

## Effect of *Ganoderma lucidum* extract on tumor necrosis factor-alpha and prostaglandin E2 levels in periodontitis model Sprague Dawley rats

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### ABSTRACT

**Background:** Periodontitis is a chronic multifactorial disease caused by microorganisms such as G-anaerobes in the periodontal tissues. It activates host defense cells and releases inflammatory mediators such as tumor necrosis factor-alpha (TNF- $\alpha$ ) and prostaglandin E2 (PGE2). *Ganoderma lucidum* is a traditional medicinal mushroom with anti-inflammatory effects against various diseases. Biologically, different levels of its active constituents, such as triterpenoids and phenolic compounds, reduce inflammation with various pathways. Furthermore, the constituents inhibit toll-like receptor 4, MyD88 receptors, and the activities of nuclear factor-kappa B, which synthesize TNF- $\alpha$  and PGE2. **Purpose:** This study aimed to analyze the extent to which *G. lucidum* extract can reduce TNF- $\alpha$  and PGE2 levels in periodontitis model Sprague Dawley rats. **Methods:** Thirty Sprague Dawley rats were randomly divided into six groups of five rats. Periodontitis inflammation was induced by the injection of *Porphyromonas gingivalis* bacteria into intrasulcular gingival incisors in the lower jaw labial section. Grouping was as follows: Group K1 (healthy control); Group K2 (negative control); Group K3 (positive control with doxycycline dose 0.27 mg/kg BW); Group P1 (*G. lucidum* extract dose 5 mg/kg BW); Group P2 (*G. lucidum* extract dose 10 mg/kg BW); and Group P3 (*G. lucidum* extract dose 20 mg/kg BW). Samples were taken from rat gingival tissue and the levels of TNF- $\alpha$  and PGE2 were examined using the enzyme-linked immunosorbent assay method. Data analysis was performed using one-way analysis of variance (ANOVA) with a confidence level of 95% ( $p < 0.05$ ). **Results:** The levels of TNF- $\alpha$  and PGE2 were the highest in the K2 group and the lowest in the K1 group. One-way ANOVA showed no significant difference in TNF- $\alpha$  and PGE2 levels between P3 and K1 group. **Conclusion:** *G. lucidum* extract can reduce TNF- $\alpha$  and PGE2 levels in Sprague Dawley rats with periodontitis.

**Keywords:** anti-inflammatory; *Ganoderma lucidum*; periodontitis; prostaglandin E2; tumor necrosis factor alpha

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### INTRODUCTION

Periodontitis is a multifactorial disease caused by microorganisms such as G-anaerobes in the periodontal tissues, which results in tooth loss.<sup>1,2</sup> Pathogenic bacteria such as *Porphyromonas gingivalis* produce endotoxins in the form of lipopolysaccharides (LPS), causing the production of local inflammatory factors, such as tumor necrosis factor-alpha (TNF- $\alpha$ ) and prostaglandin E2 (PGE2), that participate in periodontal collagen fibers destruction and alveolar bone resorption.<sup>3-6</sup> Conventional therapies such as plaque control, scaling root planning,

and host modulation therapy (HMT) are used as treatment methods. A sub-antimicrobial dose of doxycycline (SDD treatment) 20–40 mg administered two times a day suppresses cytokines such as TNF- $\alpha$ ,  $\alpha$ -amylase, and phospholipase A2 enzymes. These two enzymes are required in the biosynthesis of inflammatory mediators such as PGE2.<sup>7,8</sup> Doxycycline SDD has an anti-inflammatory effect by suppressing TNF- $\alpha$  and PGE2, but it must be consumed regularly for 3, 6, or up to 9 months to obtain optimal results.<sup>9</sup> As such, we aimed to ascertain whether an alternative anti-inflammatory drug for periodontitis might have a faster effect.

Many studies have shown that mushrooms are rich in bioactive compounds. *Ganoderma lucidum* is a medicinal mushroom known as “*Lingzhi*” in China or “*Reishi*” in Japan. It has around 400 bioactive compounds, including terpenoids, polysaccharides, polyphenols, steroids, fatty acids, proteins, and glycopeptides. The triterpenoid contents consist of ganoderic, ganoderenic, ganoderol, and lucidenic acid, which have been widely reported<sup>10</sup> to have anti-inflammatory and antimicrobial effects, and have been officially recorded in the Chinese Pharmacopoeia.<sup>11–15</sup> Polyphenol contents also have these effects,<sup>16–18</sup> while polysaccharides provide immunomodulatory and antibacterial effects.<sup>19</sup> According to Yoon et al.,<sup>20</sup> *G. lucidum* extract inhibits the inflammatory response by deactivating the toll-like receptor 4 (TLR4) pathway and suppressing nuclear factor-kappa B (NF-κB) activation in BV2 microglial cells, and significantly reduces the release of TNF-α and PGE2. Weng et al.<sup>21</sup> reported a study in which *G. lucidum* extracts were administered at a daily dose of 10 mg/kg for 7–10 days. The results showed a decreased number of inflammatory cells, treating the inflammation that induces bone resorption, and an improvement in the distance of cemento–enamel junction and alveolar bone crests was observed using micro-computed tomography and histology. Considering the results of Weng et al.’s<sup>21</sup> preliminary study, this research examines the effects of administering *G. lucidum* extract at doses of 5, 10, and 20 mg/kg BW on TNF-α and PGE2 levels in Sprague Dawley rats.

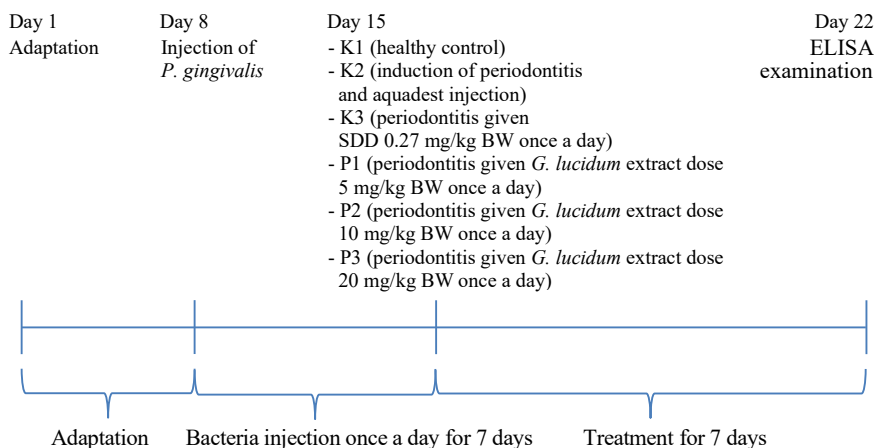
**MATERIALS AND METHODS**

The type of research used was laboratory experimental, and the type of design was post-test-only with a control group. Ethical clearance was granted by the Ethics Commission, Faculty of Medicine, Jenderal Soedirman University, Purwokerto (8079/KEPK/XI/2019). The research was conducted using 30 Sprague Dawley rats, which were randomly sampled into six groups: K1 (healthy control);

K2 (negative control, induction of periodontitis and aquadest); K3 (positive control, induction of periodontitis and sub-antimicrobial dose of doxycycline [SDD] 0.27 mg/kg BW); P1 (periodontitis with *G. lucidum* extract dose 5 mg/kg BW); P2 (periodontitis with *G. lucidum* extract dose 10 mg/kg BW); and P3 (periodontitis with *G. lucidum* extract dose 20 mg/kg BW).

The rats were adapted for 1 week in a rat cage measuring 40 x 30 x 60 cm and placed in a room with sufficient airflow and light. On the eighth day, periodontitis was induced in groups K2, K3, P1, P2, and P3 by injection of *P. gingivalis* bacteria into intrasulcular gingival incisors. Suspension of *P. gingivalis* was made using one dose of *P. gingivalis* and mixed with 2 mL of sterile BHI-B solution in a test tube. The test tube was incubated in an incubator for 24 hours at a temperature of 37°C. The solution was then diluted by adding NaCl, homogenized using a vortex mixer, and absorption measured at 1 McFarland standard (McFarland standard is a specific scale for the concentration of bacteria/ml; it is used to approximate the number of microbial cells in a liquid). The injection was administered to the lower jaw labial section at a dose of 0.2 mL, 1 McFarland standard, once a day for 7 days. Radiographic examinations were conducted to investigate the occurrence of bone resorption.

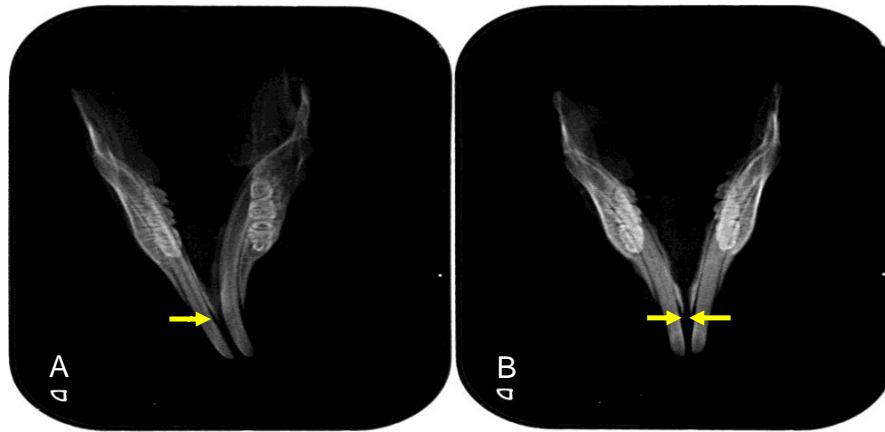
The experimental animals were given *G. lucidum* extract and SDD treatment, according to the group, intraperitoneally using a gastric sonde to compare the therapeutic effects of *G. lucidum* extract and the standard drug for periodontitis (i.e. SDD) for 7 days. The extraction of *G. lucidum* was performed through maceration with 96% ethanol. The standard dose of doxycycline in humans is 20 mg. This dose was converted to rat administration by multiplying by 0.018 to 20 mg x 0.018 = 0.36 mg for rats weighing 200 g and 150 gr / 200gr x 0.36 mg = 0.27 mg for a weight of 150 g, so that a dose of 0.27 mg / kgBB was obtained. On the 22nd day, the whole group was decapitated and samples were obtained from the gingival tissue. Tissue collection was carried out in the first 24 hours after treatment by an incision in the periodontitis



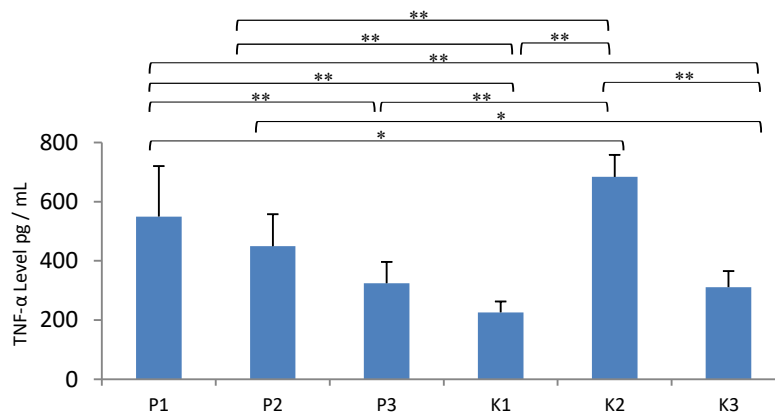
**Figure 1.** Timeline of the entire experiment.

induction area on the lower incisors. The tissues were cut and weighed, with each tissue weighing  $\pm 25$  mg. The tissue was treated with PBS liquid and then smoothed until there were no fine fibers and sonicated ( $10^{11} \times 3$  interval for 30-seconds). The supernatant was centrifuged at 10,000 rpm for 15 minutes. The supernatant was separated from the pellet and put into a new tube, then stored at  $-80^{\circ}\text{C}$ .

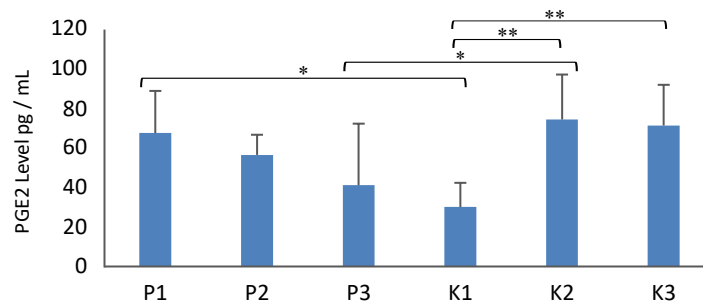
For TNF- $\alpha$  and PGE2 examination, gingival tissue was examined using the enzyme-linked immunosorbent assay (ELISA) method (Elabscience, Texas, USA), showing an absorbance wave of 450 nm in groups P1-K3. Data analysis was performed using one-way analysis of variance (ANOVA) with a confidence level of 95% ( $p < 0.05$ ). The timeline is shown in Figure 1.



**Figure 2.** (A) Radiographic image picture of healthy rat alveolar bone; (B) radiographic image of rat alveolar bone with periodontitis.



**Figure 3.** Average results for TNF- $\alpha$  levels determined using a mouse ELISA assay kit, comparing groups. Data are expressed as means  $\pm$  SDs. The statistical significance of differences between groups was determined using one-way ANOVA ( $n=4$ ; \* $p < 0.05$ ; \*\* $p < 0.01$ ).



**Figure 4.** Average results for PGE2 levels determined using a mouse ELISA assay kit, comparing groups. Data are expressed as means  $\pm$  SDs. The statistical significance of differences between groups was determined using one-way ANOVA ( $n=4$ ; \* $p < 0.05$ ; \*\* $p < 0.01$ ).

## RESULTS

In this study, induction of *P. gingivalis* was performed in a chronic periodontitis model for 7 days. Figure 2A presents a radiographic image of the lower jaw incisor alveolar bone in the healthy rat (yellow arrow). Figure 2B shows a radiographic picture where there is resorption in the alveolar bone, broken lamina dura, and widening periodontal space of the lower jaw incisors (yellow arrow).

The TNF- $\alpha$  and PGE2 levels were observed using an ELISA test with sandwich technique. The mean TNF- $\alpha$  level is shown in Figure 3. The highest TNF- $\alpha$  levels were obtained in the K2 group, with an average of 683.66 + 74.44 pg / mL, whereas the lowest were in the K1 group, with an average of 226.27 + 36.64 pg / mL. From the highest to the lowest levels, the groups were ranked as follows: K2, P1, P2, P3, K3, and K1. The test results showed significant differences between groups with a value of  $p = 0.000$  ( $p < 0.05$ ), and indicated a significant effect of *G. lucidum* extract treatment on TNF- $\alpha$  levels. There was a significant difference between the treatment groups that received doses of 5 mg / kgBW (P1) and 10 mg / kgBW (P2) and the healthy control group (K1). There was no significant difference between the group with a treatment dose of 20 mg/kg BW (P3) and the healthy controls (K1) or positive controls (K3). The mean PGE2 levels are shown in Figure 4.

## DISCUSSION

The results showed that TNF- $\alpha$  levels in periodontitis decreased with extract of *G. lucidum*. The 20 mg/kgBW dose had a better anti-inflammatory effect than the 10 mg/kgBW dose. This shows that the higher the extract dose is, the lower the TNF- $\alpha$  levels in the gingival tissue. In addition, the results showed that PGE2 levels in periodontitis decreased with extract of *G. lucidum*. The 20 mg/kgBW dose had a better anti-inflammatory effect than the 10 mg/kgBW dose. This shows that the higher the extract dose is, the lower the PGE2 level in the gingival tissue.

Ethanol extract of *G. lucidum* was investigated for its anti-inflammatory potential by stimulating murine BV2 cells with LPS. Thereafter, the amount of TNF- $\alpha$  and PGE2 in supernatant culture was quantified following Yoon et al.<sup>20</sup> The obtained results showed that *G. lucidum* extract inhibits the degradation of I $\kappa$ B- $\alpha$ , the translocation of the NF- $\kappa$ B p65 subunit, and the transcription activity of NF- $\kappa$ B. In addition, it inhibits the transcription activity of NF- $\kappa$ B in the nucleus of inflammatory cells such as macrophages, since it suppresses COX-2 and TNF- $\alpha$  expression. Its inhibition of the NF- $\kappa$ B signaling pathway decreases the regulation of proinflammatory mediators and produces anti-inflammatory effects. Similarly, Hasnat et al.<sup>22</sup> reported an increased *G. lucidum* content

on germinated brown rice (GLBR) in a colitis rat model. This is because *G. lucidum* suppressed the production of PGE2 in macrophages stimulated with LPS and decreased COX-2 and TNF- $\alpha$  mRNA expression. It also inhibited the activation of p38, Extracellular Signal-Regulated Kinase (ERK), c-Jun N-terminal Kinase (JNK), Mitogen Activated Protein Kinase (MAPK), and NF- $\kappa$ B. Colon mucosal injury was evaluated using macroscopic, biochemical, and histopathological tests. The results showed significantly decreased values after treatment with GLBR. Therefore, GLBR treats colitis through inhibition of MAPK phosphorylation and activation of NF- $\kappa$ B. Similarly, Dudhgaonkar et al.<sup>23</sup> studied the ethanol extract of *G. lucidum* by isolating triterpenoids against Lipopolysaccharides (LPS)-stimulated RAW 264.7 macrophages cells. They reported that a dose of 10–50  $\mu$ g/mL significantly suppressed TNF- $\alpha$  secretion (IC50 15.1  $\mu$ g/mL) and PGE2 (IC50 28.2  $\mu$ g/ml) and COX-2 expression. Its anti-inflammatory effect was expressed in inhibition of NF- $\kappa$ B transcription factors, as observed in decreasing NF- $\kappa$ B DNA-binding activity, suppression of p65 phosphorylation, inhibition of AP-1 DNA-binding activity, decreasing expression of c-Jun, and suppressed MAP kinase activity, as observed in decreasing regulation of ERK 1/2 phosphorylation and JNK.

Liu et al.<sup>24</sup> in 2012 isolated six derivatives of triterpenoids from *G. lucidum*, namely ganoderic acid A, C, D, J, K, and L. These derivatives were further identified based on LC-MS and NMR spectra to obtain the most effective compounds in suppressing TNF- $\alpha$  production in Lipopolysaccharides (LPS)-stimulated RAW 264.7 macrophages cells. The ganoderic acid C constituent is extremely effective in inhibiting the production of TNF- $\alpha$  from macrophages (IC50 24.5 mg/ml). It also inhibits JNK and c-Jun phosphorylation by stimulating LPS in macrophages at a dose of 20  $\mu$ g/ml. Ganoderic acid C blocks ERK activation and decreases LPS-induced nucleus translocation. Furthermore, it suppresses the production of TNF- $\alpha$ -induced LPS by regulating the MAPK, NF- $\kappa$ B, and AP-1 signaling pathways in macrophages. In 2018, Chi et al.<sup>25</sup> isolated ganoderic acid A from *G. lucidum* and reported that it decreased the release of TNF- $\alpha$ -induced LPS in cortical microglial culture cells by inhibiting the NF- $\kappa$ B signaling pathway.

Ethanol extract of *G. lucidum* has polyphenol content, known to inhibit the pathway of NF- $\kappa$ B. Two phenolic compounds were isolated, namely ganoduriporol A and B, from the body of *Ganoderma duripora* fruit, and the anti-inflammatory activity in relation to RAW 264.7 cells was tested.<sup>26</sup> These compounds can inhibit the production of TNF- $\alpha$  and PGE2 through suppression of the MAPK signaling pathway, NF- $\kappa$ B, and LPS-induced COX-2 macrophage expression. *G. lucidum* is also known to contain ingredients such as flavonoids, steroids, and Ling-Zhi 8 (LZ-8). Flavonoids show strong inhibitory activity against various enzymes, such as phospholipase A2, kinase C, tyrosine kinase, and phosphodiesterase.



They also have the potential to inhibit prostaglandins and suppress their inflammatory process. They exhibit antioxidant activity in inhibiting NO synthetase activity and excessive NO production, which results in tissue damage and inflammation. Steroid contents such as trametenolic acid, ergosterol peroxide, and ergosterol have been isolated from fungi and are reported to exhibit anti-inflammatory activity. Kobori et al.<sup>27</sup> reported that ergosterol and ergosterol peroxide in fungi suppress the secretion of TNF- $\alpha$  in RAW 264.7 cells induced by LPS. In addition, they suppress inflammatory response by inhibiting the binding activity of DNA NF- $\kappa$ B and C/EBP $\beta$  and the phosphorylation of MAPK p38, JNK, and ERK. In 2017, Chen et al. investigated the effect of LZ-8 protein isolation of *G. lucidum* on PGE2 levels in LPS-stimulated microglial BV2 cells. They reported that it interfered with the NF- $\kappa$ B signaling cascade by inhibiting I $\kappa$ B phosphorylation and translating p65 into the nucleus.<sup>28</sup> The results of flow cytometric and PCR analysis showed that LZ-8 significantly inhibited TLR-4 expression and NF- $\kappa$ B signal transduction pathways. Therefore, it suppresses the production of pro-inflammatory mediators and excessive expression of COX-2.

Based on the results of the research conducted, the conclusion can be drawn that *G. lucidum* extract can reduce TNF- $\alpha$  and PGE2 levels in periodontitis model Sprague Dawley rats. The dose was 20 mg/kg BW. The results also showed that SDD treatment could reduce TNF- $\alpha$  levels better than *G. lucidum* extract, and that it could also reduce PGE2 levels, but no better than *G. lucidum* extract. Therefore, *G. lucidum* might have potential as an alternative treatment. There were no significant differences in TNF- $\alpha$  and PGE2 levels between the 20 mg/kg BW *G. lucidum* and SDD groups.

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