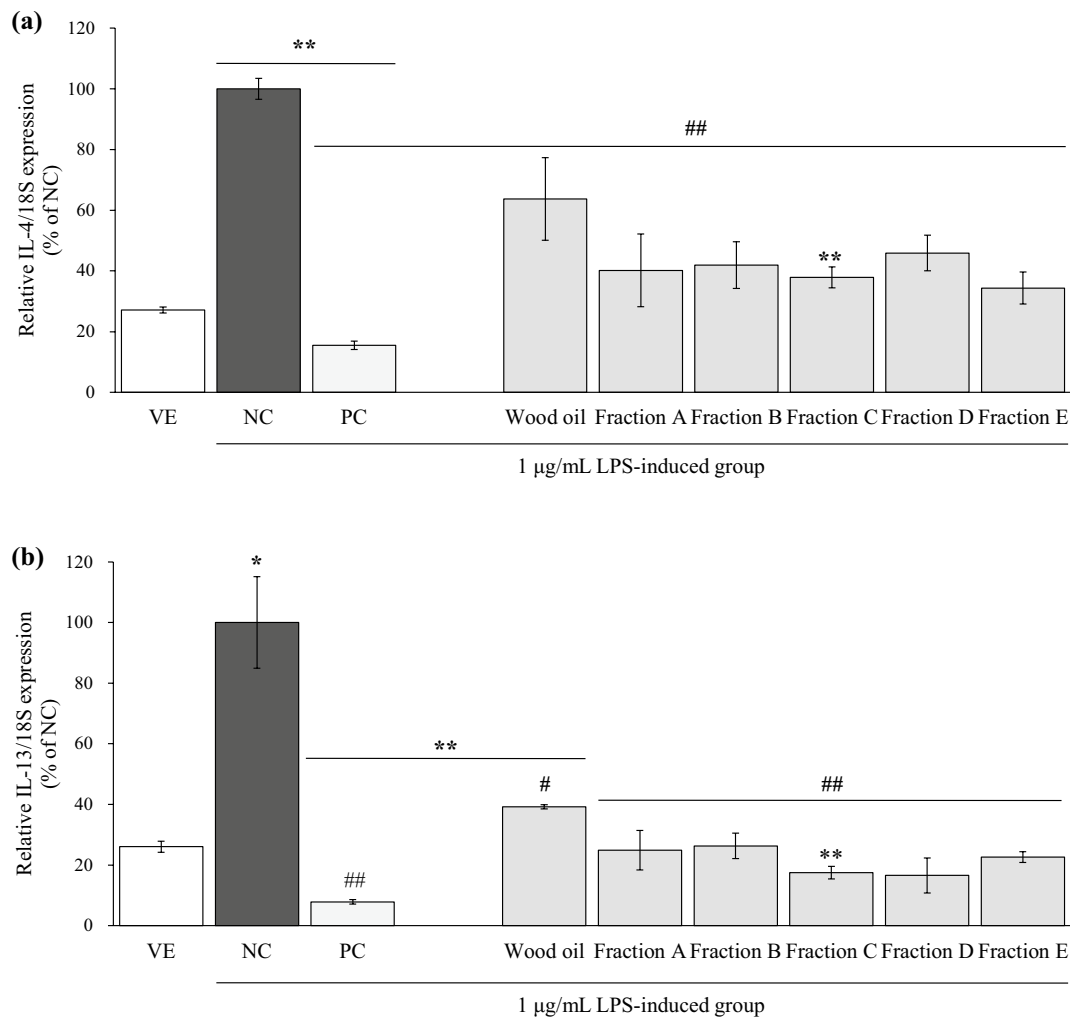
Among the available in vitro chemosensitivity testing methods, the MTT assay is very useful for analyzing cell proliferation and cytotoxicity in vitro [21]. In particular, the mitochondrial enzyme succinyl dehydrogenase reduces the yellow soluble MTT salt to the insoluble precipitate purple formazan, and the amount of formazan formed is proportional to the number of viable cells. A higher amount of formazan formed through the MTT assay can be determined by a greater absorbance, which, in turn, indicates an enhanced cell activity [22, 23].



**Fig. 2** Chemical structures of the main components in the five fraction oils (A–E)



**Fig. 3** Effects of the pine wood essential oil and its oil fractions on cell viability. VE: vehicle, DMSO. The results are presented as mean  $\pm$  standard deviations. \* $p < 0.05$  compared to VE



**Fig. 4** Effects of the wood essential oil and five oil fractions on **a** IL-4 and **b** IL-13 mRNA expression levels. VE: vehicle, DMSO; NC: negative control, 1 µg/mL LPS-treated group; PC: positive control, 100 nM dexamethasone treated group, an oil fraction (A–F) (0.08 µg/mL). Values are presented as mean ± standard deviation. \* $p < 0.05$  and \*\* $p < 0.01$  compared with VE; and # $p < 0.05$ , ## $p < 0.01$  compared with NC

#### Effect of pine wood oil on the expression of IL-4 and IL-13

The modulation of inflammatory cytokines from mast cells is a key indicator of reduced allergic symptoms, and in particular, the regulation of IL-4 and IL-13 is considered as the most important therapeutic step for allergic inflammation diseases. Therefore, in this study, it was examined whether pine wood oil could regulate the pro-inflammatory cytokines IL-4 and IL-13 in RBL-2H3 cells. Stimulation of the RBL-2H3 cells by LPS significantly increased the relative expression level of IL-4 (Fig. 4a), whereas the relative IL-4 expression was suppressed in LPS-stimulated RBL-2H3 cells treated with dexamethasone, a steroidal inflammatory drug. Pine wood oil at a 0.08 µg/mL concentration also inhibited the IL-4 secretion from the LPS-stimulated RBL-2H3 cells, but the best

inhibitory activity was observed for fraction E, which was, however, less active than dexamethasone. A remarkable increase in the IL-13 relative expression was also observed compared to the vehicle group (VE, DMSO) when the RBL-2H3 cells were treated with LPS, whereas the IL-13 relative expression levels were significantly reduced after treatment of the cells with dexamethasone, the wood essential oil, and oil fractions A–E (Fig. 4b).

Mast cells produce mediators of allergic inflammatory responses by external stimuli. Prior to stimulation, these mediators are stabilized by ionic bonds with proteoglycans in the granules. However, upon stimulation, these ionic bonds are cleaved, and the chemical constituents are released out of the cell. Antigens and IgE react with the high-affinity IgE receptor (FcεRI) on the surface of



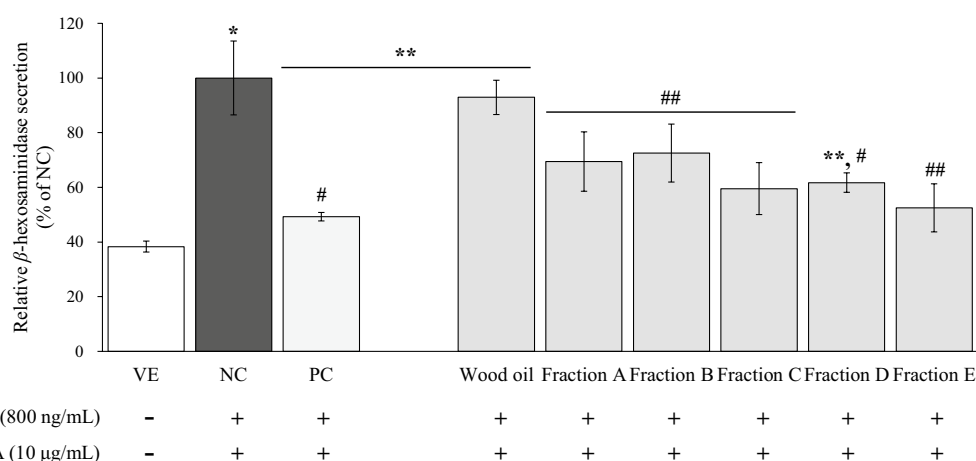
mast cells, thus inducing allergic inflammatory reactions by secreting pro-inflammatory mediators such as histamine and cytokines in granules [24, 25]. Two cytokines, namely, IL-4 and IL-13, secreted by Th2 have been reported to be directly or indirectly related to allergic

reactions and are typical markers for allergic reactions [26].

On the basis of the reason, their anti-inflammatory activity is suggested. Moreover, among the oil fractions, fraction E exhibited the best anti-inflammatory

**Table 4** Compositions of the major classes of the compounds identified in the essential oils

| Classification             | Compound               | Area % |       |       |       |       | KI    |
|----------------------------|------------------------|--------|-------|-------|-------|-------|-------|
|                            |                        | Wood   | A     | B     | C     | D     | E     |
| Monoterpene hydrocarbons   | $\alpha$ -Pinene       | 49.64  | 68.51 | 59.82 | 44.59 | 6.33  | 930   |
|                            | Camphene               |        | 1.06  |       | 0.86  |       | 943   |
|                            | $\beta$ -Pinene        | 11.87  | 14.16 | 15.92 | 16.92 | 3.38  | 971   |
|                            | Myrcene                |        |       |       | 0.90  |       | 990   |
|                            | Sabinene               | 12.97  | 10.19 | 15.44 | 24.85 | 13.53 | 1025  |
| Oxygenated monoterpenes    | 1-Heptanol             | 3.99   | 4.25  | 4.02  | 3.83  | 5.61  | 5.13  |
|                            | Fenchyl alcohol        |        |       |       |       | 1.37  |       |
|                            | Borneol                |        |       |       |       | 2.41  |       |
|                            | Terpinen-4-ol          |        |       |       |       | 4.00  |       |
|                            | <i>p</i> -Cymenol      |        |       |       |       | 1.52  |       |
| Sesquiterpene hydrocarbons | $\alpha$ -Terpineol    | 1.55   |       |       | 1.89  | 19.18 |       |
|                            | Longicyclene           |        |       |       |       |       | 2.86  |
|                            | Sativen                |        |       |       |       |       | 1.85  |
|                            | Longifolene            | 10.51  |       |       | 1.09  | 19.71 | 63.71 |
|                            | $\beta$ -Caryophyllene |        |       |       |       |       | 2.73  |
| Oxygenated sesquiterpenes  | $\delta$ -Cadinene     |        |       |       |       |       | 1.94  |
|                            | Longiborneol           |        |       |       |       |       | 4.23  |
| Diterpene                  | Cembrene               | 1.31   |       |       |       |       | 1.13  |
| Unknown compounds          |                        | 8.15   | 1.83  | 4.80  | 5.07  | 22.97 | 16.40 |

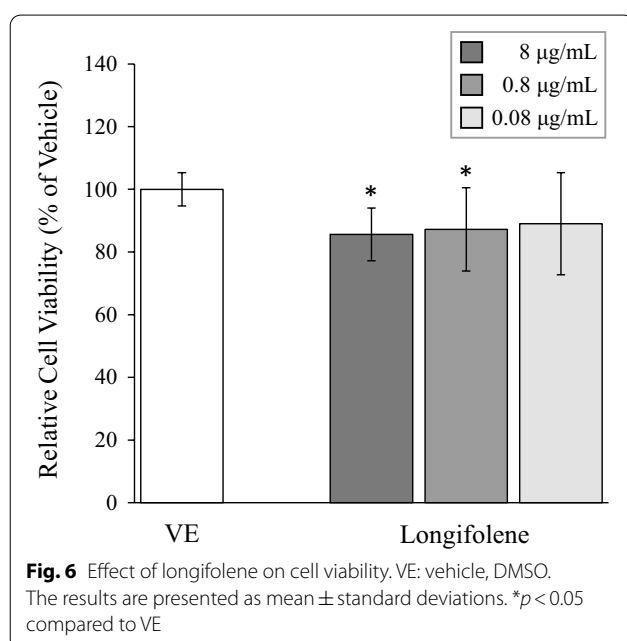


**Fig. 5** Inhibitory effect of the wood essential oil and five oil fractions on  $\beta$ -hexosaminidase release. VE: vehicle, DMSO; NC: negative control, 800 ng/mL DNP-IgE and 10  $\mu$ g/mL DNP-BSA treated group; PC: positive control, 100 nM dexamethasone treated group, an oil fraction (A–F) (0.08  $\mu$ g/mL). Values are presented as mean  $\pm$  standard deviation. \* $p$  < 0.05 and \*\* $p$  < 0.01 compared with VE; and # $p$  < 0.05, ## $p$  < 0.01 compared with NC

activity, probably because of its high content in longifolene (Table 4).

#### Effect of pine wood oil on $\beta$ -hexosaminidase release

$\beta$ -Hexosaminidase exists in granules of basophils or mast cells and is secreted out of the cells because of allergic reactions. The secretion of  $\beta$ -hexosaminidase can also be employed to indicate and quantify the extent of degranulation [27]. The effect of pine wood oil on the release of  $\beta$ -hexosaminidase is illustrated in Fig. 5. In the negative control group (NC), stimulated with DNP-BSA and with promoted degranulation, the  $\beta$ -hexosaminidase secretion was about three times higher than that of the VE. However, treatment of the inflammatory-induced RBL-2H3 cells with dexamethasone resulted in a significant decrease in the relative secretion of  $\beta$ -hexosaminidase compared to the NC. Similarly, the  $\beta$ -hexosaminidase secretion was significantly reduced upon treatment with the pine wood essential oil and oil fractions A–E. Consequently, both wood oil and oil fractions exhibited considerable anti-inflammatory activity by inhibiting the  $\beta$ -hexosaminidase secretion from the LPS-induced RBL-2H3 cells. Among them, fraction E was the most potent, with a similar anti-inflammatory effect to that of dexamethasone. Furthermore, among the 13 components of fraction E, longifolene, with a content of 64.26%, was the one that effectively inhibited the degranulation of RBL-2H3 cells and the secretion of inflammatory mediators.



#### Effect of longifolene on the IL-4 and IL-13 expression

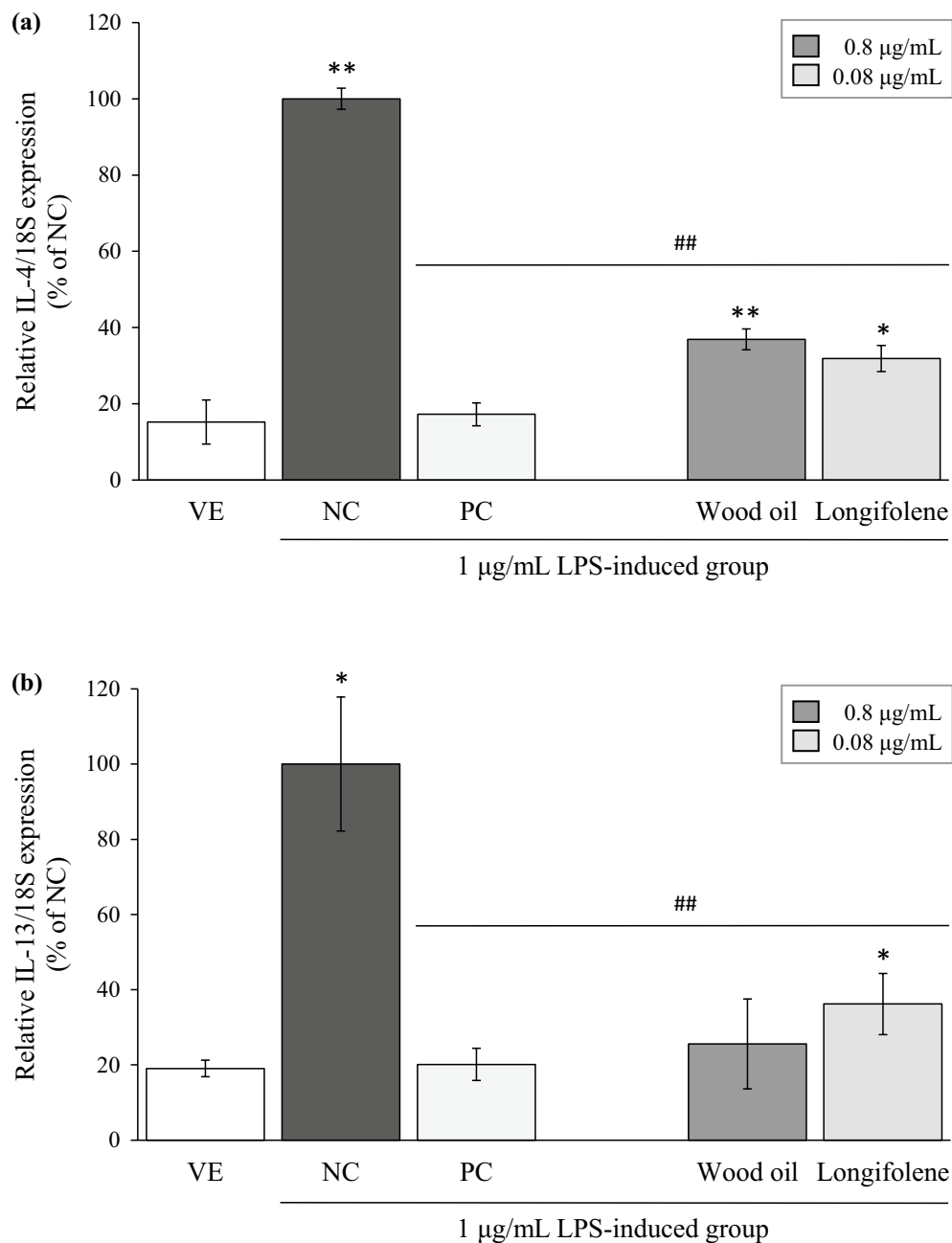
The anti-inflammatory effect of longifolene, the main component of fraction E, which had excellent anti-inflammatory activity, was investigated. In particular, to determine the effect of longifolene on the survival and proliferation of RBL-2H3 cells, the MTT assay was applied by treating the cells with a concentration range of 0.08–8  $\mu$ g/mL. However, longifolene was not cytotoxic at these concentrations when compared with the vehicle group (Fig. 6).

Therefore, a treatment concentration of 0.08  $\mu$ g/mL was used to further evaluate the anti-inflammatory effects of longifolene. Moreover, in order to keep the concentration of longifolene constant, the concentration of the used essential oil was 0.8  $\mu$ g/mL, considering that the longifolene ratio in the essential oil was 10%. Thus, the inflammatory response-induced RBL-2H3 cells were treated with pine wood essential oil and longifolene (Fig. 7). As shown in Fig. 7a, treatment with LPS (NC) increased the relative expression level of IL-4 by more than six times compared to the VE. Upon treatment with dexamethasone (PC), the IL-4 gene expression levels were similar to the VE. In addition, the relative IL-4 expression upon treatment with the pine wood essential oil and longifolene was significantly reduced compared to the NC group, whereas longifolene was more effective than the wood essential oil (Fig. 7a). Similar results were observed regarding the relative expression level of IL-13 (Fig. 7b). More specifically, the pine wood essential oil exhibited a similar activity to dexamethasone (PC), and longifolene exhibited a significant anti-inflammatory effect compared to NC. Nevertheless, in this case, longifolene was less active than the wood essential oil. These results suggest that the anti-inflammatory effect of pine wood oil can be assigned to longifolene, while implying that longifolene could also be effective in regulating Th2 cell activation and inhibiting the pro-inflammatory signal molecules expression, which is effective in allergic inflammatory response.

#### Effect of longifolene on $\beta$ -hexosaminidase release

The effect of longifolene on  $\beta$ -hexosaminidase secretion is graphically illustrated in Fig. 8. The treatment of LPS-induced RBL-2H3 cells with the pine wood essential oil and longifolene significantly suppressed the  $\beta$ -hexosaminidase secretion, indicating their anti-inflammatory activity. However, stimulation of the RBL-2H3 cells with DNP-BSA promoted degranulation and significantly increased the  $\beta$ -hexosaminidase secretion (NC). Moreover, it was proven that the inhibitory effect of longifolene was similar to that of the pine wood essential oil, thus indicating longifolene as





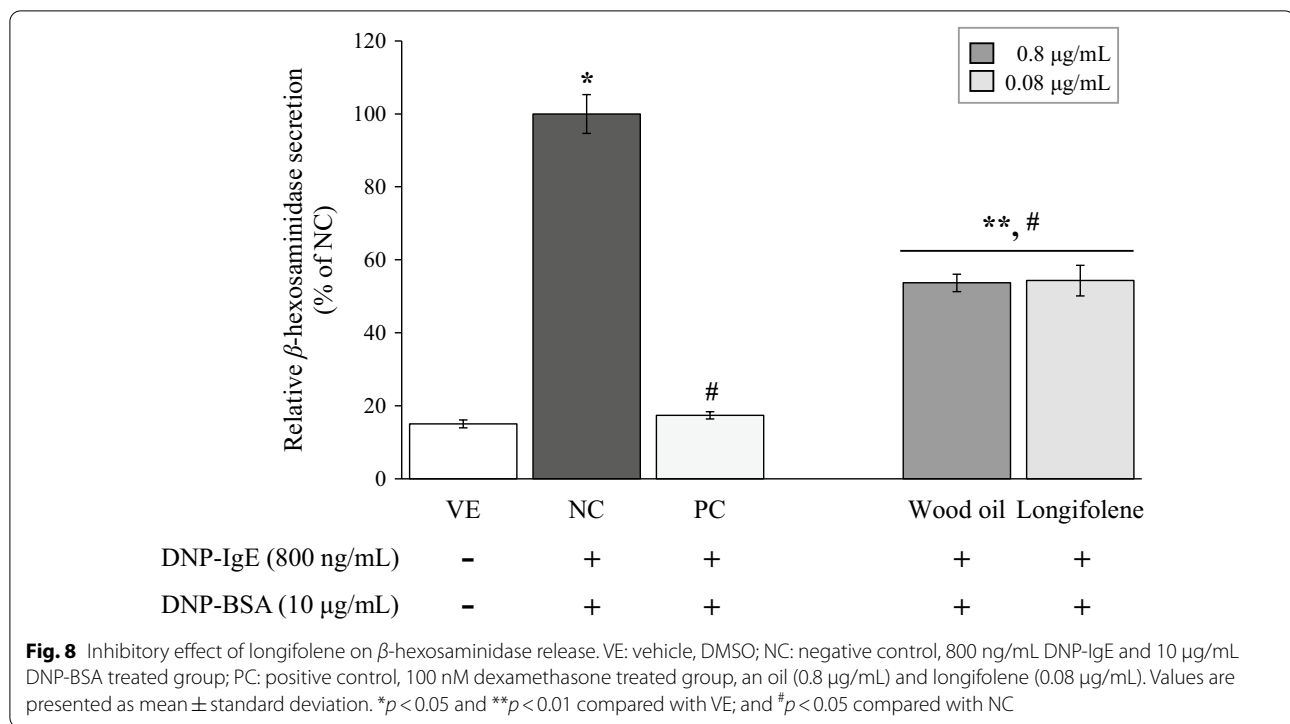
**Fig. 7** Effect of longifolene on pro-inflammatory cytokine mRNA expression. Level of **a** IL-4, **b** IL-13. VE: vehicle, DMSO; NC: negative control, 1 µg/mL LPS-treated group; PC: positive control, 100 nM dexamethasone treated group, an oil (0.8 µg/mL) and longifolene (0.08 µg/mL). Values are presented as mean ± standard deviation. \* $p < 0.05$  and \*\* $p < 0.01$  compared with VE; and ## $p < 0.01$  compared with NC

the potent constituent of the essential oil against the  $\beta$ -hexosaminidase secretion.

#### Protein quantification of anti-inflammatory cytokines

As shown in Fig. 7, the relative IL-4 and IL-13 expression were significantly suppressed in LPS-stimulated

RBL-2H3 cells treated with longifolene (0.08 µg/mL). ELISA assay confirmed that the anti-inflammatory effect of longifolene on inflammatory protein expressions. The effect of longifolene on LPS-induced IL-4 and IL-13 production was compared with wood oil extracted from *P. densiflora* (Fig. 9).



In our study, ELISA revealed that the level of IL-4 and IL-13 in cell culture supernatants were also significantly increased in NC group compared with the VE group. These results indicate that treatment with wood oil and longifolene suppresses the increase in the IL-4 and IL-13 levels. As shown in Fig. 9a, the longifolene was treated in RBL-2H3 cells in which the inflammatory response was induced, the inhibition of IL-4 protein level was similar to the wood oil. The release of IL-4, pro-inflammatory cytokine, from RBL-2H3 cells was markedly suppressed by longifolene in a dose-dependent manner. In particular, the anti-inflammatory activity of longifolene was effective in inhibiting the secretion of IL-13 protein. IL-13 level in NC group (LPS-treated group) were increased, as compared to the VE group. When longifolene was up to 0.8  $\mu$ g/mL that possessed more noticeable anti-inflammatory effect than steroidal inflammatory drug (PC) and wood oil did ( $p < 0.01$ ).

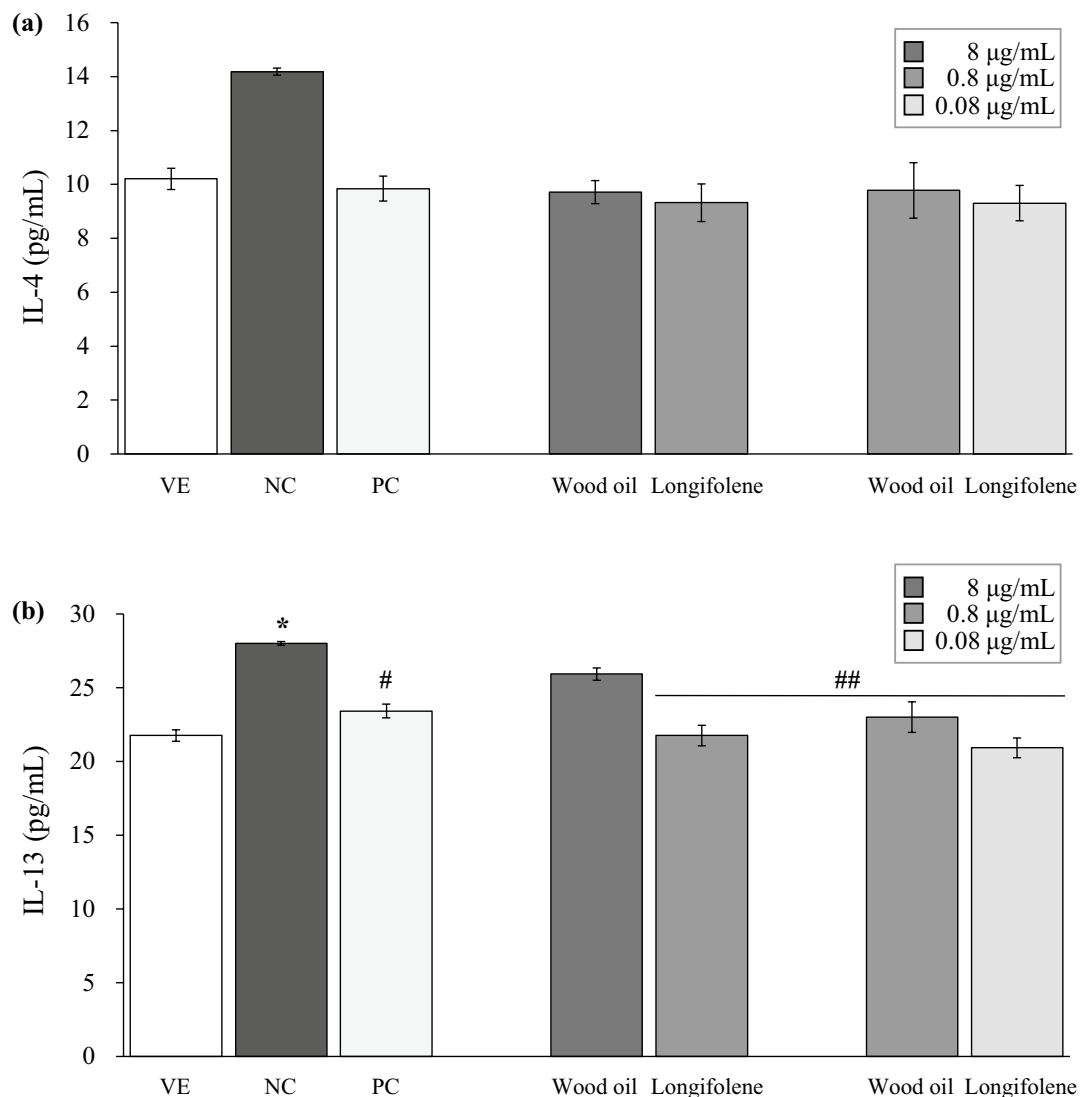
In this study, when longifolene was treated in RBL-2H3 cells in which inflammatory-induced, the protein levels were showed a different tendency than gene expression. There can be several reasons for explaining the difference in tendency between protein and mRNA expression. First, sensitive and reliable quantification of specific proteins requires specific antibodies of high affinity, whereas mRNA can be sensitive quantified by PCR using gene-specific oligonucleotide primers [28].

Second, mRNA is relatively short-lived and is reached at instant steady-state concentration, whereas the concentration of protein in the medium is a result of the protein accumulation [29].

Cytokines are significant importance to mediate inflammatory responses. Our results showed that longifolene could significantly suppressed the protein secretion of IL-4 and IL-13 than steroidal inflammatory drug did. It was indicated that longifolene is the active compound that has anti-inflammatory effect in the essential oil extracted from *P. densiflora* wood. Thus, is worthwhile to exploit longifolene and develop as a new agent for prevention and treatment of inflammatory diseases.

## Conclusion

Although several previous studies have demonstrated the anti-inflammatory effects of pine wood oil, the anti-inflammatory active constituents of the oil have not been identified. Therefore, this study aimed to investigate the constituents in the essential oil of pine wood with effective anti-inflammatory activity. On the basis of the GC-MS analysis, the chemical composition was different in the five oil fractions. The five fractions also exhibited different anti-inflammatory effect. Among the five obtained fractions, fraction E exhibited the best anti-inflammatory activity, and its main constituent, longifolene, was considered as the active compound. Further investigation



**Fig. 9** Effect of longifolene on the protein expression levels of **a** IL-4 and **b** IL-13 in LPS-stimulated RBL-2H3 cells. VE: vehicle, DMSO; NC: negative control, 1 µg/mL LPS-treated group; PC: positive control, 100 nM dexamethasone treated group. Values are presented as mean  $\pm$  standard deviation. \* $p < 0.05$  and \*\* $p < 0.01$  compared with VE; and # $p < 0.05$ , ## $p < 0.01$  compared with NC

of longifolene revealed that its anti-inflammatory activity was comparable with that of the pine wood oil. The inhibitory effect of longifolene on the expression levels of IL-4 and IL-13 and the  $\beta$ -hexosaminidase secretion was similar to that of the *P. densiflora* wood oil, indicating longifolene as the active constituent of the *P. densiflora* wood oil with immunosuppressive effects on inflammation. On the basis of the results of an earlier study, we found that the anti-inflammatory effect of the essential oil extracted from pine wood was due to its essential oil components. The anti-inflammatory activity of longifolene was also confirmed, and it may be useful for the prevention and improvement of inflammatory diseases,

while setting also the basis for the future utilization of pine wood and longifolene.

#### Abbreviations

BSA: Bovine serum albumin; cDNA: Complimentary deoxyribonucleic acid; DNP: Dinitrophenyl; ELISA: Enzyme-linked immunosorbent assay; FBS: Fetal bovine serum; FID: Flame ionization detector; GC-MS: Gas chromatography-mass spectrometry; IgE: Immunoglobulin E; IL: Interleukin; KI: Kovats retention index; LPS: Lipopolysaccharide; M-MLV: Moloney murine leukemia virus; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NIST: National Institute of Standards and Technology; NC: Negative control; NF- $\kappa$ B: Nuclear factor kappa light chain enhancer of activated B cells; OD: Optical density; PCR: Polymerase chain reaction; RBL: Rat basophilic leukemia; RNA: Ribonucleic acid; Th2: T helper type 2; VE: Vehicle.

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## Authors' contributions

All authors contributed to preparing the research protocol. JY performed the experiments and manuscript writing. W-SC performed data analysis by GC-MS. E-BJ and K-JK designed and coordinated the experiments. M-JP performed discussion and reviewed the manuscript. All authors read and approved the final manuscript.

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## Availability of data and materials

All data generated or analyzed during this study are included in this published article. Additional data are available from the corresponding author on reasonable request.

## Declarations

### Competing interests

The authors declare no competing interest associated with this manuscript.

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