ORIGINAL ARTICLE

The Changes of Immunohistochemistry in Lung Tissues, Surfactant Protein-D, eNOS, and NO in Mice Exposed to Essential Oil Vapor

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ABSTRACT

Introduction: The use of essential oils in aromatherapy is widespread. However, few studies have explored the effects of smoke from the evaporation of commonly used essential oils. While essential oils are promoted for various benefits, prolonged exposure to inhaled particles from essential oil smoke may pose potential health risks. This study aimed to examine the effects of essential oil vapors on mice.
Methods: This was an experimental study investigating the effects of different treatments on lung immunohistopathology, endothelial nitric oxide synthase (eNOS) expression, serum nitric oxide (NO) levels, and serum surfactant protein-D (SP-D) as an inflammation marker in mice. A total of 40 adult male *Mus musculus* mice (25–30 g) were randomly divided into four groups. Inflammation models were established by exposing the mice to a gas mixture containing vegetable glycerin, propylene glycol, and vitamin E acetate solution. Following inflammation induction, the mice received seven-day interventions with 0.9% NaCl solution, *Eucalyptus globulus* essential oil (EgEO), and citronella essential oil (CtEO), alongside an untreated inflammatory group.
Results: The CtEO group intervention showed significant increases in eNOS expression (P=0.001) but no significant increase in NO compared to the other groups. The correlation analysis of eNOS expression in lung cells, serum NO, and SP-D levels was not significant, P>0.05 (p=0.160; p=0.115; p=0.234).
Conclusion: Gas intervention containing 100% oxygen (O_2) and CtEO steam increased eNOS expression on the immunohistochemistry (IHC) examination of mice.

INTRODUCTION

Fragrances have piqued people's interest since the sixth millennium.[1] Numerous cultures have used essential oils for a variety of purposes.[1] Aromatherapy is an alternative medicine method that functions through the fragrance of essential oils extracted from certain plants at high concentrations.[2] Over the centuries, numerous aromatic and herbaceous plants and their essential oils have been employed in animal healthcare, particularly in ethnoveterinary medicine.[3,4] However, using essential oils in aromatherapy does not necessarily mean it is without risks. Several case reports stated that aromatherapy using essential oils could lead to acute eosinophilic pneumonia.[5,6]

The primary component of the essential oils of *Eucalyptus globulus* is 1,8-Cineole, whereas *Eucalyptus citriodora* mainly contains citronellal.[7] It stimulates breathing, eases coughing, aids in mucus ejection, and relaxes the muscles of the respiratory system. Leaf

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essential oil of *E. globulus* is utilized in folk medicine to treat respiratory issues, including colds, coughs, runny nose, sore throat, asthma, nasal congestion, bronchitis, and sinusitis.[8]

This study aimed to examine the effects of essential oil vapors on lung pathology and serum biomarkers in mice. A previous study reported that compound essential oils reduced particulate matter (PM) 2.5-induced acute lung inflammation in mice and inhibited systemic immune responses.[9] Therefore, this study sought to evaluate whether essential oil vapor intervention could influence lung pathology and inflammatory biomarkers in this induced inflammation model. As an evaluation marker, endothelial nitric oxide synthase (eNOS) expression through immunohistochemical (IHC) examination, and analysis of serum surfactant protein-D (SP-D) were determined through the enzyme-linked immunosorbent assay (ELISA) method, and the colorimetric examination method was chosen for serum nitric oxide (NO) levels examination.

METHODS

This study used a vegetable glycerin (VG)+propylene glycol (PG)+vitamin E acetate (VEA)-induced lung inflammation model to simulate inflammatory conditions before administering essential oil vapor interventions.

Collecting Sample

This experimental study involved four groups of *Mus musculus* mice. Group K1, K2, and K3 underwent inflammation induction and were subsequently treated with NaCl, *E. globulus* essential oil (EgEO), and citronella essential oil (CtEO), respectively. Group K4 served as the inflammation-induced group without any treatment. The sample size was determined using a simple random sampling technique and calculated with the Federer formula.[10]

(r-1)(t-1)	≥15
(r-1)(4-1)	≥15
r	≥ 6 mice/group

Note:
r=replication
t=treatment

A total of 40 mice were used, with a minimum of six per group. Mice were selected based on inclusion criteria (male, 10 weeks old, 25–30 g, healthy) and exclusion criteria (unhealthy, such as lethargy, alopecia, watery eyes, or nasal discharge).[11] All mice met the inclusion, exclusion, and dropout criteria, as confirmed by a veterinarian. Randomization assigned 10 mice per group, exceeding the minimum required sample size of six. Dropout criteria included incomplete data or interruptions during the gas intervention.

Intervention

The mice were housed individually in 15×15×15 cm transparent cages. The chambers were made of acrylic material (Figure 1), with a specific size (15×15×15 cm). Each chamber had holes in one of its walls connected to a reservoir and a gas line. This gas intervention chamber functioned as the mice cage equipped with a device for storing essential oil solutions, which were aerosolized using high-pressure oxygen (O_2) to allow the mice to inhale them. The solution reservoir was placed within each chamber wall, with a 5 cm-long connecting pipe leading to the reservoir and another 5 cm-long pipe leading to the chamber.

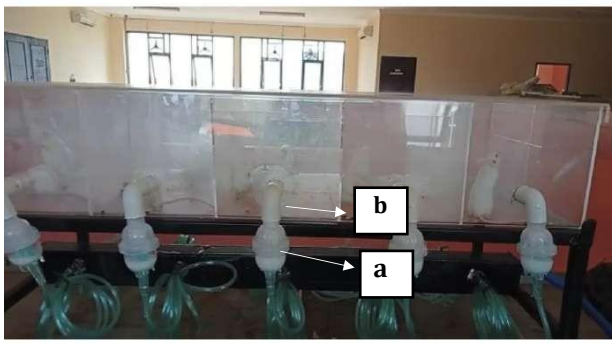


Figure 1. Mice gas intervention chamber. (a) Solution reservoir; (b) Connecting pipe

A simple device was created to disperse the solution into each mice chamber using 10 L/min of O_2. The O_2 flow rate was 10 L/min, with an outlet pressure of 1.4 psi. The liquid volume in each solution reservoir was 7 ml, and the hose length from the O_2 source to the solution reservoir was 2 m. The chamber size, liquid volume, and gas pressure were consistent across all chambers to ensure uniform exposure. The gas intervention process was monitored through the transparent cage walls. The solution to induce lung inflammation consisted of VG and PG in a 30:70 ratio and VEA at 100 µg/kg body weight.[12,13] A mixture of 84 ml of VG and 196 ml of PG was prepared, and 100 µg of VEA was added. This resulted in 2.5 µg of VEA per 7 ml of VG+PG+VEA solution. Gas intervention was conducted by administering 7 ml of this mixture into the reservoir.

After lung inflammation was induced, each group received different treatments for one hour per day over seven days. Group K1 received 7 ml of 0.9% NaCl, group K2 received 7 ml of 0.2% EgEO solution, group

K3 received 7 ml of 0.2% CtEO solution, and group K4 received no intervention. The essential oils (*E. globulus* and citronella) used in this study were locally produced. *Eucalyptus globulus* essential oil contained cineole, α-pinene, aroma dendrite, and o-cymene, while pure citronella oil was used without additional processing.

The mice received daily 1-hour gas interventions for seven days before euthanasia. The euthanasia procedure was performed following anesthesia with ketamine-xylazine. Ketamine (75 mg/kg BW) and xylazine (8 mg/kg BW) were administered.[14] Blood samples were collected for NO and SP-D level analysis, while lung tissues were collected for IHC examination to assess eNOS expression in lung cells.[15] Nitric oxide levels were measured using a colorimetric method based on chromogenic reagent color change.[16] Surfactant protein-D levels were analyzed using ELISA with reagents from Chinese Bioassay Technology. Endothelial nitric oxide synthase expression in lung tissue was assessed using an anti-eNOS antibody (Bioss USA), and eNOS-expressing cells were counted per 100 cells. The procedures were randomized using a simple random sampling technique.[17]

Data Analysis

Study outcomes included lung cells expressing eNOS and SP-D and NO serum levels in surviving mice. Data were analyzed using analysis of variance (ANOVA), followed by least significant difference (LSD) tests, univariate analysis, and correlation analysis. The principal investigator supervised allocation, experimentation, outcome assessment, and data analysis. The F-test is a statistical method to determine differences in eNOS cell expression, NO serum levels, and SP-D counts among group K1, K2, K3, and K4. This test analyzes the mean differences of a variable. The data distribution was tested using the one-sample Kolmogorov-Smirnov test with a significance level of 95% (α=0.05). Meanwhile, data homogeneity was assessed using Levene's test. After testing the data distribution and homogeneity assumptions, the next stage was conducting the F-test or ANOVA. In this study, the confidence level was 95%, with an α value of 0.05.

Ethical Clearance

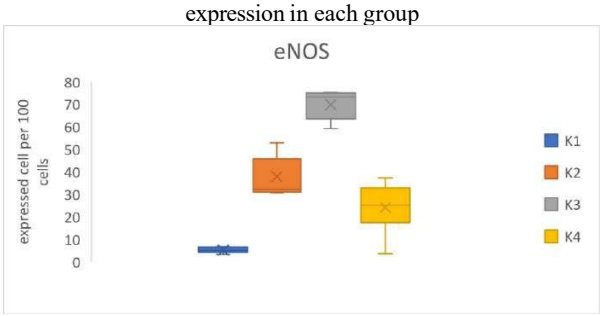
This study had received ethical approval from the ethics committee of the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya (No.2.KEH.044.04.2022).

RESULTS

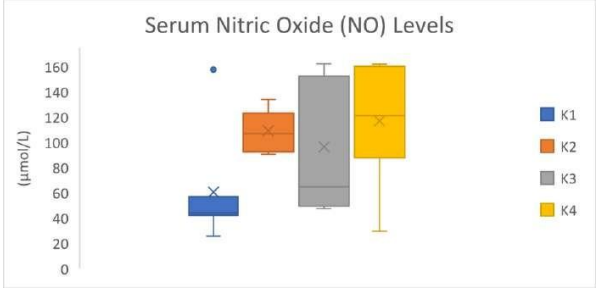
Lung inflammation in mice was induced by intervening VG+PG+VEA for one hour per day for seven days. Four mice in group K4 died from inflammation, while three mice died in group K1, K2, and K3, leaving seven mice alive, and group K4 with six mice. Serum NO, SP-D levels, and eNOS expression were used to identify inflammation. A significant difference was observed in the serum NO levels between group K1 and group K4 because the latter had the highest mean difference (p=0.028). Using the ANOVA test, a bivariate test was conducted to determine the effect of gas intervention on each treatment group. As a result, significant differences in the expression of eNOS cells were found in group K1, K2, K3, and K4, with an F value of the eNOS variable of 87.344, with a p<0.001. However, the results of the F-test showed no difference in serum NO levels and the number of SP-D in each group.

The results of the ANOVA test also concluded that the four different gas intervention methods did not produce significant differences in serum NO and SP-D levels. To determine the difference in the mean NO levels between the two groups, the LSD test was performed. The results showed that the highest NO levels were found in the inflammatory group, which was not given gas intervention, as indicated by the strongest p-value (0.028) in group K1 to group K4. As for eNOS, the greatest mean differences in eNOS were found between group K1 and group K3.

P-Plot graphics on endothelial nitric oxide synthase (eNOS) expression in each group



P-Plot graphics on nitric oxide (NO) expression in each group



P-Plot graphics on surfactant protein-D (SP-D) levels in each group

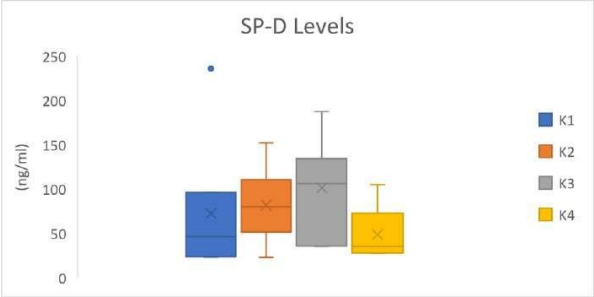


Figure 2. P-Plot graphics of serum (eNOS expressions, NO expressions, and SP-D levels) in each group

Based on the univariate analysis, in the eNOS expression, there was a mean difference between group K1 and the other groups. The most remarkable mean differences in eNOS were between group K1 and group K3. Based on the analysis, the mean eNOS expression in group K3 was 64.7 points greater than in group K1. In the P-Plot graphic shown in Figure 2, the differences in the eNOS expression between group K1 and group K3 were different. Nevertheless, it was not yet assumed that the differences between the two groups were significant. Otherwise, based on mean analysis, the smallest mean difference in eNOS expression was between group K2 and group K4. The difference was only 13.78 points. At this point, it was assumed that eNOS expression between group K2 and group K4 was similar. Group K4 had the most significant mean points in NO expressions. The 56.02-point differences between group K1 and group K4 were the most significant mean differences. The SP-D level showed that SP-D level differences in group K4 and group K3 were 52.52 points, and this was the highest difference between other groups. Table 1 shows the average value of NO levels in the inflammation group without therapy. It was interesting to see that the highest mean value of SP-D was in group K3. The interpretation of SP-D levels presents a new challenge, as does the finding of the highest eNOS expression in group K3. Determining its profitability requires further study.

Table 1. The average levels of endothelial nitric oxide synthase (eNOS), surfactant protein-D (SP-D), and nitric oxide (NO) in each group

		K1	K2	K3	K4
Mean	eNOS*	5.18	37.87	69.88	24.09
	SP-D (ng/mL)	72.56	81.96	101.39	48.87
	NO (mmol/L)	60.81	109.00	96.55	116.83

Table 2. Analysis of the relationship between nitric oxide (NO) levels, endothelial nitric oxide synthase (eNOS) expression, and serum surfactant protein-D (SP-D) levels

		eNOS	S-PD
NO	Pearson correlation	0.237	0.311
	P score	0.234	0.115

The results in Table 2 show no significant relationship between serum NO levels, eNOS expression, and serum S-PD levels with score p=0.234; p=0.115, respectively. In the correlation coefficient between NO and eNOS levels, the correlation coefficient value was 0.237, which indicated that the higher the NO level, the higher the eNOS level. The correlation coefficient between NO and S-PD levels was 0.311, which stated that the higher the NO level, the higher the S-PD level.

Figure 2 explains the calculation results of univariate analysis. The graph clearly shows the significant and non-significant groups. In this study, eNOS cells, NO-level serum, and SP-D counts were numerical variables categorized as parametric data. However, only eNOS cells differed significantly in each treatment compared to NO and S-PD. This might be due to the higher sensitivity of eNOS to inflammatory changes or interventions. Before analyzing eNOS cell expression, NO serum levels, and SP-D counts, data distribution and homogeneity were tested as assumptions for the F-test.

Based on the single-sample Kolmogorov-Smirnov test, the p-values for eNOS, NO, and SP-D were 0.076, 0.054, and 0.074, respectively. Since all three values were greater than 0.05, it can be concluded that eNOS, NO, and SP-D meet the assumption of normal data distribution. Levene's test for homogeneity of variance showed that the p-values for eNOS, NO, and SP-D were 0.050, 0.063, and 0.385, respectively. Since all values exceed 0.05, it can be concluded that the data meet the assumption of homogeneity of variance. After confirming normality and homogeneity, an F-test (ANOVA) was conducted.

This study used a 95% confidence level (α=0.05). The data consisted of four groups (K1, K2, K3, and K4) with 27 observations. The degree of freedom (df) between groups was 3, and the within-group df was 23. Thus, based on α=0.05, df1=3, and df2=23, the critical F-value from the F-table was 3.422. The ANOVA test results showed that the F-value for eNOS was 87.344, with $p<0.001$. Since the calculated F-value (87.344) was

greater than the critical F-value (3.422) and $p<0.05$, indicating a significant difference in eNOS cell expression among group K1, K2, K3, and K4. These results suggest that differences in treatment among the groups significantly impacted eNOS cell expression.

Based on the ANOVA test result, it was assumed that the eNOS expression was significantly different between all groups. There were significant differences between all groups in eNOS expression. Some immunohistochemical features of alveolar lung cells are shown in Figure 3.

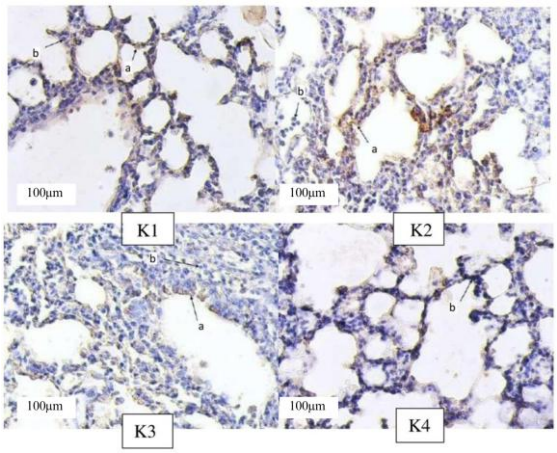


Figure 3. Immunohistochemical features of lung alveolar cells. (A) Endothelial nitric oxide synthase (eNOS) expression cell; (B) Nucleus

Figure 3 shows the appearance of the cell nucleus (b) and eNOS expression in the cytoplasm (a). The administration of gas intervention with CtEO showed a significantly higher number of cells expressing eNOS than the other groups. In contrast, the ANOVA test results on the NO and SP-D variables produced F values of 2.261 and 1.042, respectively, with p-values of 0.108 and 0.393. It shows the F-value was smaller than the F-table (3.422), and the p-value was greater than 0.05. The F test results showed no difference in the serum level of NO and the number of SP-D in each group. The results of the ANOVA test can also provide a conclusion. Four different gas intervention methods did not produce significant differences in NO and SP-D serum levels.

DISCUSSION

Considering the widespread use of aromatherapy, this study explored the effect of essential oil gas versus 0.9% NaCl solution on the lungs of mice exposed to an inflammation-inducing substance. The 0.9% NaCl solution, commonly used in intensive care unit (ICU) and ward aerosol therapies, was compared to VG+PG+VEA, an ingredient found in vape cigarettes, to induce inflammation. The significant difference in serum NO levels in group K1 and group K4 indicated that the VG+PG+VEA solution triggered increased serum NO levels in group K4. Although the statistical analysis results could not confirm that inflammation occurred in group K4, clinical observations showed that the inflamed mice without gas intervention experienced more deaths than the other groups. Intervention with 0.9% NaCl solution, as shown in Figure 2, was in the lowest position between the average values of group K2, K3, and K4, causing an increase in serum NO and SP-D levels and eNOS-expressing cells compared to those in other groups. However, only eNOS cells differed significantly in each treatment compared to NO and S-PD.

In addition to the NO serum examination, IHC was performed using antibody markers of cells expressing eNOS. The number of lung cells expressing eNOS in the lung tissue significantly differed among all groups. The highest number of cells expressing eNOS was observed in mice treated with the CtEO intervention. Nitric oxide is an essential biological mediator that functions in the lungs and regulates smooth muscle contractility, ventilation/perfusion relationships, and mucus secretion from the airway glands. It is also an essential mediator of the inflammatory response in the lung and mediates its effects through the formation of reactive nitrogen released from various inflammatory cells.[18] As such, serum NO was selected as a marker of inflammation in the lungs stimulated by intervening VG+PG+VEA solution. However, the results were inconsistent with the hypothesis in that no significant difference was observed in group K4 compared to other groups. This may be due to the reasonably short half-life of NO in serum due to its reactivity with hemoglobin and a broad spectrum of different biological compounds.[19]

In this study, gas-intervened mice with VG+PG+VEA might not have experienced severe sepsis. Therefore, it was possible that high serum NO levels could be contained. A previous study demonstrated that high amounts of NO were released during sepsis, which explained the shock observed in mice due to severe vasodilation.[20] It will be necessary to conduct further research on humans. However, suppose eNOS levels are higher in the citronella intervention group than in other groups. In that case, there is a possibility that CtEO can be used as gas therapy to induce eNOS in suitable cases. Endothelial nitric oxide synthase has potential in treating chronic lung diseases through its vasodilatory, antifibrotic, and inflammation-modulating effects.[21,22] Moreover, a previous study stated that eNOS has therapeutic potential in patients with cerebrovascular disease.[23]

The NaCl 0.9% intervention group stood out among the four groups because it had the lowest number of cells that expelled eNOS, the mean serum NO level, and serum SP-D (group K1). Intervention with NaCl 0.9% solution is widely used in the ICU as a mucolytic and increases the cough reflex, making it easier to clear the airway. However, the optimal level of NaCl in the respiratory tract is still debatable, given that various osmolarities will affect the patient.[24] Additionally, a previous study had attempted to analyze the use of saline or hypertonic saline for patients with acute respiratory distress syndrome (ARDS).[25] Endothelial nitric oxide synthase and NO production were higher in group K4 than group K1 due to the absence of intervention, allowing lung inflammation to persist continuously. Excessive inflammation triggers eNOS upregulation as a protective mechanism to increase NO production, which helps reduce leukocyte adhesion and suppress the release of pro-inflammatory cytokines. However, in uncontrolled inflammation, excessive NO production reacts with reactive O_2 species (ROS) to form peroxynitrite ($ONOO^-$), exacerbating oxidative stress and damaging lung tissue.[26,27]

The results from the four groups showed that the intervention group with CtEO significantly affected the release of eNOS expression in lung cells. There may be an effect of giving 100% O_2 in studies on eNOS, NO, and SP-D levels, but it cannot be revealed in what form the impact of O_2 is. Theoretically, giving high doses of O_2 will affect eNOS and NO levels for a specific period. It can stimulate eNOS to produce higher NO, but giving 100% O_2 for a long time will increase O_2 production, which is a radical, and will inhibit NO production.[28,29] To avoid bias, all groups in this study received high O_2 flow. Moreover, the purpose of giving 100% O_2 in this mice study was to reduce the risk of hypoxia, which could cause death. Thus, it is clear that the deaths that occurred in this study were due to the administered gas intervention.

Group K2 had lower eNOS levels than group K3, but higher serum NO levels than group K2. The results of this study suggest the following: 1) The selection of CtEO as a gas intervention increased eNOS expression in cells at the dose determined in this study; 2) An inappropriate dose of EgEO caused the goal as gas intervention to not appear in the change of biomarker level in this study; 3) The composition of the *E. globulus* used in this study contained some of the other materials as additive ingredients compared to CtEO which was a pure solution without any additive materials; 4) The use of gas intervention containing 0.9% NaCl liquid did not significantly affect the levels of the analyzed biomarkers. Endothelial nitric oxide synthase is a homeostatic regulator of several critical cardiovascular processes. Endothelial nitric oxide synthase-derived NO dilates various blood vessels by stimulating soluble guanylyl cyclase and increasing cyclic guanosine monophosphate (cGMP) in smooth muscles. Deletion of the eNOS gene increases blood pressure. Endothelial NOS inhibits leukocyte adhesion and vascular inflammation, controls smooth vascular proliferation, stimulates angiogenesis, and activates endothelial progenitor cells.[29,30] Some studies stated that eNOS helped in controlling pro-inflammatory conditions.[31,32]

Uncertainty surrounds the origin of SP-D in circulation. It is postulated to function in the cardiovascular system to control inflammatory signals. It is expressed in endothelial and smooth muscle cells (SMCs).[33] Additionally, the arterial wall produces SP-D and affects the total serum levels, and lung spillover is the primary contributor to SP-D levels in serum.[34] Based on studies that suggested the possibility that inflammation increases serum SP-D levels, especially in the lungs, this study hypothesized that the response to increased serum SP-D would increase under inflammatory conditions.[34] However, this study did not observe this, and the SP-D serum levels were similar between groups. There are several possible explanations for the aforementioned results: 1) there was no increase in alveolar permeability. Therefore, there was no significant increase in serum SP-D levels in each group; 2) this study used an inappropriate time of collection (there is a possibility that a particular time after the induction of inflammation shows peak blood levels of SP-D; 3) endothelial inflammation was not induced to high levels, which are align with the study by Colomorten, *et al*. (2019).[35]

CONCLUSION

This study indicates that CtEO may have a positive effect related to increasing the eNOS. The treatment group with citronella intervention showed a significant enhancement in eNOS expression compared to the group with 0.9% NaCl solution and EgEO intervention. The significant enhancement in eNOS expression observed in the CtEO treatment group suggests a potentially protective effect on the lungs or airways. Increased eNOS expression can lead to elevated NO production. However, careful formulation and delivery are essential to avoid airway irritation. Further studies should explore the efficacy of CtEO in preclinical models of lung disease.

LIMITATIONS OF THE STUDY

The limitation of this study is that the essential oils used were identified only by their composition, while their exact percentages remain unknown.

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Conflict of Interest

The authors declared there is no conflict of interest.

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Authors' Contributions

Collecting the participant data, sample, and manuscript draft editing: ASV, HSP, AAD. Performed and interpreted the data and edited manuscript: AR, SS. Interpreted tissue histology results: HR. Performed the statistical analysis: RFM, SSS. All authors contributed and approved the final version of the manuscript.

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