ฤทธิ์ทำลายแบคทีเรียและกลไกการยับยั้งของกรดโคจิกต่อ *Pseudomonas aeruginosa*

Bactericidal Activity and Antagonistic Mechanism of Kojic Acid against *Pseudomonas aeruginosa*

ศศิธร หล่อเรืองศิลป์ และ ปาริชาติ พุ่มขจร*

Sasithorn Lorroengsil and Parichat Phumkhachorn*

ภาควิชาวิทยาศาสตร์ชีวภาพ คณะวิทยาศาสตร์ มหาวิทยาลัยอุบลราชธานี

Department of Biological Science, Faculty of Science, Ubon Ratchathani University

*E-mail: scpariph@gmail.com

Received: Dec 31, 2022

Revised: Feb 01, 2023

Accepted: Feb 06, 2023

บทคัดย่อ

กรดโคจิกนิยมใช้ในผลิตภัณฑ์เครื่องสำอางเพื่อเพิ่มความขาวให้แก่ผิว การปนเปื้อนจุลินทรีย์เป็นหนึ่งในปัญหาที่สำคัญที่พบในเครื่องสำอาง เนื่องจากเครื่องสำอางมีส่วนประกอบหลายชนิดที่เอื้อต่อการเจริญของจุลินทรีย์ *Pseudomonas aeruginosa* เป็นแบคทีเรียที่ไม่เพียงแค่พบบ่อยว่ามีการปนเปื้อนในเครื่องสำอางเท่านั้น แต่ยังก่อโรคติดเชื้อที่ผิวหนังได้อีกด้วย การศึกษานี้มีวัตถุประสงค์เพื่อประเมินประสิทธิภาพและกลไกของกรดโคจิกในการยับยั้งการเจริญของ *P. aeruginosa* จากการทดสอบด้วยวิธี swab paper disc พบว่ากรดโคจิกสามารถยับยั้งการเจริญของ *P. aeruginosa* วิธี broth dilution แสดงให้เห็นว่าความเข้มข้นที่น้อยที่สุด (MIC) ของกรดโคจิกในการยับยั้งการเจริญของ *P. aeruginosa* เท่ากับร้อยละ 1 กรดโคจิกที่ความเข้มข้นเท่ากับ MIC มีกลไกการยับยั้ง *P. aeruginosa* แบบทำให้เซลล์ตาย โดยทำให้เซลล์ผิดรูปจากการศึกษาด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด และทำให้เกิดการรั่วไหลของกรดนิวคลีอิกและโปรตีนออกจากเซลล์ *P. aeruginosa* เมื่อศึกษาด้วยวิธีสเปกโทรโฟโตเมตรีที่ความยาวคลื่น 260 และ 280 นาโนเมตร ตามลำดับ ผลการทดลองนี้อาจเป็นประโยชน์สำหรับเป็นแนวทางในการพิจารณาปริมาณกรดโคจิกที่เหมาะสมที่จะใช้ในเครื่องสำอางเพื่อยับยั้งแบคทีเรีย

คำสำคัญ: กรดโคจิก ฤทธิ์ทำลายแบคทีเรีย กลไกการยับยั้ง

Abstract

Kojic acid is commonly used in cosmetic products to lighten the skin. Bacterial contamination is one of the serious problems in cosmetics because they contain ingredients that support the growth of microorganisms. *Pseudomonas aeruginosa* is not only the most frequently found in contaminated cosmetic products but also the major cause of skin infections. The aim of this study was to assess the effectiveness and the mechanism of kojic acid in inhibiting growth of *P. aeruginosa*. By swab paper disc method, it was found that kojic acid clearly inhibited *P. aeruginosa*. The broth dilution method revealed that the minimum inhibitory concentration (MIC) of kojic acid against *P. aeruginosa* was 1%. Kojic acid at the MIC level exhibited bactericidal effect on *P. aeruginosa* causing cell deformation examined by scanning electron microscopy and leakage of nucleic acids and proteins from *P. aeruginosa* cells evaluated by spectrophotometry at the wavelengths of 260 and 280 nm, respectively. The results may be useful as a guideline for determining the proper amount of kojic acid to be used in cosmetics for inhibition of bacteria.

Keywords: Kojic acid, Bactericidal activity, Antagonistic mechanism

1. Introduction

Kojic acid ($C_6H_6O_4$) or 5-hydroxy-2-(hydroxy methyl-pyrone) is a substance produced by fermentation of some fungi such as *Aspergillus flavus*, *Aspergillus oryzae* and *Penicillium citrinum* [1]. Many fungal-fermented food products are found to contain kojic acid including miso, shoyu and sake. Kojic acid has been used for a variety of purposes, especially in food and cosmetic industries. In food industry, it is used as a food preservative [2] and as a substance to reduce the color change of food caused by enzymatic browning [3]. Kojic acid has recently gained widespread popularity in the cosmetic industry. It is commonly used as a skin whitening agent or skin-lightening agent due to its antimelanogenic activity [4]. Other advantages of kojic acid when used as an ingredient in cosmetic products include slowing aging (anti-aging effects) by reducing wrinkles, preventing melasma and freckle and decreasing the appearance of scars [5]. Moreover, kojic acid is beneficial in medicine because it has been reported to have antimicrobial activity [6], antiviral activity [7] and anticancer activity [8].

Cosmetics are products favoring the growth of microorganisms because they contain many components supporting microbial growth, including water, lipids, polysaccharides, proteins, amino acids, glycosides, peptides and vitamins [9]. Contamination with pathogenic microorganisms in cosmetics is one of the risk factors for infection in product users. The production of cosmetics usually passes processes to ensure their safety before being sold in the market such as preservatives addition and compliance with good manufacturing practices (GMP) regulations. However, a wide variety of cosmetic microorganisms have also been reported [10]. The most common cosmetic microorganisms include *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella oxytoca*, *Burkholderia cepacia*, *Staphylococcus aureus*, *Enterobacter gergoviae*, *Serratia marcescens* and some molds and yeasts [11]. Although the skin has immune mechanisms that protect against infection to some degree, it can allow those microbes contaminated in cosmetics to invade the body and cause infection if it is torn or has minor cuts (such as abrasions, bruises, cracked skin).

Pseudomonas aeruginosa is a gram-negative bacillus that is commonly found in the environment, like in soil and in water. It is considered to be the most common cause of infections in humans compared to other *Pseudomonas* species. It is an important opportunistic pathogen that causes a variety of infection in immunocompromised patients [12]. It can cause infections in the blood, lungs (pneumonia), or other parts of the body. Patients can experience a range of clinical manifestations, from mild symptoms to critical illness depending on the location of infection, number of pathogens received and appropriateness of treatment. Since *P. aeruginosa* is found in humid environments, some cosmetic products with high water content are susceptible to be contaminated with this bacterium. This is a serious issue because it represents significant health risks to consumers who use the products. [10].

The objectives of this study were to investigate kojic acid's antimicrobial activity against *P. aeruginosa*, its mode of action and its effect on *P. aeruginosa* cells.

2. Materials and methods

2.1. Bacterium used in this study

The bacterium used in this study was *Pseudomonas aeruginosa* ATCC 27853. It was cultured in Brain Heart Infusion (BHI) medium and incubated at 37°C. The bacterial stock was prepared as a 20% (v/v) stock in glycerol and stored at -20°C.

2.2. Kojic acid

Kojic acid used in this study is the product of Dimollaure sold as 99% kojic acid powder. The stock solution of kojic acid was prepared as a 40% (w/v) solution in sterile distilled water and stored in a dark-colored bottle at 4°C.

2.3. Antimicrobial activity of kojic acid against *P. aeruginosa*

The ability of kojic acid to inhibit *P. aeruginosa* was studied by swab paper disc method. The bacterium was cultured in BHI broth for 24 h and then smeared by a sterile cotton swab throughout a BHI agar dish. A paper disc (6 mm in diameter) (Schleicher & Schuell, New Hampshire, USA) was placed in the center of the cultured dish. Ten µL of kojic acid stock solution were dropped onto the paper disc. The agar dish was incubated at 37°C for 24 h before observing an inhibition zone around the paper disc. This experiment was performed 3 times and the control experiment was performed using sterile distilled water instead of kojic acid solution.

2.4. Determination of MIC of kojic acid against *P. aeruginosa*

The determination of MIC of kojic acid against *P. aeruginosa* was performed by broth dilution assay, which was performed as follows. *P. aeruginosa* was cultured in 2 mL of BHI broth containing kojic acid at final concentrations of 2%, 1%, 0.5%, 0.25%, 0.125%, 0.063%, 0.031% and 0.016%. All culture tubes were incubated at 37°C for 24 h before visual observation of turbid growth. This experiment was performed 3 times and the control experiment was performed using sterile distilled water instead of kojic acid solution.

2.5. Inhibitory mechanism of kojic acid against *P. aeruginosa*

Inhibitory mechanism of kojic acid against *P. aeruginosa* was studied using a method adapted from the method reported previously [13]. *P. aeruginosa* was cultured in 2 mL of BHI broth containing kojic acid at the final concentration equal to the MIC value. The tube was incubated at 37°C for 24 h. An aliquote (100 µL) of each culture was inoculated in a 2 mL BHI broth tube without kojic acid and spread on a BHI agar dish without kojic acid. After incubation at 37°C for 24 h, bacterial growth was observed in the BHI broth and on the BHI agar. This experiment was performed 3 times and the control experiment was performed using sterile distilled water instead of kojic acid solution.

2.6. Effect of kojic acid on *P. aeruginosa* cells

The effect of kojic acid on *P. aeruginosa* cells was studied by observation on cell morphology change using scanning electron microscopy (SEM) and on leakage of nucleic acids and proteins from the cells.

For cell morphology study by SEM, overnight cultures of *P. aeruginosa* grown at 37°C in BHI broth without (control) and with kojic acid at the final concentration equal to the MIC value were centrifuged at 5,000 xg for 5 min. The *P. aeruginosa* cells were fixed with 2% glutaraldehyde for 2 h at 4°C, stained with 1% osmium tetroxide solution for 30 min and washed twice with phosphate buffered saline. The cells were then dried at a critical point in liquid CO_2, gold covered by cathodic spraying and examined by a scanning electron microscope (JEOL JSM-6010LV, Tokyo, Japan), provided by the Scientific Equipment Center, Ubon Ratchathani University.

Leakage of nucleic acids and proteins from *P. aeruginosa* cells was performed as follows. *P. aeruginosa* was cultured in 5 mL BHI broth and incubated at 37°C for 24 h. After centrifuge at 5,000 xg for 5 min, *P. aeruginosa* cell pellet was collected and washed with 0.1 M phosphate buffer solution (PBS) and then resuspended in 5 mL of 0.1 M PBS solution. Kojic acid was added to the cell suspension at the final concentration equal to the MIC value. For the control experiment, sterile distilled water was used instead of kojic acid. After incubation at 37°C for 4 h, supernatants of both treatment and control sets were obtained by centrifugation the corresponding cells suspensions at 5,000 xg for 5 min. The supernatants were subjected to the optical density (OD) measurement at wavelengths of 260 (OD_{260}) and 280 (OD_{280}) nm to determine the leakage nucleic acids and proteins, respectively. This experiment was performed 3 times. The data (means ± SD) were analyzed by the independent *t*-test to compare the means of both groups. Significance level was taken as $p<0.05$ level.

3. Results and discussion

3.1. Antimicrobial activity of kojic acid against *P. aeruginosa*

An investigation of the antimicrobial activity of kojic acid against *P. aeruginosa* on BHI agar by swab paper disc method showed that kojic acid was able to inhibit the growth of the bacterium indicated by an inhibition zone surrounding the paper disc treated with kojic acid. On the other hand, no inhibition zone was found in the control using sterile distilled water (Figure 1). Previous research has found that kojic acid and its derivatives have biological activity against bacteria. It has antibacterial activity against a wide variety of gram-positive and gram-negative bacteria such as *Listeria monocytogenes*, *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella typhimurium* and *Escherichia coli* [5], [14], [15]. Kojic acid has also been reported to be active against certain yeast and fungi, such as *Saccharomyces cerevisiae* and *Aspergillus niger* [15].

3.2. Determination of MIC of kojic acid against *P. aeruginosa*

In this experiment, *P. aeruginosa* was cultured in BHI broth containing different concentrations of kojic acid varying from 0.016% to 2% at 37°C for 24 h. Turbidity of the bacterial cultures was observed to determine bacterial growth. Bacterial growth was observed in the control culture (with no kojic acid) and in the cultures with kojic acid ranging from 0.016% to 0.5% (Figure 2). However, no bacterial growth was noticed in the cultures with 1% and 2% of kojic acid indicated by transparent cultures (Figure 2). Therefore, the MIC of kojic acid against *P. aeruginosa* was 1%.

The MIC values of kojic acid for different bacteria vary depending on the type of bacteria. These values indicate bacterial susceptibility to kojic acid and can be guidelines for determining the amount of kojic acid to be used for inhibition or control of certain bacteria [16]. Gram-negative bacteria were also reported to be more sensitive to kojic acid than gram-positive bacteria due to the difference between cell wall structures of both bacteria [14]. Therefore, less amount of kojic acid is required to inhibit gram-negative bacteria than to inhibit gram-positive bacteria. Antimicrobial activity of kojic acid broadens its benefits in cosmetic products. Addition of 1% of kojic acid in cosmetic products not only helps reducing melanin production and making the skin whitened [4], [17] but also preventing users from bacterial infection [12]. Moreover, it can act as a preservative to prevent bacterial contamination in cosmetic products.

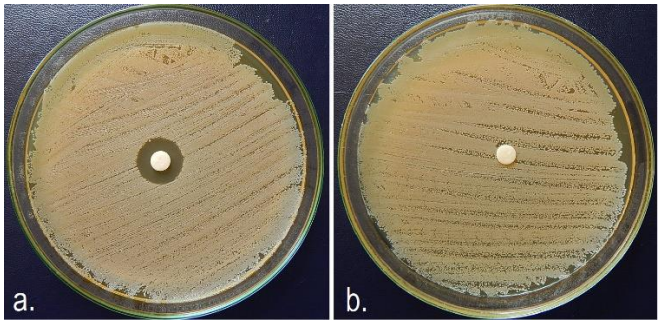

Figure 1 Antimicrobial activity (zone of inhibition) of kojic acid against *P. aeruginosa* by swab paper disc method
(a) an inhibition zone around a paper disc treated with kojic acid;
(b) no inhibition zone around a paper disc treated with sterile distilled water

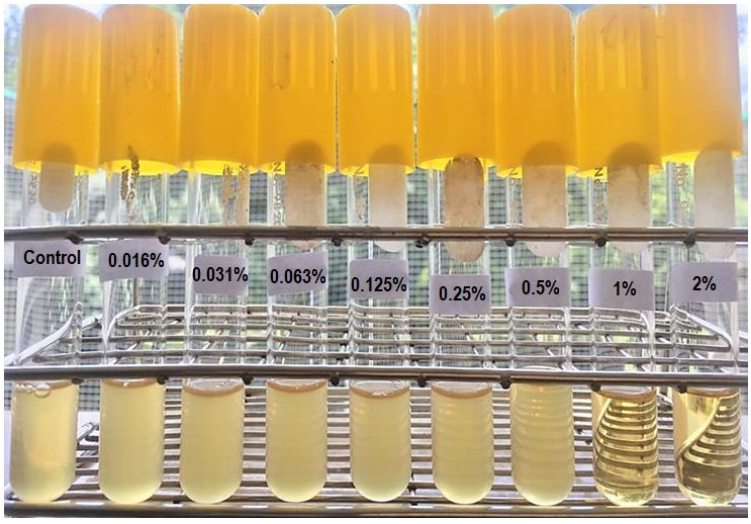

Figure 2 Growth of *P. aeruginosa* in the cultures containing different concentrations of kojic acid

3.3. Inhibitory mechanism of kojic acid against *P. aeruginosa*

Antimicrobial agents can use either bactericidal or bacteriostatic mode of action to inhibit the bacterial growth. Antimicrobial agents with bactericidal mode of action completely kill target bacteria, thereby preventing them to regrow after removal of the antagonists whereas ones with bacteriostatic mode of action just suppress but not kill the target bacteria, thereby allowing them to regenerate after removal of the antagonists [18]. In this experiment, no bacterial regeneration was observed after transferring kojic acid treated *P. aeruginosa* cells to fresh BHI broth without kojic acid. This result indicated that kojic acid exerted bactericidal mode of action against *P. aeruginosa*. Since the minimal concentration of kojic acid to inhibit and kill *P. aeruginosa* was 1%, MIC and minimal bactericidal concentration (MBC) of the substance against *P. aeruginosa* were the same which were 1%. There has been no report on MBC of kojic acid against *P. aeruginosa*. However, Wu et al.

[14] reported that MBC of kojic acid against *E. coli*, a gram-negative bacterium, was 4 times higher than MIC. The inconsistency in findings might be in part due to the difference in species used in the experiments. In the latter case, kojic acid exerted both bacteriostatic and bactericidal modes of action depending on its concentration.

3.4. Effect of kojic acid on *P. aeruginosa* cells

Antimicrobial substances with bactericidal action against bacteria have been reported not only to deform bacterial cell morphology but also to dismantle bacterial cell membrane to release subcellular contents [4], [19]. In this study, morphology study by SEM on kojic acid treated and untreated (control) *P. aeruginosa* cells revealed that untreated *P. aeruginosa* cells had rod shape with smooth surface whereas kojic acid treated *P. aeruginosa* cells had irregular shape with rough surface (Figure 3). The size of kojic acid treated *P. aeruginosa* cells was much larger than that of normal *P. aeruginosa* cells. This might be due to cell fusion or cell swelling from water influx. Further investigation is required to clarify this phenomenon. The cell deformation in kojic acid treated *P. aeruginosa* was in agreement with the finding obtained from the observation on leakage of nucleic acids and proteins from kojic acid treated and untreated *P. aeruginosa* cells. The absorbance at 260 and 280 nm of the supernatant prepared from kojic acid treated *P. aeruginosa* culture was significantly higher than those of the supernatant prepared from untreated *P. aeruginosa* culture (Table 1), indicating the leakage of nucleic acids and proteins from kojic acid treated *P. aeruginosa* cells, respectively. This finding supports previous research reporting that kojic acid had detrimental effects on bacterial membrane integrity and permeability leading to leakage of subcellular constituents such as potassium ions, nucleic acids and proteins [14].

4. Conclusion

The present study revealed that kojic acid was able to inhibit the growth of *P. aeruginosa* with a MIC value of 1% and had bactericidal inhibitory mechanism against the target bacterium. It deformed *P. aeruginosa* cell morphology and caused the leakage of nucleic acid and proteins from the cells. How these phenomena happen is still unclear but has been further investigated in our laboratory.

5. Acknowledgements

The authors wish to thank the Faculty of Science, Ubon Ratchathani University, Thailand for the support on facilities and equipment to this work. The authors gratefully acknowledge the Scientific Equipment Center, Ubon Ratchathani University, Thailand for the technical assistance on using the scanning electron microscope.

6. References

[1] Rattanachaikunsopon, P. and Phumkhachorn, P. 2021. Kojic acid: its properties and applications. **Journal of Science and Technology Mahasarakham University.** 40(3): 235-243. (*in Thai*)

[2] Brtko, J. 2022. Biological functions of kojic acid and its derivatives in medicine, cosmetics, and food industry: Insights into health aspects. **Archiv der Pharmazie.** 355(10): e2200215.

[3] Zhao, X. and et al. 2019. Storage and preservative study of tropical fruits by kojic acid. **IOSR Journal of Agriculture and Veterinary Science.** 12(11): 84-90.

[4] Singh, B.K. and et al. 2016. Kojic acid peptide: A new compound with anti-tyrosinase potential. **Annals of Dermatology.** 28(5): 555-561.

[5] Saeedi, M., Eslamifar, M. and Khezri, K. 2019. Kojic acid applications in cosmetic and pharmaceutical preparations. **Biomedicine and Pharmacotherapy.** 110: 582-593.

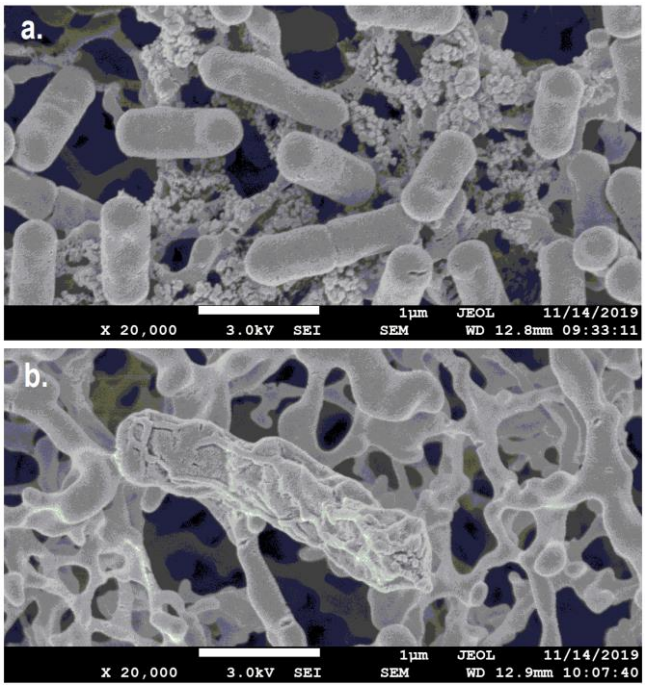

Figure 3 Scanning electron micrographs of untreated (a) and kojic acid treated (b) *P. aeruginosa* cells

Table 1 OD_{260} and OD_{280} of supernatants prepared from kojic acid treated (Test) and untreated (Control) *P. aeruginosa* cultures

Optical density	Test	Control	*t*-value	*p*-value
OD_{260}	0.537 ± 0.014	0.012 ± 0.003	61.9282	< 0.00001*
OD_{280}	0.829 ± 0.024	0.018 ± 0.004	56.8899	< 0.00001*

*Significant difference between the means of kojic acid treated and untreated *P. aeruginosa* cultures

[6] Sanjotha, G., Shivasharana, C.T. and Manawadi, S.T. 2019. An *in vitro* approach for evaluating antimicrobial activity and production of kojic acid by *Aspergillus flavus* isolated from Karwar region. **Journal of Pure and Applied Microbiology.** 13(4): 2261-2272.

[7] Zilles, J.C. and et al. 2022. Biological activities and safety data of kojic acid and its derivatives: A review. **Experimental Dermatology.** 31(10): 1500-1521.

[8] Karakaya, G. and et al. 2019. Kojic acid derivatives as potential anticancer agents: synthesis and cytotoxic evaluation on A375 human malignant melanoma cells. **Journal of Research in Pharmacy.** 23(4): 596-607.

[9] Kim, H.W. and et al. 2020. Risk factors influencing contamination of customized cosmetics made on-the-spot: Evidence from the national pilot project for public health. **Scientific Reports.** 10: 1561.

[10] Neza, E. and Centini, M. 2016. Microbiologically contaminated and over-preserved cosmetic products according Rapex 2008-2014. **Cosmetics.** 3(1): 3.

[11] Dadashi, L. and Dehghanzadeh, R. 2016. Investigating incidence of bacterial and fungal contamination in shared cosmetic kits available in the women beauty salons. **Health Promotion Perspectives.** 6(3): 159-163.

[12] Qin, S. and et al. 2022. *Pseudomonas aeruginosa*: pathogenesis, virulence factors, antibiotic resistance, interaction with host, technology advances and emerging therapeutics. **Signal Transduction and Targeted Therapy.** 7: 199.

[13] Srisutham, W. and et al. 2018. Influence of temperature on antimicrobial activity and mode of action of thymol against *Escherichia coli* O157:H7. **Journal of Science and Technology, Ubon Ratchathani University.** 20(1): 36-43. (*in Thai*)

[14] Wu, Y. and et al. 2018. Evaluation of antibacterial and anti-biofilm properties of kojic acid against five food-related bacteria and related subcellular mechanisms of bacterial inactivation. **Food Science and Technology International.** 25(1): 3-15.

[15] Liu, X. and et al. 2014. Synthesis, characterization, and antimicrobial activity of kojic acid grafted chitosan oligosaccharide. **Journal of Agricultural and Food Chemistry.** 62: 297-303.

[16] Saaaquib, S.A. and et al. 2019. Evaluation and comparison of antibacterial efficacy of herbal extracts in combination with antibiotics on periodontal pathobionts: An *in vitro* microbiological study. **Antibiotics.** 8(89): 1-12.

[17] Phasha, V. and et al. 2022. Review on the use of kojic acid-A skin-lightening ingredient. **Cosmetics.** 9(3): 64.

[18] Mogana, R. and et al. 2020. Antibacterial activities of the extracts, fractions and isolated compounds from *Canarium patentinervium* Miq. against bacterial clinical isolates. **BMC Complementary Medicine and Therapies.** 20(55): 1-11.

[19] Wijesundara, N.M. and et al. 2022. Bactericidal activity of carvacrol against *Streptococcus pyogenes* involves alteration of membrane fluidity and integrity through interaction with membrane phospholipids. **Pharmaceutics.** 14(10): 1992.