Antibacterial and Antibiofilm Activities of Thailand Propolis Against Escherichia coli

Kazuma Mukaide¹, Yuko Shimamura¹, Shuichi Masuda¹, Boonyadist Vongsak² and Shigenori Kumazawa¹

Abstract

Escherichia coli is an important bacterium for preventing food poisoning and biofilm infections. The emergence of antibiotic-resistant microorganisms necessitates the development of new antibiotics. The formation of bacterial biofilm is a drug-resistance mechanism utilized by diverse microorganisms. Therefore, it is important to identify compounds that can inhibit biofilm formation and cell survival, without triggering drug resistance. Herein, the antibacterial and antibiofilm activities of 2 types of Thai propolis (collected from Chiang Mai and Chanthaburi) against E. coli were investigated. The antibacterial activity was evaluated using the paper-disc method, while the minimum inhibitory concentration assay was performed using 2-fold serial dilution. Both types of Thai propolis and their isolated compounds showed antibacterial activity against E. coli (minimum inhibitory concentration: 32 µg/mL). The biofilm growth and development were assessed using a crystal violet solution. In particular, the extracts of the Chiang Mai propolis exhibited a significant antibiofilm formation activity against E. coli. Four prenylflavonoids, present in high proportions in the Chiang Mai propolis extracts, inhibited biofilm formation at low concentrations, contributing to the overall antibiofilm activity. These findings indicate that Thai propolis, a natural product, exhibits antibacterial and antibiofilm activities against E. coli.

Keywords

propolis, Thailand, antibacterial activity, biofilm formation, E. coli, prenylflavonoid

Received: January 31st, 2022; Accepted: April 1st, 2022.

Introduction

Propolis is a natural substance collected by Apis mellifera from the buds and exudates of certain trees and plants. Propolis has attracted attention owing to its various biological activities, including antibacterial, anti-inflammatory, antioxidant, and anti-cancer properties, and is widely used in folk medicine in many regions worldwide.¹–³ Moreover, propolis is extensively used in foods, beverages, and supplements to prevent diseases, such as inflammation, heart disease, and cancer, as well as in cosmetics as a beauty aid.⁴–⁶

The chemical composition of propolis is highly dependent on the vegetation within the collection area because honeybees preferentially target plants growing near their hives as sources of propolis. Thus, plant origin can also lead to variations in the properties of propolis, including its biological activity, texture, flavor, and color.

Propolis types that have high polyphenol contents are known to inhibit bacterial growth.⁷,⁸ However, a majority of microorganisms (bacteria, fungi, and yeast) live in 3-dimensional self-organizing communities called biofilms, which protect individual cells from starvation, antibiotic agents, and the immune system. Therefore, it is important to identify new compounds that can inhibit bacterial biofilm formation and cell survival. Recently, Okinawan propolis, which is rich in prenylated flavanones, was reported to exhibit antibiofilm formation activity against Escherichia coli.⁹

In previous studies, we isolated and reported 20 kinds of prenylated flavonoids in propolis from Chiang Mai, Thailand,¹⁰ and 9 xanthone derivatives in propolis from Chanthaburi, Thailand.¹¹ In this study, we focused on these 2 Thai propolis substances (Chiang Mai and Chanthaburi) and evaluated their antibacterial and antibiofilm activities against E. coli.

¹Graduate School of Integrated Pharmaceutical and Nutritional Sciences, University of Shizuoka, Suruga-ku, Shizuoka, Japan
²Pharmaceutical Innovations of Natural Products Unit (PhInNat), Faculty of Pharmaceutical Sciences, Burapha University, ChonBuri, Thailand

Corresponding Author:
Shigenori Kumazawa, Graduate School of Integrated Pharmaceutical and Nutritional Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan.
Email: kumazawa@s-shizuoka-ken.ac.jp

DOI: 10.1177/1934578X221095354
Results and Discussion

First, the effects of the 2 Thai propolis samples on the growth of *E. coli* were investigated. When paper discs impregnated with 100 mg/mL propolis extracts (from Chiang Mai and Chanthaburi) were cultured in a medium containing *E. coli*, the propolis extracts formed inhibition zones. However, their antibacterial activity was lower than that of the positive control (100 units/mL of penicillin G + 100 µg/mL of streptomycin) (Table 1). The minimum inhibitory concentrations (MICs) of these 2 propolis extracts were 32 µg/mL (Table 1). Therefore, both propolis samples contained substances with equivalent antibacterial activities against *E. coli*.

Next, the effects of the isolated compounds from the propolis samples were evaluated using the same method as employed for the propolis extracts. The results of the antibacterial activity test, conducted by the paper disk method using 100 mM isolated compounds, revealed that out of the 12 isolated compounds, 11 exhibited antibacterial activity (Table 2). In particular, 8-prenylnaringenin (4) exhibited the highest antibacterial activity. The antibacterial activity of 4 was almost the same as that of the positive control. The compound 4 has been previously extracted from hops, a bitter component present in beer, and exhibits various physiological activities, such as estrogen-like effect and muscle-aging inhibitory effect. In addition, 3-hydroxy-5′-methoxy-licoflavonanone (6) exhibited the second-highest antibacterial activity among the tested compounds. In these samples, the compounds with a few prenyl groups tended to exhibit relatively high antibacterial activities. Therefore, it is considered that the high-polarity compounds in the test samples diffuse easily into the aqueous agar medium and reach the bacteria cell wall, thus explaining their high antibacterial activity.

To identify compounds that can inhibit the formation of *E. coli* biofilm, the antibiofilm formation activity of the Thai propolis extracts was evaluated. Chiang Mai propolis exhibited a concentration-dependent antibiofilm formation activity at a concentration less than half of the MIC (Figure 1). These extracts inhibited the biofilm formation by 32% at 10 µg/mL. Conversely, Chanthaburi propolis extracts did not indicate any antibiofilm formation activity against *E. coli*. This is attributable to the difference in the components of each propolis, and it is suggested that prenylflavonoids, which are abundant in the propolis from Chiang Mai, inhibited the biofilm formation of *E. coli*. A structure called curli is involved in the biofilm formation mechanism of *E. coli*. Curli is a type of functional amyloid produced by microorganisms and plays an important role in biofilm formation and the infection/colonization of hosts. Previous studies have reported that prenylflavanone isolated from *Macaranga tanarius*-derived propolis, a plant-derived flavonoid myricetin, and the tea component, epigallocatechin gallate (EGCG), inhibit the biofilm formation of *E. coli* by suppressing the expression of the CSG gene group involved in curli production. Therefore, it is speculated that the prenylflavonoids in Chiang Mai propolis also inhibit *E. coli* biofilm formation through the same mechanism.

Next, we investigated the antibiofilm formation activity of the high concentration compounds in Chiang Mai propolis against *E. coli* and found the components responsible for the activity. Ten kinds of prenylflavonoids isolated from the Chiang Mai propolis were employed as test samples (abyssinone V [1], munduleaflavanone B [2], cathayanon H [3], 8-prenylnaringenin [4], flowerine [5], 3-hydroxy-5′-methoxy-licoflavonanone [6], 3′-hydroxy-5′-methoxy-glabranin [7], isolicoflavonol [8], lespedezaflavanone C [9], and 5′-methoxy-lespedezaflavanone C [10]). As 8 had the strongest antibacterial activity against *E. coli* (MIC: 156 µM), the biofilm inhibition was evaluated at the sample concentration of 25 to 100 µM less than MIC of 8. The results revealed that 1-3 and 5 inhibited *E. coli* biofilm formation by 40% or more at 50 µM (Figure 2). The activities of the above 4 compounds were almost the same as those of myricetin (IC₅₀ 46.2 µM) and EGCG (IC₅₀ 5.9 µM). The results show that these 4 compounds are the main components

Table 1. Antibacterial Activity of Thailand Propolis Against *Escherichia coli*, Using a Paper Disc and Broth Microdilution Method (MIC Values).

| Propolis    | Inhibition zone (mm) | MIC (µg/mL) |
|-------------|----------------------|-------------|
| Chiang Mai  | 9.0                  | 32          |
| Chanthaburi | 8.0                  | 32          |
| Positive control | 15.5               | 64*         |

Abbreviations: MIC, minimum inhibitory concentration; DMSO, dimethylsulfoxide.

Table 2. Antibacterial Activity of the Compounds Isolated from Thailand Propolis Against *Escherichia coli*, Using a Paper Disc Method.

| Compound                                      | Inhibition zone (mm)/6 mm disc |
|-----------------------------------------------|-------------------------------|
| Chiang Mai propolis                           |                               |
| Abyssinone V (1)                              | 8.5                           |
| Munduleaflavanone B (2)                       | 8.75                          |
| Cathayanon H (3)                              | 11.75                         |
| 8-Prenylnaringenin (4)                        | 14.5                          |
| Flowerine (5)                                 | —                             |
| 3-Hydroxy-5′-methoxy-licoflavonanone (6)      | 13.75                         |
| 3′-Hydroxy-5′-methoxy-glabranin (7)            | 8.25                          |
| Isolicoflavonol (8)                           | 10.0                          |
| Lespedezaflavanone C (9)                     | 11.5                          |
| 5′-Methoxy-lespedezaflavanone C (10)          | 11.0                          |
| Chanthaburi propolis                          |                               |
| α-Mangostin (11)                              | 9.0                           |
| γ-Mangostin (12)                              | 11.5                          |

Abbreviations: MIC, minimum inhibitory concentration; DMSO, dimethylsulfoxide.

* : Inhibition zone was not detected.
contributing to the antibiofilm formation activity of Chiang Mai propolis against *E. coli*. Compounds 7 and 9 did not exhibit any antibiofilm formation activity at 50 µM; however, they exhibited remarkable activity at 100 µM. Although 10 of the tested compounds used in this experiment were identical to the compounds used in the above-mentioned antibacterial test, when the results of the antibacterial and antibiofilm assays were compared, no correlation was found between the antibacterial and the antibiofilm formation activities. Therefore, it is suggested that Chiang Mai propolis inhibits the formation of *E. coli* biofilm by a different mechanism than that used for antibacterial activity.

Quantitative analysis of Chiang Mai propolis was performed (for the antibiofilm formation active compounds, 1-3 and 5). A calibration curve was prepared for each compound, and a good linearity (R²>0.99) was obtained over the concentration range tested (0.1-10 mg/mL). Compound 1 was used in the addition recovery test, and it exhibited a recovery rate of approximately 75%. The amount of compounds obtained in 1 mg of the Chiang Mai propolis in 70% EtOH extracts was determined, and the limit of detection (LOD) and limit of quantitation (LOQ) were calculated as shown in Table 3. Among the 4 active ingredients (1-3 and 5), 1, 2, and 3 were confirmed to be present at a very high content percent (10% or more) in the propolis extracts. Thus, it was concluded that these compounds contributed to the antibiofilm formation activity of Chiang Mai propolis.

Moreover, compounds 1-3 and 5, isolated from Chiang Mai propolis, exhibited particularly excellent antibiofilm formation activities. Quantification analysis revealed that 1-3 were present in high proportions in the 70% EtOH extracts of Chiang Mai propolis, indicating that these compounds significantly contributed to the antibiofilm formation activity. The results of this study highlight the potent antibacterial activity of Thai propolis, which was previously unknown and may lead to its effective use.

**Materials and Methods**

**Propolis Material**

The Chiang Mai propolis used in this study was collected and combined together as crude material by beekeepers from several apiaries in the Mae Rim district of central Chiang Mai Province, Thailand, in September 2018. A voucher sample of the propolis (BV20180908) studied in this paper has been deposited at the Faculty of Pharmaceutical Sciences, Burapha University, Thailand. The origin plant of this propolis is still unknown. The Chanthaburi propolis sample was collected from an orchard in Chanthaburi, Thailand in May 2016. The specimen (No. 1214003) for the same was deposited at the Faculty of Pharmaceutical Sciences, Burapha University, Thailand. The plant origin of this propolis was determined to be *Garcinia mangostana*. The propolis samples were treated with 70% ethanol, and 20 types of prenylflavonoids were isolated from Chiang Mai propolis, while 9 types of xanthone derivatives were isolated from Chantaburi propolis.

**Bacterial Strain and Media**

*E. coli* ATCC 10798 was employed in this study. The *E. coli* culture was grown at 37 °C aerobically, in brain heart infusion (BHI) broth (Nissui). When preparing the agar medium, 2% agar was added to the BHI medium.
Antibacterial Activity Analysis

The antibacterial activity was evaluated using the paper disc method. The E. coli culture (100 µL) in the BHI broth (10^8 CFU/mL) was added to a sterile petri dish, and the overnight culture was subjected to pour culture by adding 15 mL of fresh BHI broth. Each propolis was extracted with hexane and its residue was extracted with 70% EtOH for 3 h. Each propolis extract was dissolved in dimethylsulfoxide (DMSO) to afford a stock concentration of 100 mg/mL. The isolated compounds from the propolis sample were dissolved in DMSO to get a stock concentration of 100 mM. On a clean bench, 10 µL of the test sample was soaked in a paper disc (φ 6 mm), dried, and subsequently placed in a medium. Thereafter, it was cultured in an incubator at 37 °C for 24 h. Afterward, the diameter of the formed growth inhibition zones was measured, and the antibacterial activity was evaluated, where each sample was tested twice. One hundred units/mL penicillin G + 100 µg/mL streptomycin was used as the positive control, and DMSO was used as the negative control.

MIC was determined by the broth microdilution method, with some modifications. The propolis extracts were dissolved in DMSO to obtain stock concentrations of 5, 10, 20, 40, 80, 160, 320, 640, 1280, and 2560 µg/mL. Overnight cultures of E. coli in BHI broth (10^8 CFU/mL) were diluted in a 1:10 ratio with fresh BHI broth. Next, 10 µL portions of the propolis extracts (5-2560 µg/mL) were transferred into the wells. Finally, 90 µL of the bacterial suspension (10^7 CFU/mL) was added to each well. The plate was incubated at 37 °C for 24 h. Each sample was tested in duplicate. The MICs were determined as the lowest concentrations of propolis showing clear wells.

Antibiofilm Formation Assay

The biofilm formation inhibitory assay was performed using 96-well polystyrene round-bottom microtiter plates. Overnight cultures of E. coli in BHI broth were diluted in a 1:10 ratio with sterile 66% trypot-soya broth containing 0.2% glucose. Thereafter, 90 µL portions of the diluted cultures were transferred into the wells with 10 µL of propolis extracts (5-25 µg/mL) or the DMSO control. The 96-well plate was vibrated to stir the culture and propolis properly, followed by statical incubation at 37 °C for 24 h. After incubation, the suspension was removed and washed 3 times using distilled water. The wells were dripped with 100 µL of a 0.1% crystal violet solution, incubated for 30 min at room temperature, rewashed using distilled water 3 times, and dried at room temperature. Next, 100 µL of 70% ethanol was added to each well and transferred to a flat 96-well plate. Thereafter, optical density measurement was performed using a microplate spectrophotometer, at a wavelength of 590 nm. The obtained value was considered the amount of biofilm formed. This assay was carried out in triplicate. The average and standard deviations were calculated for all replications.

Quantification Analysis of the Active Compounds by High-Performance Liquid Chromatography

The quantification of the compounds with antibiofilm activity was performed by analytical high-performance liquid chromatography (HPLC). The analytical HPLC was carried out using an instrument equipped with a Jasco PU-980 pump (Jasco

| Compound                  | Contents (µg/mg 70% EtOH ext.) | LOD (µg/mL) | LOQ (µg/mL) |
|---------------------------|---------------------------------|-------------|-------------|
| Abyssinone V (1)           | 143.7 ± 3.3                     | 1.0         | 10          |
| Munduleaflavanone B (2)    | 158.5 ± 4.1                     | 1.0         | 10          |
| Cathayanon H (3)           | 113.1 ± 2.2                     | 1.0         | 10          |
| Flowerine (5)              | 19.2 ± 0.3                      | 1.0         | 10          |

Abbreviations: LOD, limit of detection; LOQ, limit of quantitation. Each value is the mean ± standard deviation (n = 3).

Figure 2. Effect of compounds isolated from Chiang Mai propolis on biofilm formation by Escherichia coli.

Table 3. Concentration of the Active Compounds in Chiang Mai Propolis.
Co., Inc.), a UV-2075 Plus detector (Jasco), and a Capcell Pak C18 reversed-phase column (5 µm, φ 4.6 × 250 mm; Osaka Soda). The 70% EtOH extract of the Chiang Mai propolis was analyzed using the following gradient program, with MeCN/H2O (in 0.1% trifluoroacetic acid) = 30:70 (0 min)–100:0 (80 min)–100:0 (90 min). The detection wavelength was set at 320 nm to detect the target compounds. Abyssinone V (1) isolated from Chiang Mai propolis was used as the standard to obtain a calibration curve for the compounds. The LOD and LOQ were determined at signal-to-noise ratios of 3 and 10, respectively. The 70% EtOH extract of the Chiang Mai propolis was analyzed 3 times, and the standard deviation was calculated. To correct for the compound loss occurring during the sample preparation, a spike test was conducted using 1. The recovery rate was calculated from the area value of the chromatogram obtained by HPLC (n = 3) and the amount of 1 added.

Acknowledgments

We thank the Pharmaceutical Innovations of Natural Products Unit (PhInNat) for coordination with beekeepers.

Author Contributions

Kazuma Mukaide contributed toward the antibacterial and antibiofilm assay and the preparation of the manuscripts. Yuko Shimamura and Shuichi Masuda supervised the experiments and checked the descriptions in the manuscript. Boonyadist Vongsak collected propolis samples. Shigenori Kumazawa supervised all the processes in the experiments and the manuscript preparation. All authors have read and approved the final manuscript.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the Japan Society for the Promotion of Science (grant number KAKENHI JP18KK0165).

ORCID iD

Shigenori Kumazawa https://orcid.org/0000-0001-9687-9619

Supplemental Material

Supplemental material for this article is available online.

References

1. Bankova VS, De Castro SI, Marcucci MC. Propolis: recent advances in chemistry and plant origin. Apidologie. 2000;31(1):3-15. doi:10.1051/apido:2000102
2. Banskota AH, Tezuka Y, Kadota S. Recent progress in pharmacological research of propolis. Phytother Res. 2001;15(7):561-571. doi:10.1002/ptr.1029
3. Touzami S, Waili AN, Meniy EN, et al. Chemical analysis and antioxidant content of various propolis collected from different regions and their impact on antimicrobial activities. Asian Pac J Trop Med. 2018;11(7):436-442. doi:10.4103/1995-7645.237188
4. Salantino A, Fernandes-Silva CC, Righi AA, et al. Propolis research and the chemistry of plant products. Nat Prod Rep. 2011;28(5):925-936. doi:10.1039/c0np00072h
5. Sforcin JM, Bankova V. Propolis: is there a potential for the development of new drugs? J Ethnopharmacol. 2011;135(2):253-260. doi:10.1016/j.jep.2010.10.032
6. Tsuda T, Kumazawa S. Propolis: chemical constituents, plant origin, and possible role in the prevention and treatment of obesity and diabetes. J Agric Food Chem. 2021;69(51):15484-15494. doi:10.1021/acs.jafc.1c06194
7. Przybylek I, Karpinski T-M. Antibacterial properties of propolis. Molecules. 2019;24(11):2047. doi:10.3390/molecules24112047
8. Sforcin JM, Fernandes Afr, Lopes CA, et al. Seasonal effect on Brazilian propolis antibacterial activity. J Ethnopharmacol. 2000;73(1–2):243-249. doi:10.1016/s0378-8741(00)00320-2
9. Lee J-H, Kim Y-G, Khadke S-K, et al. Antimicrobial and antibiofilm activities of prenylated flavanones from Macaranga tanarius. Phytomedicine. 2019;63(10):153033. doi:10.1016/j.phymed.2019.153033
10. Mukaide K, Honda S, Boonyadist V, et al. Prenylflavanoids from propolis collected in Chiang Mai, Thailand. Phytochem Lett. 2021;43(6):88-93. doi:10.1016/j.phytol.2021.03.015
11. Ishizu E, Honda S, Boonyadist V, et al. Identification of plant origin of propolis from Thailand stingless bees by comparative analysis. Nat Prod Commun. 2018;13(8):973-975. doi:10.1177/1934578x1801300813
12. Milligan S, Kalita J, Pocock V, et al. Estrogenic activity of the hop phyto-oestrogen, 8-prenylnaringenin. Reproduction. 2002;123(2):235-242. doi:10.1530/reprod.0,1230235
13. Mukai R, Honkawa H, Fujikura Y, et al. Prevention of disuse muscle atrophy by dietary ingestion of 8-prenylnaringenin in denervated mice. PLoS One. 2012;7(9):e45048. doi:10.1371/journal.pone.0045048
14. Anta-Morikawa K, Yamanaka K, Mizuoe Y, et al. Inhibitory effects of myricetin derivative on curli-dependent biofilm formation in Escherichia coli. Sci Rep. 2018;8(1):8452. doi:10.1038/s41598-018-26748-z