The Effect Of Nanoemulsion Mouthwash Containing Citronella Oil (Cymbopogon Winterianus) And Lemon Oil (Citrus Limon) On The Number Of Macrophages In Pregnant Rat Model Induced By Gingivitis

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Received 19 July 2025; Accepted 25 August 2025; Published online 25 August 2025

Keywords:

citronella oil (*Cymbopogon winterianus),* gingivitis, lemon oil (*Citrus limon*), macrophages, mouthwash,nanoemulsion

ABSTRACT

Background: *Pregnancy gingivitis is known to affect high-risk pregnancies. Therefore, preventive treatments are necessary, one of them being the use of mouthwash. Citronella oil and lemon oil have been proven to have anti-inflammatory properties. The aim of this study was to investigate the effectiveness of a nanoemulsion mouthwash combining citronella oil (Cymbopogon winterianus) and lemon oil (Citrus limon), on macrophage counts in a pregnant rat model induced with gingivitis.*
Methods: *Female Sprague Dawley rats were confirmed to be pregnant, followed by ligation of both lower incisor teeth, and then mouthwash application. The rats were routinely fed a moist diet and drinking water containing 10% sucrose. Mouthwash application was performed twice daily (morning and evening) over 7 days. Histological evaluation was conducted by counting the number of macrophages in hematoxylin-eosin preparations.*
Result: *The results indicated a reduction in the number of macrophages following the administration of a nanoemulsion mouthwash that combines citronella oil and lemon oil. The Mann -Whitney test revealed a significant difference compared to the control group ($p<0.05$).*
Conclusion: *The nanoemulsion mouthwash with 1% citronella oil and 2,77% lemon oil may help reduce the number of macrophages in pregnant rats model induced with gingivitis.*

DOI: http://dx.doi.org/10.30659/medali.7.2.162-168
MEDALI Jurnal: Media Dental Intelektual accredited as **Sinta 4 Journal** (https://sinta.kemdikbud.go.id/journals/profile/13665)
2337-6937/ 2460-4151 ©2025 National Research and Innovation Agency

How to Cite: *Rahman et al*. The Effect Of Nanoemulsion Mouthwash Containing Citronella Oil (Cymbopogon Winterianus) And Lemon Oil (Citrus Limon) On The Number Of Macrophages In Pregnant Rat Model Induced By Gingivitis. MEDALI Jurnal: Media Dental Intelektual, v.7, n.2, p.162-168, August 2025.

INTRODUCTION

Gingivitis develops due to an interaction between the human immune response and the biofilm of pathogenic microorganisms present in dental plaque.[1] The presence of bacteria or toxins in plaque stimulates an inflammatory response and immune response, which are important parts of the pathogenesis of periodontal disease. Various systemic conditions and factors modulate the interaction between microorganisms and the host's immune response.[2] Plaque-induced gingivitis can be influenced by modifications in systemic factors, one of which is pregnancy.[3] Studies report that 34% of pregnant women have shallow pockets (4–5 mm) and 2% have deep pockets (≥6mm).[4] Several studies indicate that periodontitis is strongly associated with high-risk pregnancies, such as low birth weight, preterm birth, impaired fetal growth, and preeclampsia.[5,6,7] Given the high risk associated with periodontitis in pregnant women, preventive measures are necessary. These preventive measures are necessary due to the high prevalence of gingivitis among pregnant women, and it is hoped that such measures will reduce the incidence of high-risk pregnancies. One of the preventive measures that can be taken is the regular use of mouthwash, as studied by Jiang et al.[8]

According to the Oral Health Care During Pregnancy Expert Workgroup, pregnant women are not recommended to use mouthwash containing alcohol. Therefore, it is necessary to develop herbal mouthwash that is safer for pregnant women.[9] Lemongrass oil (Cymbopogon winterianus) contains the compounds citronellal, citronellol, geraniol, and limonene.[10] Lemongrass oil has been proven to have antibacterial activity,[11] as well as anti-inflammatory effects. [12] Lemon oil (Citrus limon) contains citral, citronellal, limonene, α-terpineol, geranyl acetate, and linalool.[13] The compounds linalool and linalyl acetate exhibit antibacterial and antiviral activity.[14] The high limonene content in lemon oil also provides anti-inflammatory effects.[15]

The pathogenesis of periodontal disease is divided into four phases: initial lesion, early lesion, established lesion, and advanced lesion. Cells involved in the early lesion phase include macrophages.[16] This phase occurs on days 4-7 after plaque accumulation.[17] In gingivitis, macrophages play a role in limiting pathological changes to soft tissue or escalating the inflammatory response to the next stage. Macrophages phagocytose bacteria, produce cytokines and chemokines, and activate the adaptive immune response. The final outcome of macrophage activity can result in healing, infection containment through fibrosis formation, or healing with scar tissue formation.[18] Polyherbal mouthwash is a mouthwash composed of more than one natural ingredient. The composition of natural ingredients in polyherbal mouthwash consists of two or more natural ingredients.[19,20,21] This study aims to determine the effect of the application of a nanoemulsion mouthwash combining lemongrass oil and lemon oil on the number of macrophages in pregnant rat model induced with gingivitis.

METHODS

The production of nanoemulsion mouthwash is carried out using a low-energy method without heating, using a magnetic stirrer to mix the mouthwash ingredients until they form a nano-sized emulsion. Lemongrass oil (Cymbopogon winterianus) 1% and lemon oil (Citrus limon) 2,77% are added to a beaker placed on a magnetic stirrer, then mixed with Tween 80 and distilled water. Stirring is performed at room temperature until a homogeneous solution is obtained. Particle diameter measurements were performed using a Particle Size Analyser (PSA).

Female adult Sprague-Dawley (SD) rats, provided from the Laboratory Animal Facility at LPPT Unit IV, Universitas Gadjah Mada, approximately three months old and measuring between 200 and 250 grammes. For a week of acclimatisation, female rats in the oestrus phase were cohabited overnight with a male for mating purposes. A vaginal swab examination was performed on the female rats in the morning, 24-48 hours post-mating, to verify the occurrence of copulation. Pregnancy was determined if spermatozoa were observed in the vaginal smear during microscopic examination.[22,23]

Female rats confirmed to be pregnant are then subjected to gingivitis induction based on the studies by Suryono et al.[24] and Rahman et al.[25] Prior to this, anesthesia is administered using a 10% ketamine and 2% xylazine solution in a 2:1 ratio, with a dose of 0.12 ml/100g body weight administered intramuscularly into the posterior thigh muscle. After anaesthesia, ligation was performed using 3.0 silk ligature thread in the gingival sulcus around the lower incisor teeth. The mouthwash was applied immediately after ligation.[26] Soft pellets (moist feed) and drinking water containing 10% sucrose were provided.[27]

This study consisted of two groups: the nanoemulsion mouthwash group with lemongrass oil and lemon oil, and the control group without any treatment. The treatment was administered by irrigating the mouthwash solution on the front incisor teeth twice daily in the morning and evening, using a micropipette of 50 µl for each treatment group.[27] Each treatment group consisted of 3 rats. If ligation threads came loose during treatment administration, re-ligation was performed immediately. All procedures were approved by the Ethics Committee of the Faculty of Dentistry-Prof. Soedomo Dental Hospital, Universitas Gadjah Mada with the number 2/UN1/KEP/FKG-RSGM/EC.

On day 7, necropsy was performed after administering anaesthesia using 10% ketamine and 2% xylazine in a 2:1 ratio at a dose of 0,12 ml/100gBW intramuscularly into the posterior thigh muscle. Histological examination was performed by cutting the lower jaw. The gingival and tooth tissues from the lower jaw that were collected were then stored in 10% neutral buffered formalin for 24 hours at room temperature, followed by decalcification with ethylene diamine tetraacetic acid (EDTA) solution pH 7,4 for 4 weeks at room temperature. EDTA was replaced weekly. The tissue was then embedded in paraffin (4 µm) and prepared for hematoxylin-eosin (HE) staining. Histometric measurements were performed using a microscope at 400x magnification with an OptiLab camera. Macrophage cells were observed in three fields of view. Observation and counting of macrophage cells were performed using Image J software.

Data analysis of macrophage counts was performed using the Mann-Whitney statistical test with a significance level of $p<0,05$.

RESULT

HE staining was performed to determine the presence of macrophage cells in the gingival tissue. Macrophage cell infiltration was observed in the gingival tissue of the treatment and control groups. Most macrophages have round or oval nuclei, with one or more small nucleoli, as shown in Figure 1. Figure 2 displays the numbers of macrophage cells in the treatment and control groups.

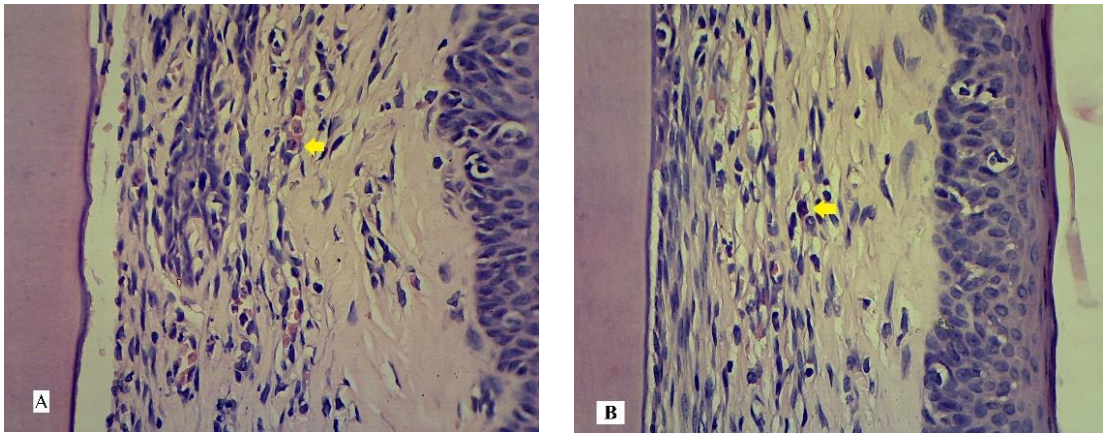

Figure 1. Representative image of macrophages (yellow arrows) in the control group (A) treatment group (B) (400×; HE)

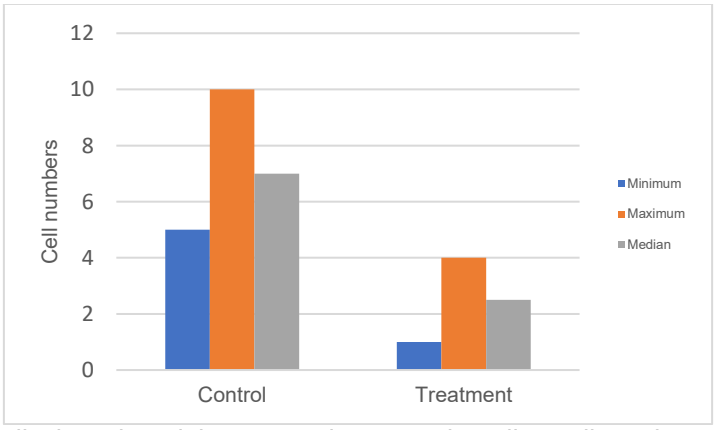

Figure 2. The bar chart displays the minimum, maximum, and median cell numbers of macrophages in both control and treatment groups.

Table 1. Results of Mann-Whitney test analysis

	n	Median (Min-Max)	p-value
Treatment	18	2,50 (1 - 4)	0,000*
Control	18	7 (5-10)	

n= sample size
*sig (*p*<0,05)

The study results showed that the treatment group using nanoemulsion mouthwash containing lemongrass oil and lemon oil had a lower number of macrophages compared to the control group. The results were further analysed using the Mann-Whitney test, which showed a significant difference between the treatment group and the control group (Table 1).

DISCUSSION

The results showed that the administration of a combination of 1% lemongrass oil and 2.77% lemon oil nanoemulsion mouthwash significantly reduced the number of macrophages in the gingival tissue of pregnant rats induced with gingivitis, compared to the control group (p=0,000). This reduction in macrophage numbers indicates a significant effect of the mouthwash on the

inflammatory response of gingival tissue.

Macrophages are one of the primary immune cells in the early lesion phase of gingivitis, which is the initial inflammatory phase occurring on days 4 to 7 after plaque formation.[16,17] Under normal conditions, macrophages play a role in bacterial phagocytosis, secretion of pro-inflammatory cytokines, and activation of adaptive immunity.[18] A high number of macrophages typically indicates an active inflammatory process and is directly related to the severity of periodontal disease.[28]

The anti-inflammatory effects of this mouthwash can be attributed to the bioactive compounds in lemongrass oil and lemon oil. Lemongrass oil contains citronellal, citronellol, and geraniol, which have been proven to have antibacterial and anti-inflammatory activity,[11,12] while lemon oil is rich in limonene, citral, and linalool, which inhibit the migration of inflammatory cells and reduce the expression of inflammatory mediators such as TNF-α and IL-6. [14,15] This mechanism is believed to contribute to reducing macrophage recruitment in inflamed gingival tissue.

The nanoemulsion mouthwash used in this study, which combines 1% lemongrass oil and 2,77% lemon oil, has an average particle size of ± 220 nm.[29] The use of a nanoemulsion delivery system enhances the efficacy of the active ingredients. Nano-sized particles increase surface area and accelerate dissolution, thereby enhancing the efficient penetration of active substances into gingival tissue.[30,31] This allows the anti-inflammatory components of essential oils to function more optimally on target tissues.

This study also supports the importance of developing herbal mouthwash as a safe alternative to non-alcoholic mouthwash for pregnant women, as recommended by the Oral Health Care During Pregnancy Expert Workgroup.[9] The high prevalence of gingivitis during pregnancy and its potential to increase the risk of high-risk pregnancies such as preeclampsia and preterm birth,[3,5,7] makes the use of natural-based anti-inflammatory agents clinically and practically relevant.

This study has limitations, the duration of treatment is only seven days. While sufficient to observe the early stages of gingivitis (early lesions), this timeframe does not yet show progression toward periodontitis. Long-term evaluation is needed to understand the long-term effects of using this mouthwash.

Overall, these results indicate that the combination mouthwash of 1% lemongrass oil and 2,77% lemon oil in nanoemulsion form is effective in suppressing gingival inflammatory responses through the reduction of macrophage numbers and has the potential to be further developed as a gingivitis-preventing agent, particularly in pregnancy.

CONCLUSION

The application of nanoemulsion mouthwash with 1% lemongrass oil and 2,77% lemon oil influences the reduction in the number of macrophages in a pregnant rat model induced with gingivitis.

ACKNOWLEDGEMENTS

The authors presented this topic orally at the TIP IPAMAGI VIII symposium on June 28, 2025. The authors deny any conflicts of interest related to this study.

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