The inhibitory effect of galo-galo honey extract (heterotrigona itama) against pseudomonas aeruginosa: experimental study

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ABSTRACT

Introduction: Pseudomonas aeruginosa is a pathogenic bacterium and a major contributing factor in nosocomial infections. Although chlorhexidine mouthwash is widely used to prevent cross-infection, its improper use can cause adverse effects. Galo-galo bee honey (Heterotrigona itama) contains various antibacterial properties, including carbohydrates, proteins, phenolics and flavonoids. The purpose of this study was to analyze the effect of Galo-galo bee honey extract in inhibiting the growth of P. aeruginosa. **Methods:** This in vitro laboratory experiment evaluated the antibacterial activity of Galo-galo bee honey extract obtained via centrifugation. The extract was tested against P. Aeruginosa at concentrations of 0.09%, 0.14%, 0.39%, 0.78%, 1.56%, 3.13%, 6.25%, 12.5%, 25% and 50% to **Results:** The Galo-galo bees honey extract significantly inhibited the growth of P. aeruginosa at higher concentrations, yielding 73% at a 12.5 % concentration, 74% at 25%, and 77% at 50%. Conversely, concentrations ranging from 0.09%to 6.25% showed no inhibitory effect against the bacteria. **Conclusion:** Galo-galo bee honey extract exhibits concentration-dependent antibacterial activity against P. Aeruginosa, with its inhibitory effects

Keywords

antibacterial, heterotrigona itama, pseudomonas aeruginosa

Efek ekstrak madu lebah galo-galo (Heterotrigona itama) dalam menghambat pertumbuhan Pseudomonas aeruginosa: studi eksperimental

ABSTRAK

Pendahuluan: *Pseudomonas aeruginosa adalah bakteri patogen yang berperan utama dalam infeksi nosokomial. Penggunaan obat kumur klorheksidin untuk menghindari infeksi silang telah dilakukan, namun penggunaan yang tidak tepat dapat menimbulkan efek samping. Madu lebah Galo-galo (Heterotrigona itama) memiliki berbagai kandungan antibakteri, yaitu karbohidrat, protein, fenolik dan flavonoid. Tujuan penelitian menganalisis efek ekstrak madu lebah Galo-galo (Heterotrigona itama) dalam menghambat pertumbuhan Pseudomonas aeruginosa.* ***Metode:*** *Penelitian ini dilakukan dengan metode eksperimental melalui pengujian di Laboratorium. Ekstrak madu lebah Galo-galo (Heterotrigon itama) yang diperoleh dengan cara sentrifugasi diuji dengan konsentrasi 0,09%, 0,19%, 0,39%, 0,78%, 1,56%, 3,13%, 6,25%, 12,5%, 25% dan 50% untuk melihat aktivitas antibakterinya terhadap bakteri P. aeruginosa.* ***Hasil:*** *Penelitian ini menunjukkan bahwa ekstrak madu lebah Galo-galo (Heterotrigona itama) menghambat pertumbuhan P. aeruginosa dengan persentase sebagai berikut: konsentrasi 12,5% (73%), 25% (74%), dan 50% (77%). Sedangkan pada konsentrasi 0,09%, 0,19%, 0,39%, 0,78%, 1,56%, 3,13% dan 6,25% ekstrak madu lebah Galo-galo (Heterotrgona itama)belum dapat menghambat pertumbuhan bakteri Pseudomonas aeruginosa,* ***Simpulan****: Efek ekstrak madu lebah Galo-galo (Heterotrigona itama) menunjukkan perbedaan dalam menghambat pertumbuhan P. aeruginosa dan daya hambat meningkat seiring dengan peningkatan konsentrasi madu.*

Kata kunci

antibakteri, heterotrigona itama, pseudomonas aeruginosa

INTRODUCTION

Nosocomial infections, or healthcare-associated infections, are infections that develop at least 48 hours or within 3 days following discharge from a healthcare facility.[1] According to World Health Organization (WHO) fact sheet, nosocomial infections become a major global health issue that affects millions of patients annually, with rapidly increasing rates in developing countries. WHO data indicates that nosocomial infections affect u to 30% of hospitalized patients in developed nations, whereas the incidence in developing countries is twice as high (CDC, 2022; WHO, 2022). These infections are frequently associated with Gram-negative bacteria that exploit immunocompromised conditions, with *Pseudomonas aeruginosa* (*P. aeruginosa)* being a primary pathogen.[2,25] The transmission of *P. aeruginosa* into oral cavity is often facilitated by dental prostheses, dental unit water lines (DUWLs), and other medical devices used during dental procedures.[4]

Pseudomonas aeruginosa is a prominent respiratory pathogen capable of extensive tissue destruction and high mortality rates; furthermore, its biofilm production causes the bacterium highly resistant to antibiotics.[5] This organism is also implicated in oral cavity infections, such as necrotizing ulcerative gingivitis, mandibular osteomyelitis and necrotic lesions the oral mucosa patients with immuno-suppression. This association establishes the oral cavity as a primary portal of entry for *P. Aeruginosa,* allowing it to progress from oral infections to respiratory tract complications, particularly pneumonia in the lungs, which can ultimately lead to death.[6,7]

Numerous preventive measures have been implemented to inhibit the growth of this bacterium. Although preprocedural mouth-washes, such as chlorhexidine, are widely used before dental procedures to prevent bacterial cross-infection, chlorhexidine use is associated with adverse effects, including tooth discoloration and altered taste perception. Therefore, alternative strategies are required to prevent the cross-infection of *P. aeruginosa.*[8,9]

Honey is one of the natural products currently being developed by communities across West Sumatera province.[10] It is a naturally sweet liquid produced by bees across various floral nectar sources. Nectar itself is a fluid secreted by plant glands that contains diverse carbohydrates and micronutrients, which contributes to honey's reputation as a promoter of health and health.[11]

Madu is produced by two main types of bees: stinging bees (Apis) and stingless bees (*Trigona*). *Trigona* bees are known by various local names throughout Indonesia; in West Sumatra, they are commonly referred to as Galo-galo bees. A key factor supportive the cultivation of Galo-galo bees in this province is the geographic proximity of local communities to hilly terrain and agricultural lands.[10]

Galo-galo (*Heterotrigona itama*) honey is a bee product exhibiting potent antibacterial activities due to its content of hydrogen peroxide, glucose, fructose, sucrose, and peptide components. Furthermore, Galo-galo bee honey possesses high antioxidant capacity, is attributed to its rich phenolic and dan flavonoid compounds.[12]

A previous study by Melia, *et al.,*[12] discovered antibacterial activities of Galo-galo bee honey against both Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus, Listeria monocytogenes, Salmonella* and *Escherichia coli*. The bioactive compounds found in this honey have prompted researchers to investigate the antibacterial efficacy of Galo-galo bee (*Heterotrigona itama*) honey as alternative therapeutic agent. This could be potentially formulated into oral care products, such as mouthwashes, toothpaste, or topical ointments, to inhibit the growth of *P. aeruginosa* in the oral cavity. However, the exact minimum inhibitory concentration (MIC) has not yet been established. Therefore, the purpose of this study is to analyze the inhibitory effect of Galo-galo bee honey extract (*Heterotrigona itama*) against the growth of *P. aeruginosa*.

METHODS

This study is an *in vitro* laboratory experiment. The study population consisted of *P. aeruginosa* ATCC 10145 cultures obtained from the Integrated Research Laboratory, Faculty of Dentistry, Universitas Gadjah Mada, Yogyakarta. The samples comprised *P. aeruginosa* ATCC 10145 suspensions adjusted to a bacterial concentration of 1.5 x 10^5 CFU/ml. The samples were divided into 13 treatment groups. The Galo-galo bee (*Heterotrigona itama*) honey extract was prepared by dissolving the honey in distilled water *(aquades)* to a final volume of 10 mL, followed by homogenization for 30 minutes.

The honey extract was then serially diluted to obtain ten distinct concentrations: 50%, 25%, 12.5%, 6.25%, 3.13%, 1.56%, 0.78%, 0.39%, 0.14% and 0.09%. Additionally, the setup included one positive control group (0.2% chlorhexidine), one negative control group (*P. aeruginosa* suspension alone) and one *Luria Bertani* (LB) media control group. Honey samples exhibiting changes in color, odor, or signs of contamination were excluded from the study.

The sample size for this study was calculated using Federer's formula, yielding a total of 39 samples (13 groups, with each group tested in triplicate). Group classification was conducted randomly using a simple randomization method without blocking or stratification. The independent variable in this study was the Galo-galo bee (*Heterotrigona itama*) honey extract, harvested from Galo-galo Bee Farm at Universitas Andalas, while the dependent variables was the growth of *P. aeruginosa.*

The study was conducted at several laboratories, including the Insect Bioecology Laboratory at Universitas Andalas for the identification of the Galo-galo bees; the Pharmacy Laboratory at Universitas Andalas for the preparation of honey extract; and the Integrated research Laboratory at the Faculty of Dentistry at Universitas Gadjah Mada for the preparation of *P. aeruginosa* and the antibacterial activity assay. The antibacterial activity was evaluated using a serial dilution method across concentrations ranging from 50% 0.09%. The resulting data were then quantified using an inhibition formula. Statistical analysis was performed using the Saphiro-Wilk test to assess normality and Levene's Test, to evaluate homogeneity of variance. If the data were normally distributed but exhibited unequal variances, Welch's *one way* ANOVA followed by the Games-Howell post-hoc test was used

RESULTS

Antibacterial activities of the Galo-galo bee (*Heterotrigona itama*) honey extract in inhibiting the growth of *P. aeruginosa* was evaluated using the broth microdilution method *at a wavelength* of λ = 600 nm, as presented in Table 1. The honey extract began to inhibit the growth of *P. aeruginosa* at a concentration of 12.5%, resulting in an inhibition rate at 73%. Furthermore, the inhibitory activity increased in a concentration-dependent manner. No adverse effect or unintended consequences were observed during the study, as no intervention involving human or animal subjects were conducted that could have resulted in harmful outcomes.

The Shapiro-Wilk normality test and Levene's test for homogeneity of variance yielded a p-value of >0.05 (Table 2) R and <0.05 (Table 3), respectively, indicating that the data were normally distributed but not homogenous (Table 2 and 3).

Table 1. Percentage inhibition of *Pseudomonas aeruginosa* growth by galo-galo bee honey (*Heterotrigona itama*) extract.

Treatment Group	Honey Extract Concentration (%)	Number of Samples (n)	Mean Percentage Inhibition (%)
Galo-galo Honey Extract 1	0.09	3	0
Galo-galo Honey Extract 2	0.19	3	0
Galo-galo Honey Extract 3	0.39	3	0
Galo-galo Honey Extract 4	0.78	3	0
Galo-galo Honey Extract 5	1.56	3	0
Galo-galo Honey Extract 6	3.13	3	0
Galo-galo Honey Extract 7	6.25	3	0
Galo-galo Honey Extract 8	12.5	3	73
Galo-galo Honey Extract 9	25	3	74
Galo-galo Honey Extract 10	50	3	77
Positive Control (0.2% Chlorhexidine)	-	3	20
Negative Control (P. aeruginosa only)	-	3	0
Media Control (LB broth)	-	3	0

Table 2. Results of the *Shapiro–Wilk* normality test

	Shapiro-Wilk		
Concentration	**Statistic**	**df**	***p-value***
50%	0.964	3	0.637*
25%	1.000	3	1.000*
12.5%	0.871	3	0.298*
6.25%	0.912	3	0.424*
3.13%	0.923	3	0.463*
1.56%	1.000	3	1.000*
0.78%	0.923	3	0.463*
0.39%	0.818	3	0.157*
0.19%	0.842	3	0.220*
0.09%	0.834	3	0.503*
Chlorhexidine	0.987	3	0.780*

Table 3. Hasil uji *Levene Test*

Variable	*Levene Statistic*	*p-value*
Bacterial growth inhibition	6.565	0.000

Note: * indicates p > 0.05

Following the normality and homogeneity tests, the data were further analyzed using the Welch's one-way ANOVA, as presented in Table 4.

Table 4. Results of *Welch's One-Way ANOVA*

Variable	*p-value*
Inhibition zone diameter (mm)	0.001*

Note: * indicates p < 0.05

The results presented in Table 4 showed a statistically significant difference (p = 0.001), leading to the acceptance of the alternative hypothesis (Ha), indicating that Galo-galo bee (*Heterotrigona itama*) honey extract had a significant inhibitory effect on the growth of *P. aeruginosa*. The data were then analyzed using the Games-Howell post hoc test, as shown in Table 5.

The Games-Howell post hoc test (Table 5) demonstrated that the 50%, 25% and 12.5% concentrations exhibited significantly greater inhibitory effects on the growth of *P. aeruginosa* ($p<0.05$) compared with the 6.25%, 3.13%, 1.56%, 0.78%, 0.39%, 0.19% and 0.9% concentrations. However, no significant difference in antibacterial activity were observed among the 50%, 25% and 12.5% concentrations themselves ($p>0.05$). Similarly, no significant differences in growth inhibition were found among the 6.25%, 3.13%, 1.56%, 0.78%, 0.39%, 0.19% and 0.9% concentrations ($p>0.05$). A significant difference ($p<0.05$) was observed between the positive control and all treatment groups receiving the honey extract.

Table 5. Hasil uji *Post Hoc Games-Howell*

Kelompok (%)	25	12.5	6.25	3.13	1.56	0.78	0.39	0.19	0.09	Chx 0.2
50	0.24	0.63	0.00*	0.00*	0.00*	0.00*	0.00*	0.00*	0.00*	0.00*
25		1.00	0.00*	0.00*	0.00*	0.00*	0.00*	0.00*	0.00*	0.00*
12.5			0.00*	0.00*	0.00*	0.00*	0.00*	0.00*	0.00*	0.00*
6.25				1.00	1.00	1.00	1.00	1.00	1.00	0.02*
3.13					0.431	0.85	1.00	0.98	1.00	0.02*
1.56						1.00	1.00	1.00	1.00	0.02*
0.78							1.00	1.00	0.84	0.02*
0.39								1.00	1.00	0.02*
0.19									0.98	0.02*
0.09										0.02*

Keterangan: : Galo-galo Honey Extract (*Heterotrigona itama*)

: Chlorhexidine (Positive control)

* : indicates $p<0.05$

DISCUSSION

The findings presented in Table 1 indicated that inhibition of *P. aeruginosa* growth was first detected at a concentration of 12.5% honey extract, with an inhibition rate at 73%. These results suggest that the Galo-galo bee (*Heterotrigona itama*) honey extract is capable of inhibiting the growth of P. aeruginosa. This finding is consistent with a previous study conducted by Melia et al.,[12] which reported that Galo-galo bee honey extract possesses antibacterial activity. This antibacterial activity is likely attributable to several bioactive components present in the honey, including carbohydrates, flavonoids and peptides. In addition, Galo-galo bee honey has a acidic pH, which may disrupt bacterial metabolic processes, thereby impairing bacterial survival and proliferation.[2,12]

The results of this study also demonstrated that Galo-galo bee (*Heterotrigona itama*) honey extract at a concentration of 6.25% was unable to inhibit the growth of *P. aeruginosa*. Similarly, a study by Kaligis et al. (2021) reported that this honey exhibited antibacterial activity only at a concentration of 25%. These findings suggest that lower concentrations may be considered sub-inhibitory concentrations. Sub-inhibitory concentrations may increase bacterial tolerance to antibacterial compounds due to elevated pyocyanin production following exposure to antibacterial agents at sub-inhibitory levels.[13]

The inhibitory activity of the honey extract against *P. aeruginosa* was subsequently compared with that of 0.2% chlorhexidine, which showed a bacterial growth inhibition rate of 20% The antibacterial effect of 0.2 % chlorhexidine was lower than that of Galo-galo bee (*Heterotrigona itama*) honey extract at concentrations of 50%, 25% dan 12.5%.

This difference in inhibitory activity is probably associated to chlorhexidine exposure in *P. aeruginosa*, which can increase the expressions of efflux pumps that expel antibacterial agents from bacterial cells, thereby reducing the amount of antibacterial substances able to reach the bacterial cell interior.[14] In contrast, honey contains flavonoids that may interrupts bacterial efflux pump activity, thus promoting the antibacterial effectiveness of honey.[1,12,15]

According to a previous study by Melia *et al.,*[12] Galo-galo bee (*Heterotrigona itama*) honey extract exhibited antibacterial activities against *Staphylococcus aureus, Listeria Monocytogenes, Salmonella* and *Escherichia coli*. The strongest inhibitory activity of the honey extract was observed against *Escherichia coli.*

Based on the results presented in Table 4, Welch's one-way ANOVA showed a significant result ($p < 0.05$), indicating that the alternative hypothesis (Ha) was accepted and that Galo-galo bee (*Heterotrigona itama*) honey extract had a significant inhibitory effect against the growth of *Pseudomonas aeruginosa*. Similarly, a study conducted by Zhu, *et al.* (2019) reported that concentrations play a major role in bacterial inhibition. However, at sub-inhibitory concentrations, the exposure of honey may instead induce bacterial adaptation and increase pyocyanin production in *P. aeruginosa,* resulting in an increased bacterial tolerance to antibacterial compounds.[13]

The inhibitory effect of Galo-galo bee (*Heterotrigona itama*) honey extract against *P. aeruginosa* is closely associated with the components found in the honey. It contains antibacterial carbohydrates oxidized by glucose oxidase to produce hydrogen peroxide, capable of killing bacteria through releasing oxygen free radicals that damage bacterial cell structure.[12,16]

Furthermore, this honey contains flavonoids that can disrupt bacterial cell membranes and inhibit efflux pump activity, as well as phenolic compounds that function as antioxidants.[11,12,15] Galo-galo bee honey was also found to possess high water content and low pH. These characteristics may contribute to its antibacterial activity by limiting bacterial survival. In addition, the acidic pH of the honey may inhibit the metabolic activity of *P. aeruginosa,* which generally grows optimally at pH values ranging from 5.5 to 9.0.[25,12,17,18]

The antibacterial components present in honey may be influenced by geographical location, bee species, environmental conditions, and harvesting time. The geographical location affects the surrounding climate, which in turn influence the species of bees and plants found in the area. The honey used in this study was obtained from stingless bees of the species *Heterotrigona itama,* resulting in honey characterized by high water content and low pH. This honey produced by the bees inhabiting an environment containing mango tress, coral vine flowers *(Antigonon leptopus)*, and papaya plants. The vegetation surrounding the bee colony may influence the flavonoid content of the honey.[19] In addition, the composition of honey is affected by harvesting time, which is closely related to its viscosity, as honey is hygroscopic in nature. Extended storage of honey within the hive promotes further water evaporation, resulting in reduced water content.[20]

The components of honey are also influenced by the harvest time, which is closely related to its viscosity, as honey has the property of absorbing water. The longer the honey remains in the hive, the more complete the evaporation of its water content.[20] This results in a more optimal inhibitory effect.

The Games-Howell's post hoc test, as shown in Table 5, indicated the honey extracts at the concentrations of 50%, 25%, and 12.5% exhibited statistically significant inhibition rates ($p<0,05$) compared to those at 6.25%, 3.13%, 1.56%, 0.78%, 0.39%, 0.19%, and 0,9%. This difference in the mean percentage inhibition can be attributed to several factors, including the concentration of antibacterial compounds presents in the honey and its low pH. Higher concentrations of honey increase the produced antibacterial components, thereby enhancing inhibitory capacity against bacterial growth.[21]

Table 5 also demonstrates that the mean inhibition rate induced by 50% extract concentration was not statistically significant compared 25% and 12.5% concentrations. Similarly, no statistically significant difference was observed when comparing the 25% to either the 50% and 12.5% concentrations. The results of this study also showed no statistically differences ($p>0.05$) among the concentrations of 6.25%, 3.13%, 1.56%, 0.78%, 0.39%, 0.19% dan 0.09%. These findings align with a study by Zhu et al., which reported that bacterial inhibition does not increase significantly at sub-inhibitory concentrations. This phenomenon may occur because bacteria possess inherent tolerance mechanism against antibacterial agents.

Bacteria may be capable of adapting to certain concentration; consequently, marginal increases in honey concentrations may not significantly inhibit bacterial growth.[22] This indicate that the percentage of bacterial growth inhibition is strongly influenced by the specific bacterial species being tested. The bacterium being evaluated in this study was *P. aeruginosa*, a Gram-negative pathogen commonly found in the environment. This organism is well known for its ability to resist antimicrobial agents through mechanisms such as low outer membrane permeability, efflux pump activity, and adaptive responses to antibacterial compounds.[31]

The findings of this study demonstrate that Galo-galo bee (*Heterotrigona itama*) hone extract has the potential to inhibit the growth of *Pseudomonas aeruginosa* in vitro. These findings are also supported by the study conducted by Melia *et al.*,[12] Nevertheless, because

this study was conducted under controlled laboratory condition, the results cannot yet be applied to clinical settings or humans without further investigation. To confirm its effectiveness and safety in more complex biological environment, such as the human oral cavity, additional studies, including in vivo studies and clinical trials, are required. Therefore, the findings of this study remain limited to the laboratory scale and cannot be broadly generalized to medical or clinical practice.

This study has several limitations that may represent potential sources of bias and inaccuracy. First, because the study was conducted in vitro, the findings may not fully represent the actual biological conditions within human body. Second, the absence of a blinding procedure for the researchers assessing the outcomes may have introduced assessment bias. In addition, no phytochemical analysis was perfomed on the honey used in this study.

Furthermore, confirmation through inoculation pn solid agar media was not conducted, limiting the ability to detect potential contamination in the honey samples. Additionally, the natural variability of Galo-galo bee honey extract, including variations in its compositions related to seasonal factors or harvesting location, was not completely controlled.

Further limitations of this study include the absence of minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC), as well as the lack of repeated evaluation under different conditions; therefore, the study findings cannot yet be broadly generalized. The complete study protocol is not publicly accessible; nevertheless, all experimental procedures and methods are described comprehensively in this manuscript. Additional information associated with the study protocols may be requested from the corresponding author through the email address provided.

CONCLUSION

Galo-galo bee (Heterotrigona itama) honey has the potential to inhibit the growth *P. aeruginosa* bacteria in vitro, with inhibitory activity increasing in a concentration-dependent manner and first being detected at a concentration of 12,5%. These findings provide a basis for the potential development of Galo-galo bee honey as a natural antibacterial agent, particularly in the field of dentistry, such as in the formulation of mouthwashes, toothpastes, or agents for prevention of cross-infection in dental clinics. This is especially important considering that chlorhexidine is associated with certain adverse effects, although further studies are still required to confirm the effectiveness and safety of honey for clinical applications.

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Conflicts of Interest: The authors declare no conflict of interest.

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