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RESEARCH ARTICLE

Protective Activity of 1, 8-Cineole against Inflammatory and Oxidative Stress Marker from Methyl Methacrylate Exposure

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Abstract

Objective: This study was aimed to determine the potential of 1,8-cineole to reduce the amount of malondialdehyde (MDA), interleukins (IL)-8 and neutrophils in MMA-exposed lung alveolar cells. Methods: Thirty mice were divided into five groups: a normal control group (K0), a negative control group (K1) and a treatment group, further subdivided into P1, P2 and P3, whose members were administered 5 mg, 10 mg and 15 mg doses of 1,8-cineole. The treatment group subjects were placed in a glass cage containing 150 ppm MMA vapor that has been nebulated by 1.8-cineole vapor for 120 minutes. The mice were subsequently terminated, and tissue removed from their lungs. Observation of MDA and expression of IL-8 was conducted during immunohistochemical examination. Neutrophils was observed using Hematoxylin Eosin (HE) staining. Results: MMA exposure significantly increased MDA, IL-8 and neutrophil counts. Meanwhile, administration of 1, 8-cineole successfully reduced MDA numbers. Fifteen milligrams of 1, 8-cineole constituted the only dose which proved capable of reducing the expression of IL-8. Administration of 1, 8-cineole in all groups also decreased the number of neutrophils. Conclusion: 1, 8-cineole, especially at a dose of 15 mg, can reduce the levels of MDA, IL-8 and neutrophil in the lung tissue of mice following exposure to MMA vapor.

Keywords: *MMA, 1, 8-cineole, Inflammation, ROS, MDA.*

Running Title: Anti-inflammatory effect of 1, 8-cineole.

Introduction

At the present day, self-curing acrylic material that consists of polymethyl methacrylate powder and methyl methacrylate (MMA) liquid monomer remains widely employed in dentistry. However, it is also known as a cytotoxic and genotoxic agent, particularly the liquid monomer which is considered less inert than polymethyl methacrylate powder [1].

MMA monomer is a volatile liquid with a pungent odor, readily released into the air and inhaled by anyone in the immediate vicinity [2]. MMA constitutes a sensory irritant to the respiratory tract where the first target tissue is in the airway epithelium [3]. Scherpereel *et al.* and Kim *et al.* reported cases of dental technicians diagnosed with hypersensitivity pneumonitis due to inhalation of MMA [4, 5] while Lyapina *et al.* highlighted the incidence of contact hypersensitivity (>24%) among members of the dental profession, students and random populations due to MMA [6].Daily use of methacrylate significantly increases the risk of asthma, nasal conditions, phlegm production and coughing [7].

Exposure to MMA causes its molecules to interact with nucleophilic targets in the cell, thus forming reactive oxygen species (ROS). If the level of ROS exceeds that of anti-ROS oxidative stress occurs [8]. Lipid peroxidase as an indicator of systemic oxidative stress can be assessed through measurement of malondialdehyde (MDA) [9].

Inflammation of the airway epithelium can be identified by measuring pro-inflammatory mediators, IL-8, IL-10 and neutrophils count [10].

To prevent oxidative stress, antioxidant can be administered as a first defense against ROS [11].1,8-cineole represents one of the largest components of eucalyptus. 1,8-cineole contains rich antioxidants and has been widely employed for medicinal purposes such as reducing pain and inflammation of mucous membranes, in addition to treating coughs, bronchitis, sinus pain and inflammation, asthma, chronic obstructive pulmonary disease (COPD) and other respiratory infections [12].

1,8-cineole has been shown to inhibit ROS production [13,14], reduce NF-κB activity in vitro [15] and affect macrophages to a greater extent than other leukocytes [12,16]. It also inhibits airway smooth muscle contraction, increases mucociliary clearance [17] and reduces lung inflammation and oxidative stress [18]. The purpose of this study was to determine the potential of 1.8-cineole to reduce the number of MDA metabolites, IL-8 expression and neutrophil counts in the respiratory tract cells of mice that had been exposed to methyl methacrylate vapor.

Material and Methods

This research was a true experimental laboratory study with a post-test only group design. Thirty 3-month-old, male BALB/c mice with a body weight of 20-30 grams and which had been declared healthy following physical examination by a veterinarian were used as research subjects. This study was granted an ethical clearance certificate (Number: 042 / HRECC.FODM / V / 2018).

Sample Preparation and Exposure

The experimental subjects were divided into five groups, namely; normal control (K0), negative control (K1) and three treatment groups (P1, P2 and P3) each of which contained six members. Prior to treatment, the subjects underwent a one-week adaptation period during which they all received basic feed and standard maintenance in order to satisfy the participation criteria for this study. The normal control group subjects were placed in cages without having received treatment, whereas those in the negative control group were exposed to MMA vapor. Initially, 20 ml of MMA (Merck, Darmstadt, Germany) was placed in a 41x49x25 cm glass cage for 90 minutes until the desired concentration of 150 ppm MMA had been achieved, at which point the subjects were placed there for 120 minutes. In the treatment group (P1, P2 and P3), the procedure for achieving MMA at a concentration of 150 ppm was the same as in the negative control group, although after 85 minutes 1.8-cineole (Merck, Darmstadt, Germany) was dissolved in a saline solution (Otsu-NS, Lawang, Indonesia) and 1% Tween-80 (Brataco, Bekasi, Indonesia).

Two milliliters of each dose was taken and placed in a medicine tank, before being nebulated with a compressor nebulizer (Beurer, Germany), at an aerosol flow = 0.2 ml / min (particle size 3.85 μm) for five minutes at a dose of 5 mg / ml in the P1 group, a dose of 10 mg / ml in the P2 group and a dose of 15 mg / ml in group P3. Post-nebulation, the subjects were placed in a glass cage for 120 minutes. After treatment, the subjects were sacrificed and airway epithelial cell specimens were excised from their lungs with a sharp blade. The tissues were subsequently immersed in a fixative solution (10% neutral buffered formalin) for further processing.

Oxidative Stress and Inflammatory Observation

Observation of MDA metabolites and IL-8 expression was conducted by indirect immunohistochemical staining. The number of MDA metabolites was calculated by counting the number of epithelial cells that reacted positively to anti-MDA and IL-8 anti-mouse monoclonal antibodies (Santa Cruz Biotechnology Inc., USA). Calculations were performed on each slide (10 fields of view), observed under a light microscope at 400x magnification and considered positive if they produced a brownish color in the cell cytoplasm.

Observation of tissue inflammation was completed through a process of hematoxylin-eosin (HE) staining which began with deparaffinization involving three immersions in technical xylol for five minutes, followed by hydration by means of successive two-minute immersion in 96% alcohol, 80% alcohol and 70% alcohol. The tissues were then rinsed with running water for ten

minutes and immersed in Meyer's Hematoxylin solution for 15 minutes. After being washed again with running water, the tissues were immersed in 1% eosin solution for 30 seconds. Dehydration was completed by placing it in 80% alcohol for two minutes and immersing it three times in 96% alcohol for two minutes. The neutrophils were counted in a 200x200 µm2 field of view under a light microscope.

Statistical Analysis

Data was analyzed with a SPSS Statistical Package for the Social Sciences Software (SPSS) 24.0 edition (SPSSTM, Chicago, United States) through a one-way ANOVA test for normal and homogeneous data, followed by a LSD multiple comparison test (α: 0.05). Data that was neither normally distributed nor homogeneous was tested by means of a Kruskal-Wallis test, while normally distributed data that was not homogeneous was subjected to multivariate comparison testing using a Brown-Forsythe ANOVA and a Games-Howell test.

Results

The results showed that MMA exposure increased the number of MDA metabolites, IL-8 expression and neutrophil count (see Table 1), whereas administration of 1, 8-sineol reduced the number of MDA metabolites, IL-8 expression and neutrophil count.

Table 1: Mean and standard deviations of MDA metabolite counts, IL-8 expression, and neutrophil counts

Group	MDA	IL-8	Neutrophils
K0	7.08 ± 6.03	4.56 ± 4.10	12.83 ± 0.98
K1	20.27 ± 7.22	33.80 ± 16.34	50.83 ± 5.04
P1	5.18 ± 2.90	16.93 ± 3.79	41.33 ± 4.32
P2	4.25 ± 2.24	17.46 ± 7.02	40.33 ± 3.56
P3	2.18 ± 1.63	16.13 ± 10.52	30.83 ± 3.66

K0: normal control; K1: negative control; P1: 1, 8-sineol 5 mg; P2: 1, 8-sineol 10 mg; P3: 1, 8-sineol 15 mg

Based on the results of a Brown-Forsythe test, the p value was determined to be 0.000 ($p < 0.05$). This meant that significant differences existed, at least between a pair of groups. The lowest level of MDA expression was found in group P3, while the highest was in K1 (see Figure 1). The mean, standard deviations and results of a Games Howell test of MDA metabolites indicating that the K1 group was significantly different from the K0, P1, P2 and P3 groups are contained in Table 1 and Figure 1.

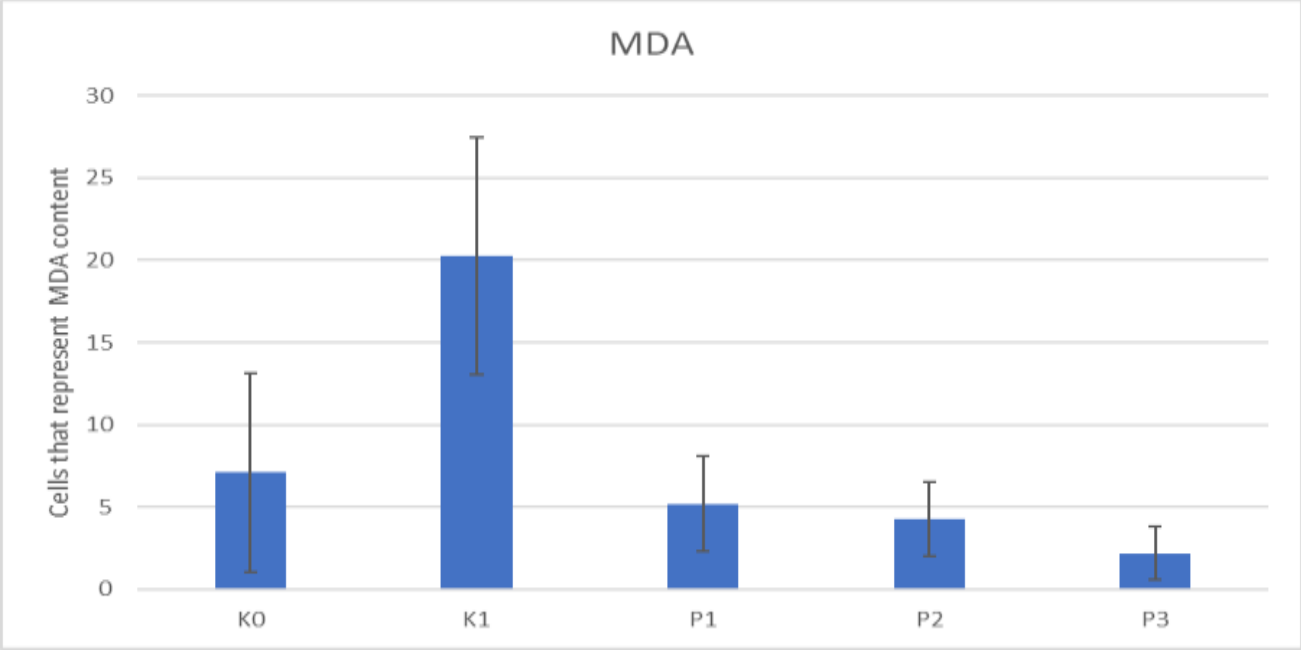

Figure 1: Mean and standard deviation of MDA metabolites

According to the Brown-Forsythe test results, the p value was 0.003 ($p<0.05$) indicating that significant differences existed, at least between a pair of groups. The lowest number of IL-8 expressions was found in the K0 group (normal control), while the highest was

found in the K1 group (negative control). The mean, standard deviation and LSD test results for IL-8 expression can be seen in Table 1 and Figure 2. The K0 group (normal control) differed significantly from other groups with the exception of the P3 group. Although not significantly different from the P1 group, the K1 group was significantly different from P2 and P3 groups.

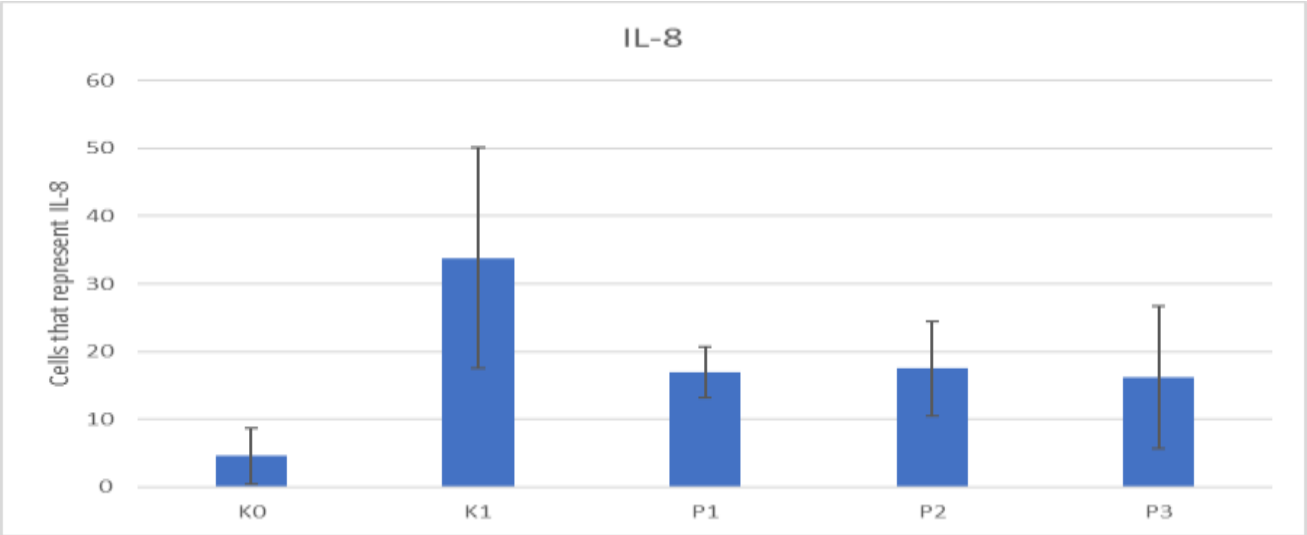

Figure 2: Mean and standard deviation of IL-8 expression

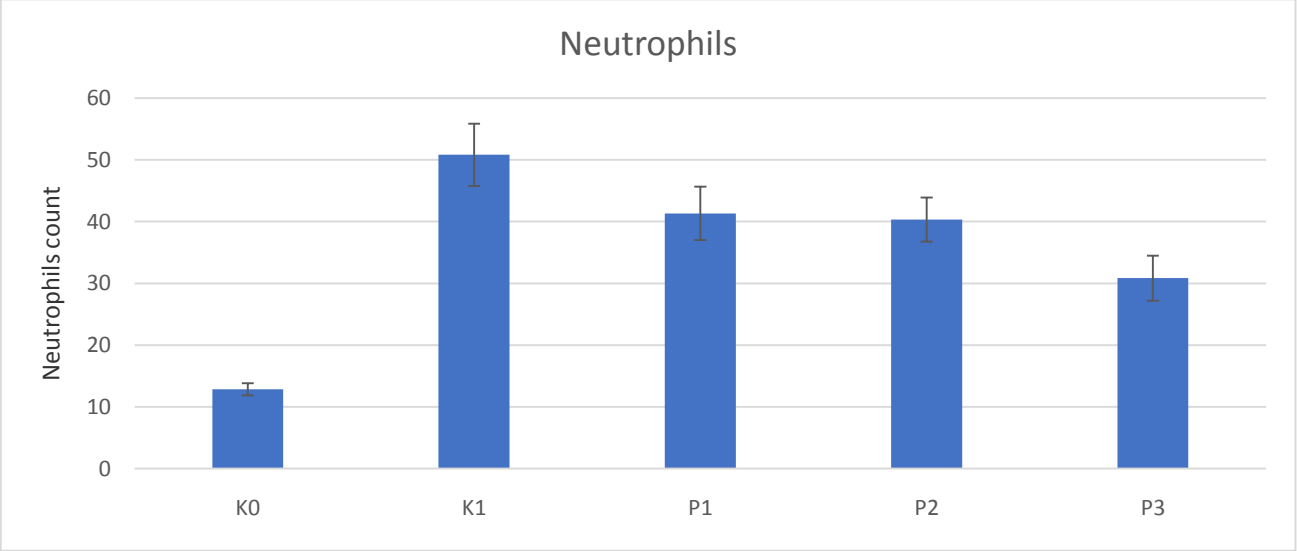

Figure 3: Number of neutrophils in the lungs of mice

The lowest neutrophil count was found in the K0 group, while the highest was in the K1 group. Significant differences between groups existed, with the exception of the P1 and P2 groups (Figure 3).

Discussion

The results of this study indicated that administration of 1, 8-cineole significantly reduced the number of MDA metabolites in all treatment groups. Exposure to Methyl Methacrylate and Tween-Saline solution (MMA+TS) produced a significant increase in MDA metabolites ($p = 0.000$). Research on lung cell cultures showed that MMA exposure reduced cell viability when both the duration and concentration of exposure increased [19]. Based on its morphology, MMA has been proven to be toxic to cells and increase ROS production. Exposure to MMA causes superoxide dismutase (SOD) activity to increase, with the result that H_2O_2 / hydroxyl radicals formed through the Fenton reaction will also proliferate and initiate lipid peroxidation [20]. This chain reaction results in an increase in MDA, a lipid peroxidation product that can trigger cell damage which, in turn, will produce cell debris which forms Damage Associated Molecular Patterns (DAMPs) [21].

Research by Aydin *et. al.* also showed that MDA increases as a result of MMA exposure [9]. Groups that were administered 5 mg, 10

mg or 15 mg doses of 1.8-cineole demonstrated a significant reduction in MDA metabolites (p = 0.000). 1,8-cineole, acting as an anti-inflammatory agent, was able to reduce redox marker levels (MDA) [22]. In this case, 1,8-cineole also played a role as an antioxidant agent by reducing ROS which, in turn, produced a decrease in the number of MDA metabolites.

However, these did not differ significantly from those of the normal control group, although the lowest reduction was achieved by the administering of a 15 mg dose. IL-8 secretion is an important mediator within the innate immune response system achieved through biochemical chain reactions. IL-8 is a pro-inflammatory cytokine which, under acute conditions, acts as a neutrophil chemotactic factor attracting neutrophils from bone marrow to the peripheral blood vessels [23].

The results presented above indicate an increased expression of IL-8 in MMA exposure (K1 group). Greater expression of IL-8 can occur due to an increase in MDA metabolites which leads to cell damage as a result of which cell debris play a role as DAMPs and induces TLR2. Furthermore, induction of the IKk complex occurs resulting in phosphorylation that will cause degradation of IkB, thereby inducing the release of NF-κB. Increased expression of NF-κB will increase the formation of active NF-κB which then translocates to the cell nucleus.

This induces DNA with the result that transcription occurs, translating and producing pro-inflammatory cytokines such as IL-1ß, IL-6, TNF-α and IL-8. MMA exposure also causes an increase in the number of neutrophils that act as an innate defense. IL-8 is an important activator and chemoattractant of PMN leukocytes and is involved in inflammatory reactions [24]. In addition, higher levels of MDA can also increase IL-8 [25]. IL-8 expression tends to decrease due to the administration of 1.8-cineole but does not reach a level as low as that in the normal control (K0) group.

However, this finding might be due to the relatively limited of research conducted with the result that the effect remains to be proved. Research by Juergens *et al.* showed that therapeutic concentrations of 1,8-cineole (1.5 µgr / ml = 10-5 M) were capable of reducing the production of IL-8 cytokines in human monocyte cell cultures by as much as 65% 26. 1, 8-cineole is a strong inhibitor of TNF-α and IL-1ß, while also affecting chemotactic cytokines (IL-8). 1, 8-cineole also plays a role in controlling airway mucus hypersecretion through cytokine inhibition and in the future could be used as a therapy to treat severe asthma, sinusitis and COPD.

The results of this study support the proposition that 1,8-sineol suppresses IL-8 secretion in LPS-induced subjects through a decrease in NF-κB p6525 phosphorylation in Der-p induced subjects by modulating TLR-4 [23]. In this study, inflammation was measured by the number of neutrophils which rose significantly (p = 0.000) after exposure to MMA. Neutrophil counts increased as a result of innate defenses [27].

IL-8 is an important activator and chemoattractant for PMN leukocytes and is involved in inflammatory reactions [24]. The anti-inflammatory effect of 1,8-cineol has been widely proven in experimental subjects to which LPS [28, 29] cerulean [30] and cigarette smoke [18] have been administered. The results of this study indicate that administration of 5 mg, 10 mg and 15 mg doses of 1, 8-cineol reduced the number of neutrophils (see Table 4).

The number of neutrophils tends to be higher than that of the K0 group, possibly due to the timing of the examination which was conducted immediately post-treatment. Nevertheless, the tendency for inflammatory markers to decrease supports the proposition that 1,8-cineol produces an anti-inflammatory effect. The limitation to this research consists of the implemented termination time immediately after exposure with the result that the measurement peak, specifically with regard to the number of neutrophils, was not reached.

Conclusion

1, 8-cineol can reduce the amount of MDA metabolites, IL-8 expression and neutrophils, particularly at a dose of 15 mg.

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References

1. Alzarea BK (2014) Exposure to Methyl Methacrylate Upregulates the Expression of INOS in the Tongue Tissue of Rats. Eur. Sci. J., 10(33):48-54.
2. Marquardt W, Seiss M, Hickel R, Reichl FX (2009) Volatile methacrylates in dental practices. J. Adhes. Dent., 11(2):101-107.
3. Gosavi SS, Gosavi SY, Alla RK (2010) Local and systemic effects of unpolymerised monomers. Dent Res. J. (Isfahan). 7(2):82-87.
4. Scherpereel A, Tillie-Leblond I, Pommier de Santi P, Tonnel AB (2004) Exposure to methyl methacrylate and hypersensitivity pneumonitis in dental technicians. Allergy, 59(8):890-892.
5. Kim Y-H, Chung YK, Kim C, Nam E Suk, Kim H-J, Joo Y (2013) A Case of Hypersensitivity Pneumonitis with Giant Cells in a Female Dental Technician. Ann Occup. Environ. Med., 25(1):19.
6. Lyapina M, Dencheva M, Krasteva A, et al (2014) Health Risk Assessment in Exposure to Methacrylic Monomers in Dental Practice. J. Sci. Res. Reports, 3(22):2848-2863.
7. Jaakkola MS, Leino T, Tammilehto L, Ylöstalo P, Kuosma E, Alanko K (2007) Respiratory effects of exposure to methacrylates among dental assistants. Allergy, 62(6):648-654.
8. Soykut B, Erdem O, Akay C, Pişkin B (2017) Investigation of the oxidative stress condition for occupational exposure to methyl methacrylate. Toxicol. Ind. Health, 33(1):61-66.
9. Aydin O, Attila G, Dogan A, Aydin MV, Canacankatan N, Arzu K (2002) The Effects of Methyl Methacrylate on Nasal Cavity , Lung , and Antioxidant System (An Experimental Inhalation Study). Toxicol. Pathol., 30(3):350-356.
10. Muttray A, Gosepath J, Brieger J, et al (2015) No acute effects of an exposure to 50 ppm methyl methacrylate on the upper airways. Int. Arch. Occup. Environ. Health, 88: 1043-1051.
11. Lodovici M, Bigagli E (2011) Oxidative stress and air pollution exposure. J Toxicol. 2011: 487-074.
12. Sadlon AE, Lamson DW (2010) Immune-modifying and antimicrobial effects of Eucalyptus oil and simple inhalation devices. Altern. Med. Rev., 15(1): 33-47.
13. Ryu S, Park H, Seol GH, Choi I-Y (2014) 1,8-Cineole ameliorates oxygen-glucose deprivation/reoxygenation-induced ischaemic injury by reducing oxidative stress in rat cortical neuron/glia. J. Pharm. Pharmacol., 66(12):1818-1826.
14. Porres-Martínez M, González-Burgos E, Carretero ME, Gómez-Serranillos MP (2015) Major selected monoterpenes α-pinene and 1,8-cineole found in Salvia lavandulifolia (Spanish sage) essential oil as regulators of cellular redox balance. Pharm. Biol., 53(6): 921-929.
15. Greiner JF-W, Müller J, Zeuner M-T, et al (2013) 1,8-Cineol inhibits nuclear translocation of NF-κB p65 and NF-κB-dependent transcriptional activity. Biochim Biophys Acta - Mol. Cell Res., 1833(12):2866-2878.
16. Santos FA, Rao VS (2000) Antiinflammatory and antinociceptive effects of 1,8-cineole a terpenoid oxide present in many plant essential oils. Phytother Res, 14(4):240-244.
17. Juergens U (2014) Anti-inflammatory Properties of the Monoterpene 1.8-cineole: Current Evidence for Co-medication in Inflammatory Airway Diseases. Drug Res (Stuttg)., 64(12):638-646.
18. Kennedy-Feitosa E, Okuro RT, Pinho Ribeiro V, et al (2016) Eucalyptol attenuates cigarette smoke-induced acute lung inflammation and oxidative stress in the mouse. Pulm Pharmacol Ther., 41:11-18.
19. Ganesh Kumar A, Jagdish S, Nandagopal S, Joshua Daniel Egan L(2014) Effect of Methyl Methacrylate and Methacrylic Acid on ROS Production and Cellular Antioxidants. Res J. Pharm. Biol. Chem. Sci., 5(6):388-396.
20. Soykut B, Erdem O, Akay C, Piskin B (2016) Investigation of the oxidative stress condition for occupational exposure to

methyl methacrylate. Toxicol. Ind. Health, 1-7.

21. Di Marzo N, Chisci E, Giovannoni R (2018) The Role of Hydrogen Peroxide in Redox-Dependent Signaling: Homeostatic and Pathological Responses in Mammalian Cells. Cells, 7(10):156.
22. Kennedy-Feitosa E, Cattani-Cavalieri I, Barroso MV, Romana-Souza B, Brito-Gitirana L, Valenca SS (2019) Eucalyptol promotes lung repair in mice following cigarette smoke-induced emphysema. Phytomedicine, 55: 70-79.
23. Lee H, Park D, Song W, et al (2016) Effect of 1.8-Cineole in Dermatophagoides pteronyssinus-Stimulated Bronchial Epithelial Cells and Mouse Model of Asthma. Biol. Pharm. Bull., 39(6):946-952.
24. Qazi BS, Tang K, Qazi A (2011) Recent advances in underlying pathologies provide insight into interleukin-8 expression-mediated inflammation and angiogenesis. Int. J. Inflam., 2011:908468.
25. Raghavan S, Subramaniyam G, Shanmugam N (2012) Proinflammatory effects of malondialdehyde in lymphocytes. J. Leukoc. Biol., 92(5):1055-1067.
26. Juergens UR, Engelen T, Racké K, Stöber M, Gillissen A, Vetter H (2004) Inhibitory activity of 1,8-cineol (eucalyptol) on cytokine production in cultured human lymphocytes and monocytes. Pulm. Pharmacol. Ther., 17(5):281-287.
27. Rosales C, Lowell CA, Schnoor M, Uribe-Querol E (2017) Neutrophils: Their Role in Innate and Adaptive Immunity 2017. J. Immunol. Res., 2017: 9748345.
28. Yadav N, Chandra H (2017) Suppression of inflammatory and infection responses in lung macrophages by eucalyptus oil and its constituent 1,8-cineole: Role of pattern recognition receptors TREM-1 and NLRP3, the MAP kinase regulator MKP-1, and NFκB. Khan MF, ed. PLoS One, 12(11):e0188-232.
29. Linghu K-G, Wu G-P, Fu L-Y, et al (2019) 1,8-Cineole Ameliorates LPS-Induced Vascular Endothelium Dysfunction in Mice via PPAR-γ Dependent Regulation of NF-κB. Front Pharmacol., 10:178.
30. Lima PR, de Melo TS, Carvalho KMMB, et al (2013) 1,8-cineole (eucalyptol) ameliorates cerulein-induced acute pancreatitis via modulation of cytokines, oxidative stress and NF-κB activity in mice. Life Sci., 92(24-26):1195-1201.

