

**THE EFFECT OF ANTIOXIDANT ACTIVITY, CYTOTOXICITY, AND
INFLAMMATORY CYTOKINE PRODUCTION OF ELEVEN ESSENTIAL OILS**Sang Nam Park^{1,a}, Yong Lim^{2,a}, Un Kyu Park¹, Ji Hye Oh¹, Man Kyu Huh^{3*} and Seock-Yeon Hwang^{1*}¹Department of Biomedical Laboratory Science, Daejeon University, Daejeon 34520, Republic of Korea.²Department of Clinical Laboratory Science, Dong-eui University, Busan 47340, Republic of Korea.³Food Science and Technology Major, Dong-eui University, Busan 47340, Republic of Korea.^aThese authors contributed equally to this study.***Corresponding Author: Dr. Man Kyu Huh and Dr. Seock-Yeon Hwang**

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ABSTRACT

Essential oils are complex mixtures of several components endowed with a wide range of biological activities. The objectives of this study were to chemically characterize and evaluate the antioxidant activity, cytotoxicity, and inflammatory cytokine production of eleven essential oils. Cypress, Pine, and Japanese polyunsaturated oil had about 90-110% of the elimination ability of free radical scavenging ability. Cypress oil showed about 80% concentration of NO decrease compared to the control. Lavender, Japanese whites, and Korean whitening oil reduced reactive oxygen species (ROS) by about 50%. Cypress and lavender oil showed low cytotoxicity and limonene oil showed about 70% cytotoxicity. Eucalyptus oil, lavender oil, mint oil, and limonene were decreased LPS-induced IL-1 β production. At 1 % concentration of essential oil, eucalyptus, lavender, mint, pine sylvestris, paddy pollen, Japanese whitening, and Korean whitening oil decreased to more than 50% LPS-induced IL-6 production. Cypress, eucalyptus, limonene oil, pine Siberian, pine, Japanese honeybee, and Korean oleaceous oil at 0.1% have about 50% of inhibition LPS-induced TNF- α production.

KEYWORDS: Antioxidant activity, cytotoxicity, essential oils, inflammatory cytokine production**INTRODUCTION**

Essential oil is a fragrant chemical compound called wood fragrance, a unique aroma secreted by wood. Volatile terpene is an organic chemical substance produced by plants for self-defense and is a physiologically active natural substance having various functions such as antibacterial, insect, and deodorization.^[1-3]

Essential oil is most effective in relieving stress, and it is known that inhaling cedrol, an essential oil component extracted from α -pinene, limonene and cedar, suppresses the activity of parasympathetic activity and sympathetic activity.^[4] This action is brought into contact with the nasal mucous membrane of the nose of the human body, passing through the olfactory nerve to the cerebral limbic system, relaxed, and so on to cope with stress.

Essential oil is a form that relaxes the body. It is used in situations such as sleeping and massage treatment, which can cause drowsiness or blur concentration in daily life.^[5] Health effects include mental illness treatment, abnormal behavioral correction, relaxation, diuretic, genomic, augmentation, and sedative effects, as well as self-confidence and agility in older children with autism and depression.^[6]

Essential oil's antioxidant activity has the mechanism in which the essential oil component electronically stabilizes free radical of DPPH by giving up radical or hydrogen radical or donating electron to reduce toxicity of free radical.^[7]

Active oxygen, DPPH radical scavenging effect, nitrogen monoxide inhibition and lipid peroxidation were evaluated in the combination of 4 essential oils.^[8]

Currently, there are tocopherols, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate, tertiarybutyl hydroxy quinone, and ascorbic acid, which can increase the in vivo antioxidant defense system or regulate reactive oxygen species. BHT and BHA are highly effective antioxidant compounds and they are widely used as tocopherol and vitamin C,^[9] because they have excellent efficacy and low cost. However, they inhibit energy production, metabolism, and respiration.^[10] Their strong toxicity, moreover, is a huge problem. Thus, in recent years, the researches for antioxidant defense systems or direct elimination of ROS have been increasing by using secondary metabolites extracted from natural plants which have no side effects on human body.^[11] The purpose of this study was to evaluate essential oil in the

means of stress reduction, attention, and arousal effects.

MATERIALS AND METHODS

Materials

The test substances were 11 kinds of essential oil and supplied by Company B as the test substance (Table 1).

Table 1: The lists of eleven essential oil in this study.

No.	Oil Name	Supplier
1	Cypress oil	Charabot
2	Eucalyptus oil	Charabot
3	Lavender oil	Charabot
4	Mint oil	Charabot
5	Tea tree oil	FKA
6	Pine Siberian	Charabot
7	Pine Sylvestris	Seoul fragrance
8	Korean pine oil	Solgo
9	Hinoki oil	Kiso
10	Limonene	Florida Chemical
11	Hinoki oil	GnG

Preparation of sample

The results were obtained through the first, second and third experiments. In the first screening, 11 essential oils were diluted in ethanol and tested by disk diffusion method (antibiotic), SOD, and catalase experiment (antioxidant). Samples were diluted 1: 1 in 99% ethanol in oil, and then diluted in culture medium or 99% ethanol.

DPPH assay

Both 150 μ l of a 0.2 mM DPPH solution dissolved in ethanol and the solution dissolved in 11 μ l of essential oil were mixed at a concentration of 10%, respectively, and 100 μ l of each was mixed and reacted in a dark room at 37°C for 30 minutes. After the reaction, the absorbance was measured at 517 nm. In the control group, distilled water was added instead of the sample solution, and ethanol was added instead of the DPPH solution to obtain a correction value.

Nitric oxide

NO concentration was measured by using Griess reagent system. Raw 264.7 cells were plated in 96 well plates at 5×10^4 cells / well and cultured for 24 hours. After cultivation, the medium was replaced with a new culture medium. Eleven species of essential oil were treated with a concentration of 0.1, 1% and 1 μ g / ml of LPS, and cultured again for 24 hours. 50 μ l of N1 buffer was added to each well and reacted at room temperature for 10 minutes. Then, 50 μ l of N2 buffer was added to each well and reacted for 10 minutes. After the reaction, the absorbance was measured at 540 nm. The concentration of NO in the culture medium was determined using the standard curve of the nitrite standard.

Measurement of ROS production in RAW264.7 cells using DCF-DA

Measurement of ROS production in RAW264.7 cells using DCF-DA-2', 7'-dichlorofluorescein diacetate (DCF-DA) was used to measure reactive oxygen species (ROS) in Raw 264.7 cells. Raw 264.7 cells were seeded at 5×10^4 cells / well in 2 well plates. After incubation for 4 hours, 11 kinds of essential oil were treated at a concentration of 0.1, 1 (%) and 1 μ g / ml of LPS, and cultured in an incubator (37°C, 5% CO₂) for 24 hours. After centrifugation, the collected cells were washed twice with cold PBS, added with DCF-DA 10 μ M, and stained at room temperature for 15 minutes. After washing them with cold PBS, centrifugation was carried out at 1,200 rpm for 5 minutes. The supernatant was removed, and 400 μ l of PBS was added thereto. The fluorescence intensity was measured using a flow cytometer (BECTON Dickinson, Franklin Lakes, NJ, USA).

MTT assay

Raw 264.7 cells were plated in 96 well plates at 5×10^4 cells / well and cultured for 24 hours. After cultivation, the medium was replaced with fresh medium, and 11 kinds of essential oil were treated at 0.1% and 1.0% concentration and cultured again for 24 hours. After incubation, 10 μ l of WST solution was added and reacted in an incubator (37°C, 5% CO₂) for 30 minutes. After the reaction, concentration of cells was measured at 450 nm the absorbance, and their survival rates of the control group were expressed as a percentage.

Inflammatory cytokine assay using Luminex

Luminex (Lumixex 200, Thermo fisher, US) was used to measure inflammatory cytokines in Raw 264.7 cells. Raw 264.7 cells were plated in a 12-well plate at 5×10^6 cells / well. After culturing for 24 hours, eleven species of essential oil were treated at a concentration of 0.1, 1% and 1 μ g / ml of LPS, and cultured in an incubator (37°C, 5% CO₂) for 24 hours. After centrifugation, IL-1 β , IL-6, TNF- α and MCP-1 were measured as supernatants using Luminex instrument.

Statistical analysis

Each value represents the mean \pm S.D. from 3 independent experiments. The experimental results were statistically analyzed using student's t-test, and significance was tested at $p < 0.05$, $p < 0.01$, and $p < 0.001$.

RESULTS

DPPH scavenging ability

In essential oil, about 10% of Eucalyptus oil and 30% of limonene and Korean whitening oil showed free radical scavenging ability. In contrast, lavender, pine Siberian, and pine sylvestris oil showed about 60% reduction, while cypress, pine, and Japanese polyunsaturated oil had about 90 ~ 110% of the elimination ability (Fig. 1).

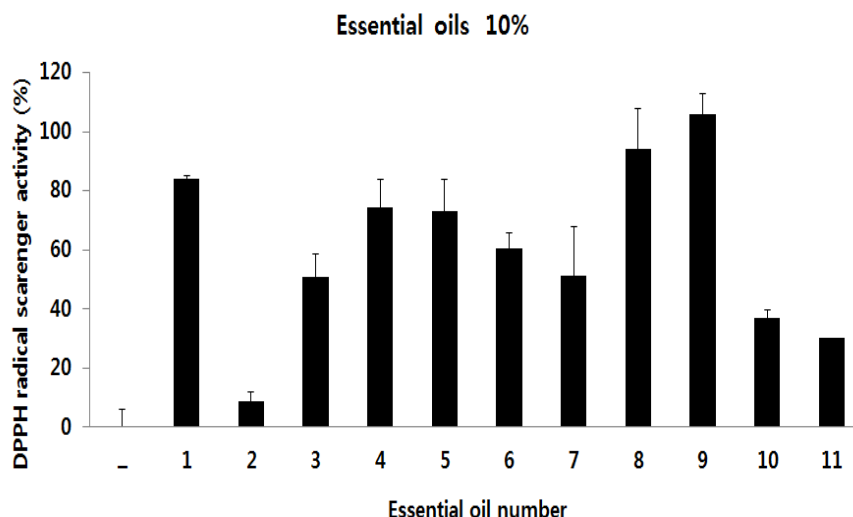


Figure 1. DPPH free radical scavenging activity of essential oils at various concentration.

The results are expressed as mean±S.D. from three independent experiments.

Production of nitric oxides induced by LPS

At the concentration of essential oil 0.1 %, there was no significant decrease in nitric oxide compared to the control group (Fig. 2A). At the concentration of 1%,

cypress oil showed about 80% decrease compared to the control, and lavender, pine cone and Korean whitening oil decreased about 40% (Fig. 2B). Other oils showed no significant decrease.

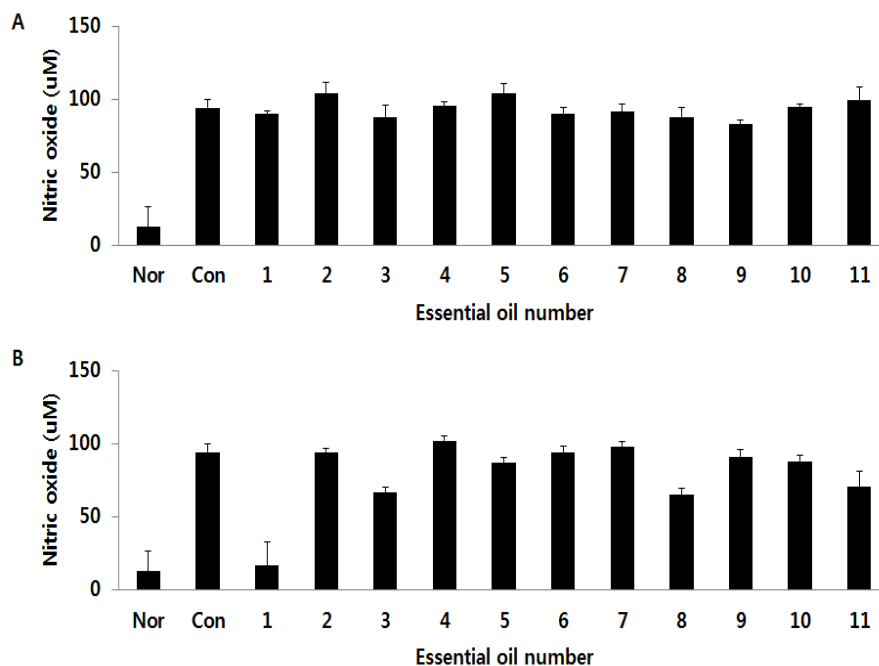


Figure 2. Effects of essential oils on LPS-induced NO production in Raw 264.7 cells.

A: Essential oils 0.1%, B: Essential oils 1%.

LPS-induced ROS generation

In general, the lower the concentration of oil, the less the removal ability of ROS appears. At 0.1% of essential oil, Pine Sylvestris oil reduced the ROS by about 20% and Lavender and Limonene oil by about 10% compared with the control (Fig. 3A). However, other oils showed

no significant reduction in ROS (Fig. 3B). In the concentration of 1 % of essential oil, lavender, Japanese whites, and Korean whitening oil reduced ROS by about 50%. Eucalyptus and tea tree were shown 25% reduction of ROS compared to the control group (Fig. 3B).

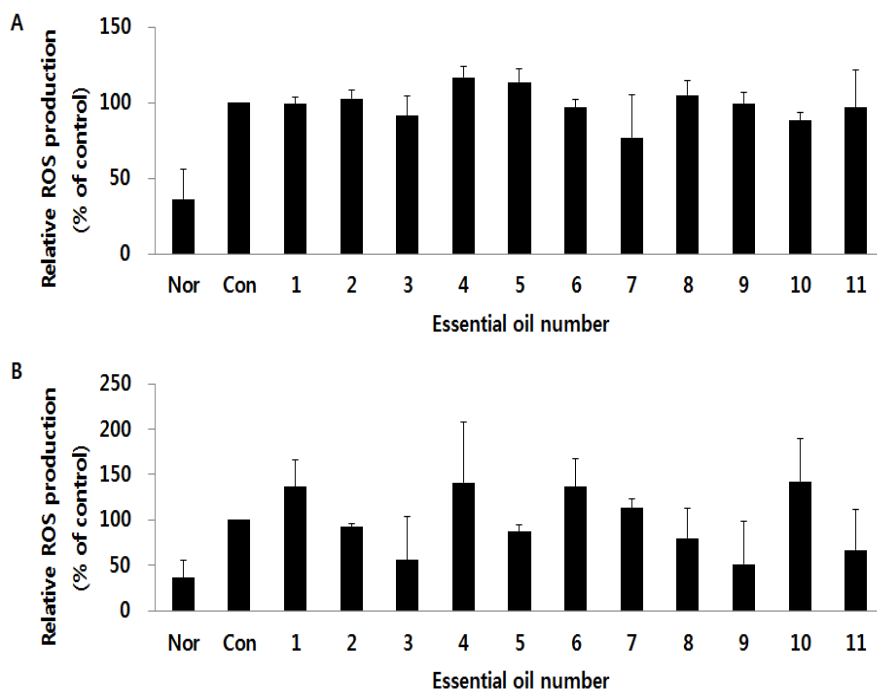


Figure 3. Effect of essential oils on ROS product in RAW 264.7. A: Essential oils 0.1%, B: Essential oils 1%.

Cytotoxicity measurement of eleven essential oil

No cytotoxicity was observed at concentrations of 0.1% of 11 essential oils (Fig. 4A). At 1 % concentration, cypress and lavender oil showed low cytotoxicity, and

limonene oil showed about 70% cytotoxicity (Fig. 4B). There was no cytotoxicity less than 0.1% of essential oil or is insignificant.

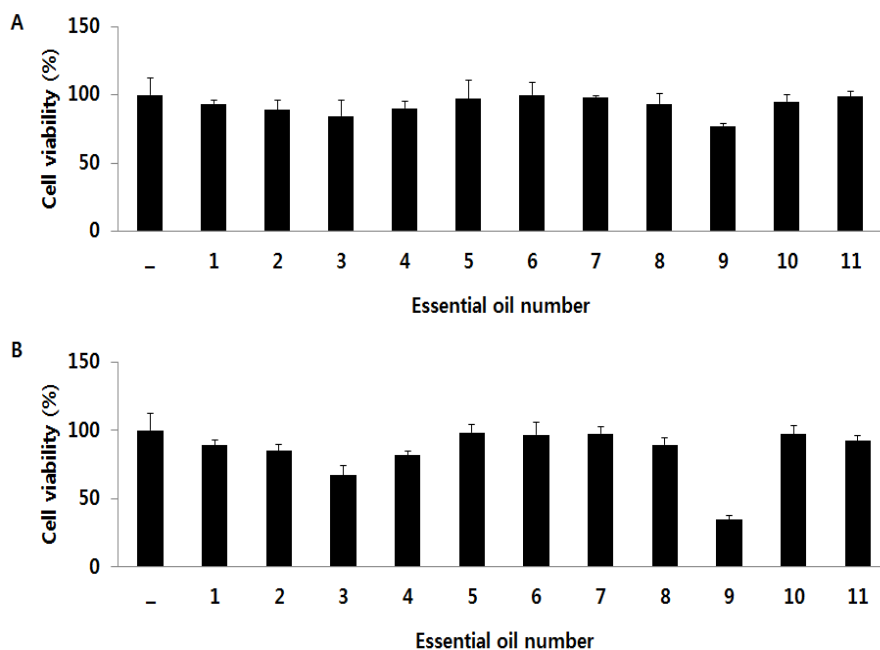


Figure 4. Cell viability of essential oils treatment on Raw 264.7 cells.

A: Essential oils 0.1%, B: Essential oils 1%.

Inflammatory cytokine production induced by LPS

Some essential oil 0.1% and 1% concentrations (eucalyptus oil, lavender oil, mint oil, and limonene) showed decrease in LPS-induced IL-1 β production. In

some other cases, the tendency to increase was rather pronounced, and no significant trend was observed in the effect on concentration deviations (Fig. 5).

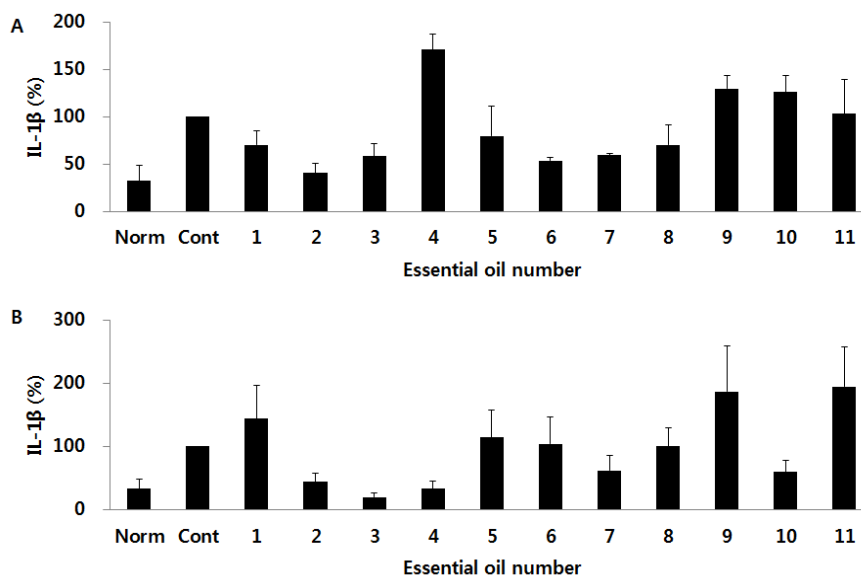


Figure 5. Effect of LPS-induced IL-1β production by essential oils in Raw 264.7 cells.

A: Essential oils 0.1%, B: Essential oils 1%.

At the concentration of essential oil 0.1%, all 11 kinds of oil showed decrease in LPS-induced IL-6 production compared to the control group (Fig. 6A). At 1% concentration of essential oil, LPS-induced IL-6 production by eucalyptus, lavender, mint, pine sylvestris,

paddy pollen, Japanese whitening, and Korean whitening oil decreased to more than 50%. Production by cypress, tea tree, pine Siberian, and limonene oil increased (Fig. 6B).

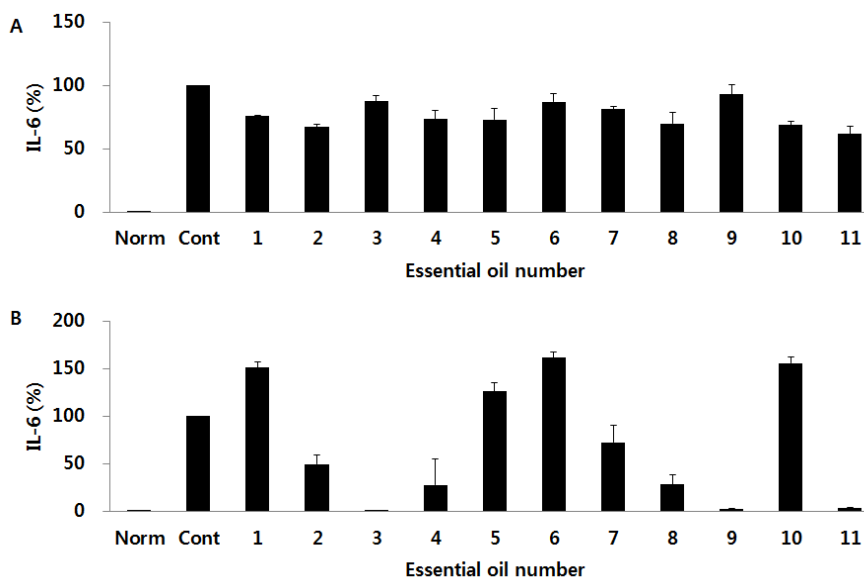


Figure 6. Effect of LPS-induced IL-6 production by essential oils in Raw 264.7 cells.

A: Essential oils 0.1%, B: Essential oils 1%.

3) TNF-α

Cypress, eucalyptus, limonene oil, pine Siberian, pine, Japanese honeybee, and Korean oleaceous oil at 0.1% have about 50% inhibition of LPS-induced TNF-α production. Mint, tea tree, and pine Sylvestris oil were reduced by about 30% LPS-induced TNF-α production, but they were slightly increased in lavender oil (Fig. 7A).

At 1% concentration of essential oil, cypress, tea tree, and pine Siberian oil showed about 60% decrease compared to control, and eucalyptus, Pine sylvestris, pine pollen and limonene oil showed about 70% decrease. Lavender, mint, Japanese white flour and Korean white flour oil showed about 95% decrease in IL-1β (Fig. 7B).

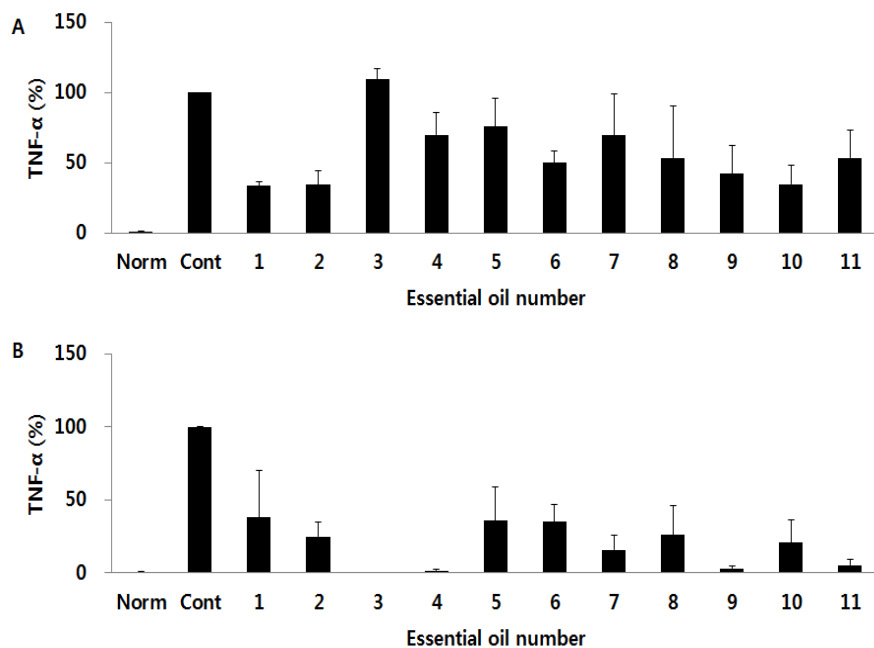


Figure 7. Effect of LPS-induced TNF- α production by essential oils in Raw 264.7 cells.

A: Essential oils 0.1%, B: Essential oils 1%.

DISCUSSION

Nature has given indigenous gifts to the earth, one of which is plants. Essential oils are among those bioactive compounds which bear properties of phytomedicines. Essential oils contain many biochemical constituents including terpenes, alcohols, esters, coumarins, ketones, aldehydes, phenols etc. Essential oil acts like a hormone that exists inter-cells in plants and regulates the physiological function. Especially, the anti-microbial and anti-oxidant activities of essential oils as well as their potential anti-cancer activity have been investigated in recent years.^[12,13] Although no cytotoxicity was observed at concentrations of 0.1% of 11 essential oils (Fig. 4A), Cypress and lavender oil showed low cytotoxicity and limonene oil about 70% cytotoxicity at 1% concentration (Fig. 4B). Ten essential oils such as mint and lemon exhibited strong cytotoxicity towards PC-3 cells at a concentration of 0.2% (v/v).^[14] Many plants such as Cypress contain monoterpenes or sesquiterpenes.^[15] Two sesquiterpenes found in guaçatonga (β -caryophyllene and α -humulene) presented similar results to the ones verified as the plant essential oils, indicating that they might be responsible for the cytotoxic action verified. These sesquiterpenes have already been described as cytotoxic components of other plant oils.^[16-18] Sesquiterpenes are antiseptic and anti-inflammatory. They work as a liver and gland stimulant and contain caryophyllene and valencene. Cassia oil (CO) inhibited mRNA expression of pro-inflammatory cytokines and chemokines and these mediators included TNF- α , IL-1 β , IL-6, monocyte chemoattractant protein-1, and macrophage inflammatory protein-1 α in LPS-activated cells.^[19] In this study essential oil compound not only inhibited inflammatory mediators but also activated anti-inflammatory mediators in LPS-activated Raw 264.7

cells.

Many essential oils have been used in folk medicine as antiseptic and antihelminthic and as a facilitator of wound healing, and one of them called Hinoki essential oil is supported by experimental data reporting the antifungal and antimicrobial activities of the phytocomplex as well as its ability to increase oxidative metabolism in Raw 264.7 cells.

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