Indonesian Journal of Chemical Research

http://ojs3.unpatti.ac.id/index.php/ijcr Indo. J. Chem. Res., 10(3), 177-182, 2023

Antibacterial Activity and Toxicity of Honey Derived from Bone, South Sulawesi, Indonesia

Zakaria[1*], Misriyani[2], Ayun Dwi Astuti[3], Ayu Masyita[3]

[1]Faculty of Tarbiyah, Institut Agama Islam Negeri Bone, Watampone 92733, South Sulawesi, Indonesia.
[2]Medical Faculty, Alkhairaat University, Jl. Diponegoro Palu 94221, Central Sulawesi, Indonesia.
[3]Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Hasanuddin, Makassar 90245, South Sulawesi, Indonesia.

[*]*Corresponding author: zakaria28041970@gmail.com*

Received: November 2022
Received in revised: December 2022
Accepted: January 2023
Available online: January 2023

Abstract

Honey is a sweet substance produced by honey bees from the nectar of flowers or other parts of plants. Honey obtained from Bone, in South Sulawesi, has been extracted and tested for antibacterial activity and toxicity. Honey was macerated with methanol to obtain a crude extract. Methanol crude extract was then partitioned successively with n-hexane and ethyl acetate to obtain ethyl acetate and methanol fraction. Antibacterial activity test was performed by agar diffusion method against *Escherichia coli* and *Staphylococcus aureus*. Methanol extract, ethyl acetate fraction, and methanol fraction showed an inhibition zone against *E. coli* at 10.10, 10.05, and 8.40 mm, respectively with amoxicillin as a positive control (20.05 mm). Also against *S. aureus*, inhibition zone was obtained at 11.90, 9.30, 8.60, and 13.70 mm for methanol extract, ethyl acetate fraction, methanol fraction, and amoxicillin, respectively. The greatest inhibition zone was obtained from methanol extract against *E. coli* and *S. aureus*, both including the strong category. The LC_{50} value of methanol extract and methanol fraction was 273.57 µg/ml and 765.66 µg/ml, respectively, categorized as toxic against *Artemia salina*, while ethyl acetate fraction was not toxic.

Keywords: Agar diffusion, Antibacterial, Honey Bones, Multifloral, Toxicity

INTRODUCTION

Honey generally has a sweet taste due to the sugar constituent such as glucose sucrose and fructose, which is approximately 80% of its weight, with water composing the remaining 20%. The profile of the sugars is influenced by the geographical origin (Agus, Agussalim, Sahlan, & Sabir, 2021). Flavonoids, phenolic acids, minerals, vitamins, amino acids, and enzymes are also present in honey (Almasaudi, 2021). The source of the bee's food determined the composition of honey produced (Eteraf-Oskouei & Najafi, 2013a).

Honey can inhibit the growth of pathogenic bacteria such as *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Streptococcus pyogenus* (Mullai & Menon, 2005), *Listeria monocytogenes* (Mundo, Padilla-Zakour, & Worobo, 2004), *Shigella sonnei* (Lusby, Coombes, & Wilkinson, 2005), *Helicobacter pylori* (Manyi-Loh et al., 2010), *methicillin-resistant Staphylococcus aureus* (MRSA) (Jenkins, Burton, & Cooper, 2014), and yeasts like *Candida albicans* (Irish, Carter, Shokohi$, & Blair, 2006). The antibacterial activities are attributed to some parameters such as low water activity (Molan, 1992), high sugar content (Molan, 1992), low pH (Molan, 1992), H_2O_2 content (Brudzynski, 2011), and polyphenolic compounds (Wahdan, 1998). These parameters are likely to depend on the apiary in which the colonies lived, the climate, and the composition of the nectar (Aal, El-Hadidy, El-Mashad, & El-Sebaie, 2007).

Monofloral honey from manuka tree (*Leptospermum scoparium*), a native tree from New Zealand, has antimicrobial activity against some gram positive and negative strains, including MRSA (Sherlock et al., 2010). The Ulmo honey from *Eucryphia cordifolia* tree, native to Chile, has better antibacterial activity against MRSA, *P. aeruginosa*, and *E. coli* compared to Manuka honey (Sherlock et al., 2010). Tualang honey is one of the multi floral honey from Malaysia which is more effective against pathogen microorganism in burn wounds than Manuka honey (Nasir, Halim, Singh, Dorai, & Haneef, 2010). Stagos et al (2018) reported the different antibacterial activity of 21 types of honey obtained from Mount Olympus in Greece (Stagos et al., 2018).

Bone is one of the cities in South Sulawesi which has 27 districts. Most of these sub-districts are located

in the highlands and have forests that are still being preserved. The bees found in the Bone forest, especially in the village of Liliriawang, are *Apis trigona* species with multiflora vegetation dominated by cocoa (*Theobroma cacao*), teak (*Tectona grandis*), mango (*Mangifera indica*) and short-term crops such as corn (*Zea mays*). Honey from Bone is a polyfloral honey that contains almost all major classes of secondary metabolites (Stevenson, Nicolson, & Wright, 2017). Research on honey from Bone has not been reported. Therefore this study was conducted to determine the antibacterial activity and toxicity of honey from Bone, in South Sulawesi, Indonesia.

METHODOLOGY

Materials and Instrumentals

The honey was collected from Liliriawang village, Bone, South Sulawesi, Indonesia and has been stored in the Pharmacognosy-Phytochemistry Laboratory, Hasanuddin University with the number of specimen Z-IAINB-01. The antibacterial activity of the honey extract was measured using the disc diffusion method with paper disc against *S. aureus* ATCC 25923 and *E. coli* ATCC 25922. Amoxicillin was obtained from Novapharin. Methanol, n-hexane, and ethyl acetate were obtained from Merck.

Figure 1. Sampling location

Methods

Extraction and Fractionation

Fifty grams of honey was macerated with 250 mL of methanol for 24 hours. The top layer was concentrated using an evaporator until the thick reddish brown methanol crude extract was obtained. A portion of the crude extract was partitioned with n-hexane, resulting in two layers of the solution. The top layer was n-hexane soluble fraction while the bottom layer was n-hexane insoluble fraction (residual methanol fraction). The n-hexane insoluble fraction was then added with water drop by drop to increase its polarity (methanol : water ratio is (7:3)). After that, ethyl acetate was added and formed two layers, the top layer was ethyl acetate fraction while the bottom layer was methanol fraction. Then, the methanol crude extract, ethyl acetate fraction, and methanol fraction were concentrated using a rotary evaporator and stored in a tightly closed container for further analysis.

Antibacterial Assay

The antibacterial activity of honey extract was tested using the disc diffusion method. Briefly, 50 µL of inoculate was distributed in a petri dish containing 20 mL of nutrient agar media using a spreading triangle. Furthermore, the disk paper (6 mm) was moistened with a solution of methanol crude extract, ethyl acetate fraction, methanol fraction, amoxicillin, and methanol in 10, 100 and 1000 µg/ml, then placed on media. Petri dishes are tightly closed and incubated anaerobically in a candle jar at 37 ^{o}C. After 24 hours, the diameter of a clear area that formed was observed and measured using a calliper. The clear zone was regarded as the growth inhibition zone of microorganisms.

Toxicity Assay

To evaluate the toxicity of the samples, we performed the brine shrimp lethality test, according to Meyer et al (1982) (Meyer et al., 1982). *Artemia salina* eggs were hatched in artificial seawater for 48 hours (nauplii) under 40 watts of an incandescent lamp. Ten nauplii were transferred into vials that contain diluted sample tests (methanol crude extract, ethyl acetate fraction, and methanol fraction at concentrations 10, 100, and 1000 µg/ml). Each vial was incubated at room temperature for 24 hours. The number of dead and surviving nauplii was counted at each concentration. This procedure was done in triplicate. Determination of half-maximal lethal concentration (LC_{50}) was done using probit analysis and regression equation.

RESULTS AND DISCUSSION

Honey extraction is done with maceration by adding 250 ml of methanol which functions to separate impurities found in the honey. The impurities will separate and be in the lower layer of the honey solution, while the upper layer was methanol crude extract which will proceed to the partitioning stage, antibacterial test, and toxicity test (Figure 2). Maceration is one of the extraction methods that have many advantages such as being simple, cheap, and easy to do. Maceration also can be used to extract all types of simplisia, both heat, and non-heat-resistant

(Dali et al., 2022). In the partitioning stage, firstly, n-hexane is used to separate non-polar compounds from the honey. N-hexane fraction was not continued in anti-bacterial and toxicity tests, because based on the literature, it is known that non-polar compounds have poor antibacterial (Mere, Bintang, & Safithri, 2021) and low toxicity (Astuti, Yasir, Subehan, & Alam, 2019). The n-hexane insoluble fraction (residual methanol fraction) was added to water to increase its polarity so that when mixed with ethyl acetate in the second partition stage, a solution with good separation can be formed. The top layer was ethyl acetate fraction and the bottom layer was methanol fraction, which will be used for antibacterial and toxicity assay along with methanol crude extract.

Figure 2. Extraction of honey from Bone

Antibacterial activity

The methanol crude extract, ethyl acetate fraction, and methanol fraction of honey from Bone were tested for their antibacterial activity against *S. aureus* (represent for gram-positive bacteria) and *E. coli* (represent for gram-negative bacteria) using the disk diffusion method (Figure 3). The formation of inhibition zones indicates an obstacle to bacterial growth. The wider inhibition zone indicates higher antibacterial activity (Mere et al., 2021).

The use of honey in the treatment of various infectious diseases has been recognized since ancient times. Natural honey has antibacterial activity against microorganisms such as *E. coli*, *Shigella sp*, *Helicobacter pylori*, and *Salmonella sp* (Eteraf-Oskouei & Najafi, 2013b). Table 1 shows the antibacterial activity of the honey. All the samples exhibit inhibition against *S. aureus* and *E. coli*. We found that the ability of the honey to inhibit the growth of *S. aureus* (gram positive) was higher than *E. coli* (gram negative). This result is in line with the research of Tajik and Jalali (2009) which reported *S. aureus* is the most sensitive microorganism to honey compared to *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *E. coli*, and *Bacillus subtilis* (Tajik & Jalali, 2009). This is influenced by the differences in the chemical structure of bacterial cell walls that determine the penetration, bonding, and activity of antibacterial compounds to the bacteria. Gram negative has an additional outer bilayer membrane consisting of phospholipids and lipopolysaccharide, while *S. aureus*, which is a gram positive, has a simpler structure of cell wall making it easier for antibacterial compounds to enter into bacterial cells (Green, 2002).

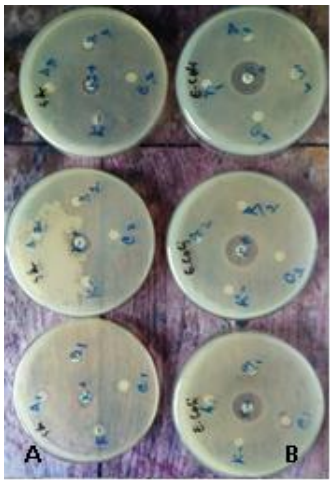

Figure 3. Disk diffusion method for antibacterial activity test: (A) *S. aureus*, (B) *E. coli*

Many factors play a role in the antibacterial activity of honey, such as osmolarity, low pH, H_2O_2 levels, and phytochemical factors (Almasaudi, 2021). The most common factor causing growth inhibition in bacteria is the peroxide effect, which is also a derivative compound from bees. The peroxide effect decreases with honey processing such as extraction, evaporation, and storage (Libonatti, Varela, & Basualdo, 2014). The inhibition zone was found maximum in methanol crude extract against *S. aureus* followed by *E. coli* at 1000 µg/ml. Methanol crude extract of the tested honey showed the greatest inhibition compared to the fraction. This may be due to compound content which still has many compounds, such as polar and non polar compounds (Mandey, Handayani, Nanda, & Noor, 2019). While, the n-hexane fraction, based on other research, only have tannin and alkaloid, making it have poor antibacterial activity than other fraction (Mandey et al., 2019).

The inhibition zone of methanol crude extract against *S. aureus* was 11.90 mm and *E. coli* was 10.10

mm at a concentration of 1000 µg/ml. Methanol crude extract still contains non polar and polar compounds (Mandey et al., 2019), while methanol fraction only contains only some compounds because it has been partitioned with n-hexane and ethyl acetate. Based on research conducted by Vaquero et al (2007) (Vaquero, Alberto, & de Nadra, 2007), this diameter shows that the tested honey has strong antimicrobial activity against both gram-positive and gram-negative. However, the tested honey was less effective as an antibacterial compared to amoxicillin (25 µg) as a positive control

Table 1. Antibacterial activity of honey from Bone, South Sulawesi, Indonesia

Honey Samples	Inhibition Zone (mm) of *S. aureus*			Inhibition Zone (mm) of *E. coli*		
	10 µg/ml	100 µg/ml	1000 µg/ml	10 µg/ml	100 µg/ml	1000 µg/ml
Methanol crude extract	7.15	8.80	11.90	7.65	7.40	10.10
Ethyl acetate fraction	9.05	7.75	9.30	7.55	7.75	10.05
Methanol fraction	7.80	7.75	8.60	7.05	7.30	8.40
Amoxicilin 25 µg	12.80	13.70	13.50	18.85	19.50	20.05

Table 2. The lethal concentration of extract and fraction of honey

Sample test	% Mortality depends on concentration (µg/ml)			LC_{50} (µg/ml)
	10	100	1000	
Methanol crude extract	10.00	16.70	80.00	273.57
Ethyl acetate fraction	6.70	10.00	50.00	1648.77
Methanol fraction	0.00	20.00	36.70	765.66

Toxicity

In our toxicity assay, we assessed early using simple methods, Brine Shrimp Lethality Test which is expressed by the LC_{50} value (Carballo, Hernández-Inda, Pérez, & García-Grávalos, 2002). LC_{50} value <1000 µg/ml was categorized as toxic while >1000 µg/ml was non-toxic (Meyer et al., 1982). In the ethyl acetate fraction, the value of LC_{50} was 1648.77 µg/ml, indicating this fraction was not toxic to *Artemia salina*, while the methanol fraction and methanol crude extract have an LC_{50} value of 765.66 µg/ml and 273.57 µg/ml, respectively. This value showed both are toxic. The high toxicity of methanol crude extract against *Artemia salina* when compared to ethyl acetate fraction and methanol fraction may be due to a large number of compounds in the methanol crude extract, resulting in a synergistic effect. This theory is supported by Mandey et al (2019) who reported that both original honey and methanol crude extract of the honey contains all the major compounds of secondary metabolites such as saponins, steroids, alkaloids, flavonoids, and tannins. (Mandey et al., 2019), different from methanol fraction, which is the remainder of the separation of the n-hexane and ethyl acetate fractions. These results are also in line with studies that have been reported by Astuti et al (2019), which show that methanol extract by macerated has the highest toxicity (55 µg/ml) than ethanol (105 µg/ml) and water extract (47241 µg/ml) using brine shrimp lethality assay (Astuti et al., 2019).

CONCLUSION

In conclusion, this study was the first to examine the antibacterial activity and toxicity of honey types derived from Bone. The antibacterial activity of tested honey was included in a strong category with inhibition zones of 10.10 mm against *E. coli* (gram negative) and 11.90 mm against *S. aureus* (gram positive), although it is less effective than amoxicillin. Methanol crude extract and methanol fraction were toxic against *A. salina* with LC_{50} values of 273.57 µg/ml and 765.66 µg/ml, respectively, while ethyl acetate fraction was not toxic with LC_{50} value of 1648.77 µg/ml. Accordingly, warrant a further investigation is needed to identify and elucidate the bioactive compounds of the tested honey for improving their potential antibacterial activity.

ACKNOWLEDGMENT

The authors are grateful to the head of the Microbiology Laboratory, Medical Faculty of Hasanuddin University for supporting the antibacterial test.

REFERENCES

Aal, A. M. A.-E., El-Hadidy, M. R., El-Mashad, N. B., & El-Sebaie, A. H. (2007). Antimicrobial Effect of Bee Honey in Comparison to Antibiotics on Organisms Isolated From Infected Burns. *Annals of Burns and Fire Disasters*, *20*(2), 83.

Agus, A., Agussalim, Sahlan, M., & Sabir, A. (2021). Honey sugars profile of stingless bee Tetragonula laeviceps (Hymenoptera : Meliponinae). *Biodiversitas*, *22*(11), 5205–5210.

Almasaudi, S. (2021). The antibacterial activities of honey. *Saudi Journal of Biological Sciences*, *28*(4), 2188–2196.

Astuti, A. ., Yasir, B., Subehan, & Alam, G. (2019). Comparison of two varieties of Plectranthus scutellarioides based on the extraction method, phytochemical compound, and cytotoxicity. *Journal of Physics: Conference Series*, *1341*, 1–7. https://doi.org/10.1088/1742-6596/1341/7/072012

Brudzynski, K. (2011). Effect of hydrogen peroxide on antibacterial activities of Canadian honey. *52*(12), 1228–1237. https://doi.org/10.1139/W06-086

Carballo, J., Hernández-Inda, Z., Pérez, P, & García-Grávalos, M. (2002). A comparison between two brine shrimp assays to detect in vitro cytotoxicity in marine natural products. *BMC Biotechnology*, *2*, 1–5.

Dali, N., Dali, S., Chairunnas, A., Ayu Melvi Amalia, H., Ayu Andini Puspitasari, S., & Sultra Jl Mayjen Katamso Lr Satya Kencana, U. (2022). Extraction of The Chemical Components of Dengen Leaves (Dillenia serrata Thunb) by MAE Method and Activity Test as Antioxidant and Toxicity. *Indonesian Journal of Chemical Research*, *10*(2), 74–82.

Eteraf-Oskouei, T., & Najafi, M. (2013a). *Traditional and Modern Uses of Natural Honey in Human Diseases: A Review*. Retrieved from www.mums.ac.ir/basic_medical/en/index

Eteraf-Oskouei, T., & Najafi, M. (2013b). Traditional and Modern Uses of Natural Honey in Human Diseases: A Review. *Iranian Journal of Basic Medical Sciences*, *16*(6), 731.

Green, D. W. (2002). The bacterial cell wall is a source of antibacterial targets. *Expert Opinion on Therapeutic Targets*, *6*(1), 1–20.

Irish, J., Carter, D. A., Shokohi$, T., & Blair, S. E. (2006). Honey has an antifungal effect against Candida species. *Medical Mycology*, *44*(3), 289–291.

Jenkins, R., Burton, N., & Cooper, R. (2014). Proteomic and genomic analysis of methicillin-resistant Staphylococcus aureus (MRSA) exposed to manuka honey in vitro demonstrated down-regulation of virulence markers. *Journal of Antimicrobial Chemotherapy*, *69*(3), 603–615.

Libonatti, C., Varela, S., & Basualdo, M. (2014). Antibacterial activity of honey: A review of honey around the world. *Journal of Microbiology and Antimicrobials*, *6*(3), 51–56.

Lusby, P. E., Coombes, A. L., & Wilkinson, J. M. (2005). Bactericidal Activity of Different Honeys against Pathogenic Bacteria. *Archives of Medical Research*, *36*(5), 464–467.

Mandey, F. W., Handayani, E., Nanda, W. E., & Noor, A. (2019). Extraction, Fractionation, And Antioxidant Examination Of Polyfloral Honey Originated From Bone Prefecture South Sulawesi Province. *Indonesian Journal of Chemical Research*, *7*(1), 86–91.

Manyi-Loh, C. E., Clarke, A. M., Munzhelele, T., Green, E., Mkwetshana, N. F., & Ndip, R. N. (2010). Selected South African Honeys and Their Extracts Possess In Vitro Anti-Helicobacter pylori Activity. *Archives of Medical Research*, *41*(5), 324–331.

Mere, J. K., Bintang, M., & Safithri, M. (2021). Antibacterial Effectiveness of Syzygium cumini (L.) Skeels Leaves to Escherichia coli pBR322. *Indonesian Journal of Chemical Research*, *9*(1), 8–14.

Meyer, B. N., Ferrigni, N. R., Putnam, J. E., Jacobsen, L. B., Nichols, D. E., & McLaughlin, J. L. (1982). Brine shrimp: A convenient general bioassay for active plant constituents. *Planta Medica*, *45*(1), 31–34.

Molan, P. C. (1992). The Antibacterial Activity of Honey. *Bee World*, *73*(1), 5–28.

Mullai, V., & Menon, T. (2005). Antibacterial activity of honey against Pseudomonas aeruginosa. *Indian Journal of Pharmacology*, *37*(6), 403.

Mundo, M. A., Padilla-Zakour, O. I., & Worobo, R. W. (2004). Growth inhibition of foodborne pathogens and food spoilage organisms by select raw honeys. *International Journal of Food Microbiology*, *97*(1), 1–8.

Nasir, N. A. M., Halim, A. S., Singh, K. K. B., Dorai, A. A., & Haneef, M. N. M. (2010). Antibacterial properties of tualang honey and its effect in burn wound management: A comparative study. *BMC Complementary and Alternative Medicine*, *10*.

Sherlock, O., Dolan, A., Athman, R., Power, A.,

Gethin, G., Cowman, S., & Humphreys, H. (2010). Comparison of the antimicrobial activity of Ulmo honey from Chile and Manuka honey against methicillin-resistant Staphylococcus aureus, Escherichia coli, and Pseudomonas aeruginosa. *BMC Complementary and Alternative Medicine*, *10*(47).

Stagos, D., Soulitsiotis, N., Tsadila, C., Papaeconomou, S., Arvanitis, C., Ntontos, A., … Mossialos, D. (2018). Antibacterial and antioxidant activity of different types of honey derived from Mount Olympus in Greece. *International Journal of Molecular Medicine*, *42*(2), 726–734.

Stevenson, P. C., Nicolson, S. W., & Wright, G. A. (2017). Plant secondary metabolites in nectar: impacts on pollinators and ecological functions. *Functional Ecology*, *31*(1), 65–75.

Tajik, H., & Jalali, F. S. S. (2009). In vitro Evaluation of Antimicrobial Efficacy of Natural Honey in Comparison with Sulfonamide Derivatives. *Journal of Animal and Veterinary Advances*, *8*(1), 23–25.

Vaquero, M. J. R., Alberto, M. R., & de Nadra, M. C. M. (2007). Antibacterial effect of phenolic compounds from different wines. *Food Control*, *18*(2), 93–101.

Wahdan, H. (1998). Causes of the antimicrobial activity of honey. *Infection*, *26*(1), 26–31.