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ORIGINAL ARTICLE

# Antibacterial activity of *Trigona* sp. propolis extract against *Aggregatibacter actinomycetemcomitans* in vitro

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**ABSTRACT**

**Background:** *Aggregatibacter actinomycetemcomitans* is a primary pathogen in aggressive periodontitis. Plaque control uses chemical and natural agents. Propolis inhibits growth of bacteria, viruses, and fungi. This study assessed the in vitro antibacterial activity of *Trigona* sp. propolis extract against *A. actinomycetemcomitans*.

**Methods:** This true experimental laboratory study used a post-test-only control group design. Samples were *A. actinomycetemcomitans* bacteria in 8 groups (n=5 each). The dilution method tested antibacterial activity. Data analysis used Kruskal-Wallis and Mann-Whitney tests.

**Results:** Mean *A. actinomycetemcomitans* colony counts (mean ± SD) were 5.60 ± 1.52, 11.60 ± 4.83, 15.60 ± 5.03, 22.20 ± 2.86, 25.40 ± 6.43, and 141.80 ± 5.40 for propolis concentrations of 50, 25, 12.5, 6.25, 3.125, and 1.56, respectively. Negative control (DMSO) showed uncountable colonies; positive control (chlorhexidine) showed none. Kruskal-Wallis test indicated significant differences (p=0.001). Mann-Whitney test showed differences between most groups (p<0.05), except 25 vs. 12.5% (p=0.421), 12.5 vs. 3.125% (p=0.056), and 6.25 vs. 3.125% (p=0.151).

**Conclusion:** *Trigona* sp. propolis inhibited and showed bactericidal potential against *A. actinomycetemcomitans* in vitro, with minimum inhibitory concentration at 3.125% and minimum bactericidal concentration at 50%.

**Keywords:** MIC, MBC, *Aggregatibacter actinomycetemcomitans*, *Trigona* sp. propolis

## Introduction

In 2018, the Indonesian Basic Health Research (RISKESDAS) reported that 57.6% of the Indonesian population had oral and dental health problems; dental caries had the highest prevalence at 88.8%, followed by periodontal disease at 74.1%.[1] The high prevalence of periodontal disease is associated with low oral hygiene awareness, infrequent dental visits, low socioeconomic status, and high illiteracy rates.[2,3] Periodontitis is an inflammatory disease that affects the tooth-supporting structures; it is caused by specific microorganisms and results in progressive destruction of the periodontal ligament and alveolar bone, as well as periodontal pocket formation or gingival recession.[4] The American Academy of Periodontology classifies periodontal disease into chronic periodontitis, aggressive periodontitis, and periodontitis as a manifestation of systemic diseases.[5] Specific gram-negative microorganisms in dental plaque biofilm, such as *A. actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Fusobacterium nucleatum*, contribute to periodontitis development.[6,7]

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*Aggregatibacter actinomycetemcomitans* (Aa) is a primary pathogen associated with aggressive periodontitis. This gram-negative, nonmotile, facultative anaerobic short rod induces progressive destruction of periodontal tissues.[8] Periodontal therapy aims to restore the balance between the host and plaque bacteria through mechanical and chemical plaque control, including antiseptic mouthwashes such as chlorhexidine.[9,10] Although chlorhexidine is the gold standard antimicrobial agent in dentistry, long-term use can cause adverse effects, including taste alteration, tooth and mucosal staining, and IgE- and histamine-mediated allergic reactions. These effects encourage the search for safer natural alternatives.[11–13]

One potential natural antimicrobial agent is propolis, a resinous substance that bees collect from plant sources. It consists mainly of flavonoids and phenolic compounds, which have anti-inflammatory, antiviral, antifungal, and antibacterial properties.[14–16] In Indonesia, propolis from *Trigona* sp. (stingless bees) is widely cultivated because of its easier maintenance and lack of a stinger.[17,18] Flavonoids are the most active components in propolis and comprise approximately 50% of its content. They function as antibacterial agents by inhibiting nucleic acid synthesis, disrupting cell membrane integrity, and suppressing bacterial energy metabolism.[19] Several studies have demonstrated the therapeutic potential of propolis in periodontal treatment. For example, propolis paste application led to clinical improvement in 90% of chronic periodontitis cases after one week.[20,21] Propolis flavonoid extracts inhibit the growth of *A. actinomycetemcomitans* and *P. gingivalis*.[22]

This study evaluated the in vitro antibacterial activity of Trigona sp. propolis extract against *Aggregatibacter actinomycetemcomitans* by determining minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values. The hypothesis stated that the extract exhibits inhibitory and bactericidal activity against *A. actinomycetemcomitans*.

## Method

This study is a true experimental laboratory study using a posttest-only control group design to analyze the in vitro antibacterial activity of Trigona sp. propolis extract against *Aggregatibacter actinomycetemcomitans*. The study was conducted from August to September 2025. Propolis extract concentrations were prepared at the Laboratory for Research and Development of Medicinal Plants, whereas phytochemical testing, bacterial cultivation, and antibacterial testing were performed at the Integrated Laboratory, Universitas Prima Indonesia.

The samples included pure cultures of *A. actinomycetemcomitans* from an e-commerce supplier and Trigona sp. propolis extract from a honey beekeeper. Samples were assigned to eight groups: six propolis extract concentrations (1.56%, 3.125%, 6.25%, 12.5%, 25%, and 50%), one positive control (chlorhexidine), and one negative control (DMSO).

The independent variable was *Trigona* sp. propolis extract concentration. The dependent variable was bacterial growth, assessed by colony counts to determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Controlled variables were Brain Heart Infusion (BHI) medium and 24-hour incubation. Equipment included a Buchner funnel, electric scale, blender, Erlenmeyer flask, rotary evaporator, test tubes, sterile inoculating loop, measuring pipette, water bath, Petri dishes, incubator, and electric stove. Materials were propolis extract, *A. actinomycetemcomitans* culture, 96% ethanol, BHI medium, Tryptic Soy Agar (TSA) medium, DMSO, aluminum foil, and chlorhexidine.

Propolis was extracted by maceration in 96% ethanol for 5 days, following a modified method from Firdaus et al. (2023). In total, 500 g of propolis was blended, soaked in ethanol with periodic stirring, filtered, and concentrated using a rotary evaporator and water bath to yield a viscous extract. The extract was diluted in DMSO to concentrations of 1.56%, 3.125%, 6.25%, 12.5%, 25%, and 50%. Phytochemical analysis identified alkaloids, flavonoids, saponins, and tannins, using a modified method from Kurniawati et al.[23].

*A. actinomycetemcomitans* was subcultured on BHI medium and incubated at 37°C for 24 hours, then adjusted to $10^8$ CFU/mL per the McFarland standard. Antibacterial assays used tube dilution: each tube contained 1 mL bacterial suspension and 1 mL extract (per concentration), followed by 37°C incubation for 24 hours. MIC was assessed by medium turbidity; MBC was determined by streaking on TSA and checking colony growth.

Data underwent normality and homogeneity testing with the Shapiro-Wilk test. As data were not normally distributed and homogeneity assumptions were unmet, the Kruskal-Wallis test was followed by the Mann-Whitney U test. Analysis used SPSS version 25 for Windows.

## Results

Visual observation revealed turbidity in all dilution tubes containing *Trigona* sp. propolis extract at concentrations of 50%, 25%, 12.5%, 6.25%, 3.125%, and 1.56%. This turbidity resulted from the dark color and viscosity of the extract, particularly at higher concentrations, which prevented direct determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

Figure 1. Turbidity levels of dilution tubes in all groups

The positive control (chlorhexidine) tube was clear, confirming its inhibition of *A. actinomycetemcomitans* growth, whereas the negative control (DMSO) tube was turbid, indicating no antibacterial activity from DMSO alone. Turbidity assessment provided an initial step for MIC and MBC evaluation.

Table 1. Turbidity of *A. actinomycetemcomitans* after treatment with various concentrations of *Trigona* sp. propolis

| Group | Replication I | Replication II | Replication III |
|---|---|---|---|
| DMSO | Turbid | Turbid | Turbid |
| K+ | Clear | Clear | Clear |
| 50% | Turbid | Turbid | Turbid |
| 25% | Turbid | Turbid | Turbid |
| 12.5% | Turbid | Turbid | Turbid |
| 6.25% | Turbid | Turbid | Turbid |
| 3.12% | Turbid | Turbid | Turbid |
| 1.56% | Turbid | Turbid | Turbid |

Mean colony counts of *A. actinomycetemcomitans* increased as Trigona sp. propolis concentration decreased. Counts at 50%, 25%, 12.5%, 6.25%, 3.125%, and 1.56% were 5.60 ± 1.52, 11.60 ± 4.83, 15.60 ± 5.03, 22.20 ± 2.86, 25.40 ± 6.43, and 141.80 ± 5.40, respectively. The positive control (chlorhexidine) showed no growth (mean = 0), while the negative control (DMSO) had uncountable colonies, excluded from mean and standard deviation calculations.

Shapiro-Wilk normality testing yielded $p < 0.05$ for 50% ($p = 0.044$), 25% ($p = 0.043$), and 3.12% ($p = 0.004$) groups, indicating non-normal distribution. Groups at 12.5% ($p = 0.298$), 6.25% ($p = 0.087$), and 1.56% ($p = 0.633$) had $p > 0.05$, indicating normal distribution. Levene's homogeneity test showed $p = 0.090$ ($> 0.05$), confirming homogeneous variances across groups.

Table 2. Normality and homogeneity test results

| Group | Shapiro-Wilk p-value | Levene's p-value |
|---|---|---|
| DMSO | - | 0.090 |
| K+ | - | |
| 50% | 0.044 | |
| 25% | 0.043 | |
| 12.5% | 0.298 | |
| 6.25% | 0.087 | |
| 3.12% | 0.004 | |
| 1.56% | 0.633 | |

The Kruskal-Wallis test indicated significant differences in mean *A. actinomycetemcomitans* colony counts among groups ($p = 0.001$; $p < 0.05$). This supports the inhibitory and bactericidal potential of Trigona sp. propolis extract against *A. actinomycetemcomitans* in vitro.

Table 3. Mean *A. actinomycetemcomitans* colony counts among groups

| Group | I | II | III | IV | V | $\bar{x} \pm SD$ | p-value |
|---|---|---|---|---|---|---|---|
| DMSO | TNTC | TNTC | TNTC | | | - | - |
| K+ | 0 | 0 | 0 | 0 | 0 | 0 | |
| 50% | 6 | 7 | 6 | 3 | 6 | 5.60 ± 1.52 | |
| 25% | 9 | 8 | 10 | 20 | 11 | 11.60 ± 4.83 | 0.001* |
| 12.5% | 19 | 20 | 18 | 13 | 8 | 15.60 ± 5.03 | |
| 6.25% | 22 | 22 | 20 | 27 | 20 | 22.20 ± 2.86 | |
| 3.12% | 29 | 27 | 28 | 14 | 29 | 25.40 ± 6.43 | |
| 1.56% | 142 | 135 | 140 | 142 | 150 | 141.80 ± 5.40 | |

(TNTC = too numerous to count)

Mann-Whitney U testing showed significant differences ($p < 0.05$) in colony counts for most group pairs. Non-significant differences occurred between 25% and 12.5% ($p = 0.421$), 12.5% and 3.125% ($p = 0.056$), and 6.25% and 3.125% ($p = 0.151$).

Table 4. Mann-Whitney U test p-values between groups

| Groups | K+ | 50% | 25% | 12.5% | 6.25% | 3.125% |
|---|---|---|---|---|---|---|
| K+ | - | 0.008* | 0.008* | 0.008* | 0.008* | 0.008* |
| 50% | | - | 0.008* | 0.008* | 0.008* | 0.008* |
| 25% | | | - | 0.421 | 0.016* | 0.016* |
| 12.5% | | | | - | 0.016* | 0.056 |
| 6.25% | | | | | - | 0.151 |
| 3.125% | | | | | | - |
| 1.56% | 0.008* | 0.008* | 0.008* | 0.008* | 0.008* | 0.008* |

## Discussion

In this study, *Trigona* sp. propolis extract served as the test agent to evaluate its antibacterial activity against *Aggregatibacter actinomycetemcomitans* (Aa). *Trigona* sp. propolis, widely produced in Indonesia, has a favorable safety profile, including biocompatibility with human tissues, low toxicity, and low hypersensitivity incidence, supporting its use in health applications.[24] *Trigona* sp. propolis contains bioactive compounds with antibacterial, antifungal, antiviral, anti-inflammatory, antioxidant, and anticancer activities. These properties position it as a candidate for alternative antibacterial therapies.[25] Phytochemical screening identified secondary metabolites in the propolis extract, including alkaloids, tannins, flavonoids, and saponins, which contribute to its biological activities. These results align with reports by Kabani et al.[26] and Tukan et al.[27] of alkaloids, flavonoids, phenolic compounds, and tannins in Trigona sp. propolis.

*Aggregatibacter actinomycetemcomitans*, a Gram-negative bacterium associated with aggressive periodontitis, disrupts osteoblast activity and bone remodeling, promoting alveolar bone resorption.[28] Diffusion methods (disc or well) measure inhibition zones but yield inaccurate results with viscous extracts like propolis, as they preclude quantitative assessment. The dilution method was thus selected for its precision in determining minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values against A. actinomycetemcomitans.[29,30]

Broth dilution turbidity was obscured by the extract's dark color at all concentrations (50%, 25%, 12.5%, 6.25%, 3.125%, 1.56%), preventing visual MIC and MBC determination. Agar dilution with colony counting confirmed activity: mean *A. actinomycetemcomitans* colony counts were 5.60 ± 1.52, 11.60 ± 4.83, 15.60 ± 5.03, 22.20 ± 2.86, 25.40 ± 6.43, and 141.80 ± 5.40, respectively. The MIC was 3.125%; MBC exceeded 50%, indicating dose-dependent inhibition.[29,31] At 1.56%, colony counts remained high due to insufficient active compounds for cell wall or membrane disruption. *A. actinomycetemcomitans'* lipopolysaccharide-rich outer membrane further limits low-concentration efficacy. At 3.125% (MIC), colony counts decreased via bacteriostatic effects, with growth persisting up to 50%, confirming MBC >50%.[29]

Antibacterial efficacy depends on bacterial characteristics, bioactive composition (varying by bee species, resin sources, environment), and methods (extraction, solvents, inoculum density, incubation). *A. actinomycetemcomitans'* outer membrane reduces compound penetration. Wulandari et al.[32] reported MBC of 10% (100 mg/mL) against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Results align with Sinaga et al.[33] showing greater propolis activity against Gram-negative than Gram-positive bacteria due to lipopolysaccharide interference. Gram-negative cell walls feature thin peptidoglycan surrounded by lipoproteins, lipopolysaccharides, and phospholipids, impeding small molecules.[29]

Kruskal-Wallis testing showed significant colony count differences across groups ($p < 0.05$), suitable for non-normal data with >2 groups. This supports Hidayat et al.[34] on *Trigona* spp. propolis against *Staphylococcus aureus* and Maghfiroh[35] on fungicidal effects against *Candida albicans*. Flavonoids disrupt membranes, reduce fluidity, cause leakage, ATP depletion, metabolic inhibition, and lysis.[35] Alkaloids inhibit peptidoglycan synthesis; tannins increase wall instability; saponins compromise integrity, promoting leakage and lysis.[27,32] Mann-Whitney U testing confirmed significant differences between propolis concentrations and chlorhexidine positive control, appropriate for non-normal data.[36]

Dimethyl sulfoxide (DMSO) dissolved propolis as negative control, showing no activity and confirming effects from propolis compounds. Chlorhexidine, the oral hygiene standard, acts via electrostatic binding, membrane disruption, leakage, and lysis but risks discoloration, taste changes, irritation, and resistance with prolonged use. This highlights the value of alternatives like *Trigona* sp. Propolis.[36,37]

## Conclusion

The results of this study demonstrate that *Trigona* sp. propolis extract exhibits in vitro antibacterial activity against *Aggregatibacter actinomycetemcomitans*, as indicated by a reduction in bacterial colony counts with increasing extract concentrations. The minimum inhibitory concentration (MIC) of *Trigona* sp. propolis extract was determined to be 3.125%, whereas the minimum bactericidal concentration (MBC) was greater than 50%.

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