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Antibacterial effect of *Melastoma malabathricum* leaves extract against locally isolated bovine mastitis pathogens

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Abstract

Mastitis is the most frequently diagnosed disease in dairy cattle and responsible for the major economic losses. To date, antibiotics are the most common treatment for this disease. However, the use of antibiotic was reported to be the main contributor to milk contamination and frequent use of this therapy will lead to microbial antibiotic resistance. Furthermore, the antibiotic withdrawal time and mastitis therapy will cause a huge profit loss to dairy farmers. Therefore, alternative plant-based treatments should be explored to replace the use of antibiotics. The aim of this study was to evaluate the antibacterial activity of *Melastoma malabathricum* (MM) extract against eight common bovine mastitis pathogens isolated from a local commercial dairy farm, namely *Staphylococcus aureus* (SA), *Staphylococcus chromogenes* (SC), *Staphylococcus haemolyticus* (SH), *Streptococcus uberis* (SU), *Streptococcus agalactiae* (RK3C), *Pseudomonas aeruginosa* (PA), *Klebsiella pneumoniae* (HS09A) and *Escherichia coli* (GN9B). MM aqueous extracts showed antibacterial activities against all pathogens, with RK3C having the highest antibacterial efficacy at an effective extract concentration of 3.12 mg/mL, followed by SA (6.25 mg/mL), SU (6.25 mg/mL), SH (12.5 mg/mL), PA (12.5 mg/mL), GN9B (12.5 mg/mL), SC (50 mg/mL), and HS09A (50 mg/mL). Most of the pathogens (SA, SH, SU, RK3C and PA), especially Gram-positive bacteria were killed within the half hour when incubated either with 25 or 50 mg/mL concentration of MM aqueous extract. In this study, the results demonstrated bactericidal effect of the MM extract against all pathogens, reflecting the potential of MM aqueous extract as new antibacterial agent against bovine mastitis pathogens.

1. Introduction

To date, antibiotics are considered the best treatment for bovine mastitis disease. Antibiotics either stop bacteria from reproducing or destroy them by targeting different parts of the bacteria cells. However, there is a worldwide concern of antibiotics being overused. In the dairy industry, antibiotics are one of the main reasons for milk contamination and frequent use of antibiotic therapy leads to microbial antibiotic resistance (Virto *et al*., 2022). Moreover, mastitis antibiotic treatment and subsequent withdrawal time will lead to the loss of profit for dairy farmers, as post-treatment milk is not marketable.

Mastitis can be divided into two types of origin; contagious and environmental (Cobirka *et al*., 2020). Contagious mastitis usually caused by pathogens such as *S. aureus*, *S. agalactiae*, and *S. uberis* (Sharif *et al*., 2009), which are originated from the rumen, genitals

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areas, and the rectal, transported to the mammary gland. The pathogens spread when the contaminated milk is in contact with the uninfected mammary gland during the milking process (Petersson-Wolfe *et al*., 2010). Meanwhile, environmental mastitis pathogens include *K. pneumonia*, *E. coli*, *E. aerogenes*, *P. aeruginosa*, as well as *S. uberis*. Besides these, other bacteria such as *S. aureus*, *S. agalactiae* can also be considered as environmental pathogens (Klaas and Zadoks, 2017). These pathogens have been transmitted from the environment through multiple ways, such as contagious infected udder, poor hygiene equipment, bedding, urine, and faeces that present from the cows' surrounding area (Cobirka *et al*., 2020). The first barrier of immune response in the mammary gland is at the teat canal. Sphincter in teat canal may open for two hours post-milking. This opens an opportunity for bacteria to enter and infect the mammary gland. According to Idriss *et al*. (2013), infections usually occur during milking or two hours post-milking.

Initially, the pathogens damage the inner tissues of the teat and gland cisterns within the quarter. The bacteria then spread to the duct cells and establish infection in the alveoli (Jones, 1998). This is followed by the formation of abscesses that wall off certain bacteria to avoid detection by the immune system and to prevent antimicrobial agents from reaching the bacteria. The destruction of alveolar and duct cells results in reduction yield of milk. All damaged cells will clog the milk within the mammary gland that drains the alveolar, subsequently detected as lumps at the udder. The activities of bacteria-host interaction will result in high somatic cell count, destruction of mammary tissue and might be recurrent in clinical mastitis (Jones, 1998). Due to the ability of microbes to form abscesses and to hide in among the host cells (Jones, 1998), it might be difficult to kill the pathogen thoroughly as drugs might not able to penetrate to all infected area.

Mastitis bacterial infection had caused the extensive use of antibiotics, either intramammary or systemic (Krömker *et al*., 2017). Approximately 90% of the antibiotics usage in a dairy farm came from mastitis treatment (Erskine *et al*., 2003). The frequent prescription of antibiotics even for non-bacterial infection, as well as unregulated use of antibiotics, was quoted as one of the reasons for antimicrobial resistance in dairy cattle. Subsequently, the degree of cure from mastitis pathogens decreased to as low as 20% cure rate only or might not be effective at all (Dingwell *et al.*, 2003).

Among the bovine mastitis pathogens reported to be resistant to antibiotic are *Enterobacter sakazakii, Klebsiella osytaca,* and *E. coli* which has resistant to clavulanic acid, amoxicillin, and erythromycin (Kovačević *et al*., 2021*).* Besides, Supre *et al.* (2014)'s study also found out that a range of bacteria such as *Streptococcus* spp*., S. aureus, S. uberis, S. dysgalactiae, E. coli,* and *Klebsiella* sp*.* are resistant to ampicillin, amoxicillin/clavulanic acid, tetracycline, erythromycin, marbofloxacin, and trimethoprim/ sulfamethoxazole. Similar research from Cheng *et al.* (2019)'s finding mentioned the resistance of *S. aureus, E. coli, Klebsiella* spp*.,* and *Streptococcus* spp*.* towards penicillin, tetracycline, clindamycin, and amoxicillin / clavulanic acid. The research conducted by Ameen *et al.* (2019) also highlighted the resistance of some main bovine mastitis pathogens, such as *S. aureus, E. coli,* and *P. aeruginosa* to common antibiotics (penicillin and oxacylin).

The ability of the bovine mastitis pathogens to develop resistance and the incidents will impact on human and animal health had become a global problem (WHO, 2015). There are possibilities of these antibiotic-resistant pathogen strains being transmitted out from the farm, eventually increasing the possibilities of these bacteria strains and antibiotic residues entering the human food chain (Gupta *et al*., 2020). Therefore, plant based, natural based and other remedies should be explored to substitute current antibiotics. Herbals treatments have been found to be comparable effective as conventional antibiotics in some situations for treating bacterial infection (Mushtaq *et al*., 2018).

To date, the use of plant metabolites as bovine mastitis treatment in Malaysia has not been extensively explored. Our previous research indicated that the crude extract of our local herbs harbours the potential in controlling mastitis. *Melastoma malabathricum* (MM) is a kind of medical herb, has been reported for its antimicrobial (Choudhury *et al*., 2011), anti-inflammation (Mazura *et al*., 2007) and antioxidant activities (Kumar *et al*., 2013). However, MM has not been used as a traditional remedy for bovine mastitis even though it has been proven to harbour antimicrobial properties against mastitis pathogens (Alwash *et al*., 2013). In tropical countries, where MM can grow widely, the plant is considered a famous medical herb with a wide range of usage (Hanafiah *et al*., 2011; Gani *et al*., 2020; Apridamayanti *et al*., 2021; Isnaini *et al*., 2021; Lestari *et al*., 2021).

When applying drugs to livestock, there will be a withdrawal time between the administrations and slaughter or milking for human consumption period. The period varied from days to weeks according to the type of antibiotic used. In dairy industries, farmers used to have three to 21 days of withdrawal period after

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application of mastitis antibiotics before the milk was allowed to drink or to be released to the market. However, as far as we are concerned, there is no reported evidence that the herbal product applied to livestock requires withdrawal time prior to slaughter. Furthermore, ethno-veterinary in bovine mastitis will be the solution for producers of organic farms that treat mastitis with a variety of alternative remedies, such as botanical, whey-based, vitamin supplement and homeopathy (Ruegg, 2009). Therefore, alternative therapy to antibiotics for mastitis treatment and control must be developed, such as the use of herbs or natural products to inhibit microbial growth in dairy cows. This study aims to evaluate the antibacterial activity of MM extract against eight common bovine mastitis pathogens isolated from a local commercial dairy farm.

2. Materials and methods

2.1 Plant materials

Fresh MM leaves were collected from the green house at the Headquarter of Malaysian Agricultural Research and Development Institute (MARDI), Malaysia (location at longitude 101° 41' 26.2284" and latitude: N 2° 59.8573') where they were cultivated under semi-controlled environmental conditions. The plant was deposited at UPM with the voucher number SK3338/18. Freshly harvested leaves were washed and dried in drying oven (50°C) overnight. Dried leaves were ground using an ultra grinder (RETSCH ZM 200 Ultra Centrifugal Mill). The ground powder was kept in the freezer at temperature of -20°C prior to extract.

2.2 Crude extract

The ground leaves powder was extracted using distilled water (aqueous), hot distilled water (hot aqueous), and 80% ethanol solvent (v/v). The crude aqueous and 80% ethanol extracts were prepared by soaking 80 g of the plant material in 1600 mL water and 80% ethanol solution, respectively and shake at 200 rpm for 2 hrs. The hot aqueous extraction was done by using boiling distilled water and continuing shake at 200 rpm for 2 hrs. At the end of shaking, all the crude extracts were filtered using Whatman No. 1. The solution was freeze dried to yield the crude extract powder. The percentage of yield was calculated based on the formula:

Percentage of Yield (%) = Dry weight of extract/Dry weight of plant material × 100

Prior to use, the crude extracts were dissolved in distilled water to a final concentration of 200 mg/mL.

2.3 Mastitis pathogens source

A total of eight mastitis pathogens were used in the study. Five isolates from Gram positive bacteria which identified as *Staphylococcus aureus* (SA), *Staphylococcus chromogenes* (SC), *Staphylococcus haemolyticus* (SH), *Streptococcus uberis* (SU), *Streptococcus agalactiae* (RK3C) and three isolates from Gram negative bacteria which identified as *Pseudomonas aeruginosa* (PA), *Klebsiella pneumoniae* (HS09A) and *Escherichia coli* (GN9B)]. Three isolates, RK3C, HS09A and GN9B from mastitis cow, were provided by Microbial Culture Collection, Institute of Bioscience, Universiti Putra Malaysia.

2.4 Minimum inhibitory concentration

Antimicrobial activity of the crude extract against mastitis bacteria was assessed using microdilution method to determine the minimum inhibitory concentration (MIC). The herbs were diluted in sterile distilled water to a stock concentration of 200 mg/mL. The 96-wells microdilution cell culture plates (SPL Life Sciences, Korea) and MH broth (Oxoid, United Kingdom) were used as culture media for the tests. Each well was filled with 200 µL of mixture at the different concentrations. The initial of the first well contained 100 mg/mL of herbs extract, followed by 50, 25, 12.5, 6.25, 3.13 and 1.53 mg/mL. The inoculums density in each well was adjusted to approximately more than 10^8 colony forming unit (CFU/mL). All the samples tested were prepared in triplicates. The plates were incubated at 37°C for 24 hrs to determine the minimum inhibitory concentration of herbs that inhibited the growth of bacteria.

2.5 Minimum bactericidal concentration

The MBC is characterized as the minimum concentration of herb samples required to kill the bacteria after the incubation. A total of 10 µL from each well of cultures were inoculated at MH agar (Oxoid, United Kingdom) to define the concentration of MBC. The lowest concentration of extract that exhibits complete killing or inhibited the visible growth of the microorganism was considered as the MBC.

2.6 Determination of bactericidal or bacteriostatic

The MBC/MIC ratio of the extract was calculated as described by Mogana *et al.* (2020) to elucidate whether the observed antibacterial effects were bactericidal or bacteriostatic. When the ratio of MBC/MIC was ≤ 4.0, the extract was considered bactericidal or otherwise bacteriostatic.

2.7 Time kill assay

The bactericidal concentration was tested for time kill behaviour. Tubes that contained a mixture of sterile MH broth, a final concentration of approximately 10^8 cfu/mL inoculums suspension, and MM extract were prepared. All pathogens were tested based on the

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concentration of MBC of MM extract. The mixture was incubated at 37℃ for 24 hrs. At the interval time of 0.5, 1, 2, 4, 8, 12, 16, and 24 hrs, a total of 200 µL of cell suspension from each tube was collected for microbial growth performance analysis. Serial dilution was conducted before the cell was grown on MH agar. After 24 hrs, the visible colonies were counted. Experiments were done in triplicates.

2.8 Statistical analysis

The data of each group were compared among each treatment. Analysis of the associations between the group was conducted using SAS software SAS/STAT® 9.4 (SAS Institute Inc. 2011). The *p*-value of less than 0.05 is regarded as statistically significant.

3. Results

3.1 Melastoma malabathricum leaves extract

Three types of extraction herb methods (aqueous, hot aqueous and 80% ethanol) were successfully performed and the yield of each MM extract is summarized in Table 1. The extraction yield obtained by conventional aqueous method (8.48±1.15%) showed the lowest percentage yield. Comparatively, the yield of hot aqueous extract (19.21±0.33%) and 80% ethanol (19.50±1.52%) extract was significantly higher (p<0.05). Extraction using hot aqueous solution and 80% ethanol represented the highest yield among all extraction methods.

Table 1. Extraction yield of MM by aqueous, hot aqueous and 80% ethanol extraction.

Plant	Solvent	Percentage of Yield (%)
Melastoma malabathricum	Aqueous	8.48±1.15[a]
	Hot aqueous	19.21±0.33[b]
	80% Ethanol	19.50±1.52[b]

Values are presented as mean±SD. Values with different superscripts are statistically significantly different (p<0.05).

3.2 Pathogens strains

Pathogens were isolated from clinical mastitis milk samples. Eight pathogens were selected as inoculums in the study as shown in Table 2 and Figure 1. The five pathogens isolated and were identified *via* 16S sequencing were *Staphylococcus aureus* (SA), *Staphylococcus chromogenes* (SC), *Staphylococcus haemolyticus* (SH), *Streptococcus uberis* (SU), and *Pseudomonas aeruginosa* (PA) and the sequence were deposited in NCBI GenBank with the accession numbers as listed in Table 2.

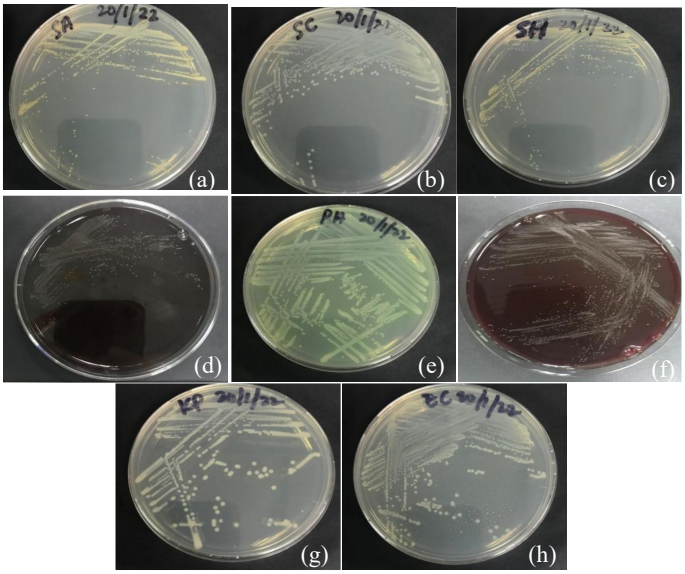

Figure 1. Eight pathogens isolated from bovine clinical mastitis milk were used as inoculums in the study. (a) *Staphylococcus aureus* (SA) (b) *Staphylococcus chromogenes* (SC), (c) *Staphylococcus haemolyticus* (SH), (d) *Streptococcus uberis* (SU), (e) *Pseudomonas aeruginosa* (PA), (f) *Streptococcus agalactiae* (RK3C), (g) *Klebsiella pneumonia* (HS09A), (h) *Escherichia coli* (GN9B).

3.3 Antibacterial study

The effectiveness of MM extract from different extraction methods were tested against mastitis pathogens by measuring the minimum inhibitory concentration (MIC). In this study, the MIC result indicated that MM extract is effective against all eight pathogens. The extract was more effective towards gram positive bacteria with MBC value of ≤12.50 mg/mL in SA, SH, SU and RK3C. On the other hand, SC, PA, HS09A and GN9B required 12.5-50.00 mg/mL MBC values (Table 3). Hot aqueous MM extract showed similar antimicrobial effectiveness with 80% ethanol MM extract, and better when compared to aqueous MM extract in inhibiting all selected pathogens. In a nutshell, all pathogens can be inhibited by 50 mg/mL of hot aqueous MM extract, stands a better chance as one of potential antimicrobial agents to tackle bovine mastitis problem. Besides, the MBC/MIC ratio was determined in

Table 2. Pathogens isolated from clinical mastitis's milk.

Lab ID	Source	Bacteria species isolated	Source / NCBI accession number
SA	Clinical mastitis 9259 (83)	*Staphylococcus aureus*	Clinical mastitis / OM392067
SC	Clinical mastitis N49	*Staphylococcus chromogenes*	Clinical mastitis / OM386845
SH	Clinical mastitis N40	*Staphylococcus haemolyticus*	Clinical mastitis / OM386852
SU	Clinical mastitis No2	*Streptococcus uberis*	Clinical mastitis / OM386870
PA	Clinical mastitis No15	*Pseudomonas aeruginosa*	Clinical mastitis / OM386871
RK3C	Clinical mastitis	*Streptococcus agalactiae*	Clinical mastitis / UPM
HS09A	Clinical mastitis	*Klebsiella pneumoniae*	Clinical mastitis / UPM
GN9B	Clinical mastitis	*Escherichia coli*	Clinical mastitis / UPM

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the range of 1.5–2.0 and confirmed the potential of MM herb as bactericidal agent (Table 3).

3.4 Time killed study

In the time kill study, the antimicrobial efficacy of hot aqueous MM extracts were determined by examining the pathogen microbes growth performance (CFU/mL) at 0.5, 1, 2, 4, 8, 12, 16 and 24 hrs under the specific incubation time. The summary of the result was presented in Figure 2 in terms of $\log_{10}$ CFU/mL of viable colonies changes, which indicated the MM extract has bactericidal activities. The bactericidal activity was defined as being equal to $\log_{10}$ CFU/mL or more reduction in the viable colony from the initial inoculums (Scheetz *et al*., 2007). Most of the Gram positive bacteria, which are SA, SH, SU and RK3C were killed at the first half hour of incubation time in both 25 and 50 mg/mL concentration of MM extract. At the concentration of 25 mg/mL MM extract, the SC, PA, RK3C, HS09A and GN9B showed 100% potent killing effect after incubated for > 24, 4, 0.5, 24 and 24 hrs, respectively. However, by increasing the MM extract concentration to 50 mg/mL, the SC, PA, RK3C, HS09A and GN9B were killed at shorter periods of time, which were 12, 0.5, 0.5, 8 and 12 hrs, respectively. Based on overall results, this finding indicated MM extract at the concentration of 50 mg/mL was effective to control the viability of all eight pathogens.

4. Discussion

Due to negative impacts from the use of antibiotics, scientists had been working very hard in the search for substitutes to antibiotics. The utmost important criteria are the issue of antimicrobial resistance. In the long run

Table 3. The MBC and MIC of crude extract of MM from aqueous, hot aqueous and 80% ethanol extraction against different clinical mastitis pathogens.

Pathogens	Type solvent MBC±SE (mg/ml) MIC±SE (mg/ml) (MBC / MIC ratio) Bactericidal (BC); Bacteriostatic (BS)		
	Aqueous	Hot aqueous	80% ethanol
Staphylococcus aureus (SA)	12.50±0.00 6.25±0.00 (2) (BC)	6.25±0.00 3.12±0.00 (2) (BC)	3.123±0.00 1.56±0.00 (2) (BC)
Staphylococcus chromogenes (SC)	100.00±0.00 50.00±0.00 (1.5) (BC)	50.00±0.00 25.00±0.00 (2) (BC)	50.00±0.00 25.00±0.00 (2) (BC)
Staphylococcus haemolyticus (SH)	6.25±0.00 3.12±0.00 (2) (BC)	6.25±0.00 3.12±0.00 (2) (BC)	12.50±0.00 6.25±0.00 (2) (BC)
Sreptococcus uberis (SU)	3.12±0.00 1.56±0.00 (2) (BC)	3.12±0.00 1.56±0.00 (2) (BC)	3.12±0.00 1.56±0.00 (2) (BC)
Pseudomonas aeruginosa (PA)	25.00±0.00 12.50±0.00 (2) (BC)	12.50±0.00 6.25±0.00 (2) (BC)	12.50±0.00 6.25±0.00 (2) (BC)
Streptococcus agalactiae (RK3C)	6.25±0.00 3.12±0.00 (2) (BC)	3.12±0.00 1.56±0.00 (2) (BC)	3.12±0.00 1.56±0.00 (2) (BC)
Klebsiella pneumonia (HS09A)	50.00±0.00 25.00±0.00 (2) (BC)	25.00±0.00 12.50±0.00 (2) (BC)	50.00±0.00 25.00±0.00 (2) (BC)
Escherichia coli (GN9B)	50.00±0.00 25.00±0.00 (2) (BC)	25.00±0.00 12.50±0.00 (2) (BC)	25.00±0.00 12.50±0.00 (2) (BC)

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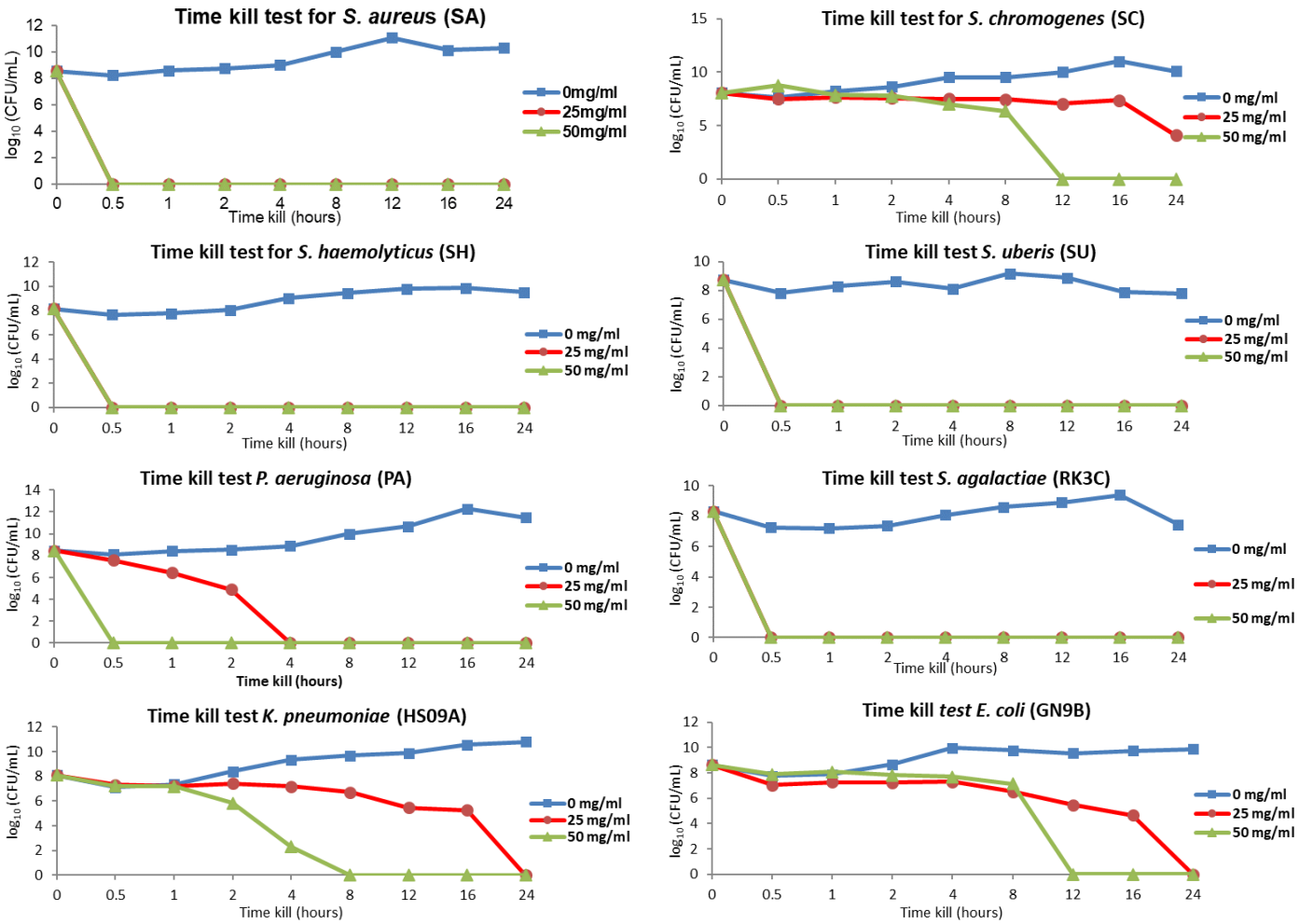

Figure 2. Time kill curves of 25 and 50 mg/mL of MM hot aqueous extract against bovine mastitis pathogens.

of antibiotics application, it was proven that bacteria tend to develop antimicrobial resistance in mastitis treatment's drugs (Gupta *et al*., 2020). Besides, in drug application on the livestock, there will be a withdrawal time between the administrations and slaughter or milking for human consumption period. The period varied according to the type of products. In dairy industries, farmers used to have several days of withdrawal period after application of mastitis antibiotics before the milk was allowed to drink or sell (Martins *et al*., 2016).

Comparatively, plant based antimicrobial and plant essential oil, which have varied modes of actions by the presence of multiple active compounds, which is less likely to stimulate resistance in bacteria when compared to the antibiotics that are mainly isolated specific active compound (Cos *et al*., 2006; Langeveld *et al*., 2014). Interestingly, plants can modulate the immune response, apoptosis, and signal transduction from time to time, as they have the ability to prevent protein interaction (Koehn and Carter, 2005). Bacteria have the difficulties to develop resistance in complex phytochemicals in plant extracts as plant phytochemicals will change accordingly to adapt when the surrounding change in order to survive under conclusive environment. One such plant, MM is a well-known medicinal herb for its antimicrobial (Choudhury *et al*., 2011), anti-inflammation (Mazura *et al*., 2007) and antioxidant activities (Kumar *et al*., 2013). However, MM is not being explored yet for bovine mastitis study and its antimicrobial properties against some mastitis pathogens were investigated in this study.

4.1 Melastoma malabathricum leave crude extracts

The effectiveness of antimicrobial properties of crude MM extracts were compared among three different extraction methods, namely aqueous, hot aqueous and 80% ethanol. The yield of hot aqueous extraction is similar to the yield of 80% ethanol extraction but showed higher yield than aqueous extraction technique (Table 1). During hot aqueous MM extraction, the temperature of water was increased to manipulate the dielectric constant to imitate organic solvents. The physicochemical properties change significantly as the temperature increases. The dielectric constant, viscosity, and surface tension decrease, while the diffusion coefficient is improved (Zhang *et al*., 2020). The water dielectric constant (ε = 80) was decreased to value nearer to ethanol (ε = 24) (Vergara-Salinas *et al*., 2015). The changes of the dielectric constant make it capable of extracting more constituents from MM, thus contributing to a better extraction process (Zhang *et al*., 2020). This

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characteristic of subcritical water allows it as the sole extraction fluid without the involvement of harmful or costly co-solvents, such as methanol, and acids. Considering its cost-effectiveness, environmental benign, non-toxic, non-flammable, and pollution prevention, for living beings, hot aqueous extraction was the best solution recommended in the study. This technique maintains the processes as green chemical extraction, as it only involves aqueous as nontoxic solvent. Solvents such as methanol, ethanol, n-hexane, petroleum ether, diethyl ether, chloroform, ethyl acetate, and glycerol are frequently used to speed up the extraction process and decrease extraction time, but these organic solvents are flammable, volatile, costly, and harmful (Cheng *et al.*, 2021), which are not advisable to use.

4.2 Mastitis pathogens and antibacterial study

The present study found that Gram negative bacteria were particularly more resistant to hot aqueous MM extract when compared to Gram positive bacteria. In fact, almost all Gram positive pathogens in the study (SA, SH, SU and RK3C), were killed within the first 30 mins at the concentration of 25 mg/mL, as opposed to Gram negative pathogens (PA, HS09A and GN9B) which took 4-24 h (Figure 2). This phenomenon can be explained by the presence of the hydrophilic surface at the outer membrane of Gram negative bacteria, which is composed of lipopolysaccharides compounds provide a barrier to penetration. Furthermore, the presence of enzymes in the periplasmic space can break down any unknown molecule from outside. The presence of outer membrane permeability barrier of Gram negative bacteria limits the access of the antimicrobial agents to the target in the bacterial cell (Tavares *et al*., 2020) and provides an explanation on why Gram positive bacteria are more sensitive than Gram negative bacteria.

Based on the chromatography analysis from previous studies, many bioactive compounds of MM have identified includes flavonoids, saponin, tannins, steroid, terpenoid and some detected alkaloid (Sarbadhikary *et al*., 2015; Hainil *et al*., 2021; Mayasari *et al*., 2021). The presence of hydroxyl groups in different rings of flavonoid compounds in MM extract was reported efficiently against Gram positive bacteria (Alwash *et al*., 2013). This might explain why the extracts are more effective against Gram positive bacteria (Table 3). The interaction depends on the position and number of hydroxyl groups. The flavonoid derivative in the hydroxyl group in the β ring is more active against microorganisms than in the 2-hydroxyl group (Maftuch *et al*., 2016), which suggests that the target of this component is a lipophilic compound that can penetrate through bacterial membrane. Flavonoids can form complexes with soluble extracellular proteins or bacterial cells and reduce the membrane fluidity of bacteria cells in the upper part of the membrane by entering the interior of lipid bilayers in the inner membrane, subsequently damage the cell wall membrane and lysed the cells (Tsuchiya and Iinuma, 2000; Ulrih *et al.*, 2010; Bilal *et al*., 2017). Through the microscopic images, Alwash *et al.* (2013) revealed that the shapes and membranes were badly ruptured, with the loss of integrity after being treated by flavonoids.

Saponins, another common bioactive component found in MM. It has strong haemolytic agents and exhibits soap-like properties (Hainil *et al*., 2021). Saponins increase the permeability of bacteria cell membranes by binding to the outer membrane (Jacob *et al*., 1991; Arabski *et al*., 2009) and degrading the cell wall, subsequently disrupting the proteins and cytoplasm membrane. Eventually, bacteria will be killed after the cell content being exposed as a consequent of cell wall degradation (Dong *et al*., 2020). On the other hand, the antimicrobial metabolism of tannins is associated with microbial adhesion inactivation, whereby the enzyme cell enveloped transport protein, caused toxicity in bacterial filaments, and binds to protein walls to inhibit bacterial growth (Pandey and Kumar, 2013). Tannins could pass through the cell wall to reach the internal membrane of bacteria and interfere the cells metabolisms, and ultimately destroy the bacterial cell. Besides, tannins also inhibit the adhesion of bacteria to surfaces (Vance *et al*., 2011). The lack of the surface attachment will result in bacteria cell death. In addition, bacteria cell growth is limited by tannins by amino acid inhibition and sugar uptake (Pandey and Negi, 2017). Alkaloids, another antibacterial agent, can intercalate with double helix DNA and uncouple respiration (Bilal *et al*., 2017). This component can also interfere with the integrity of the peptidoglycan component of bacterial cells and cause killing effect (Hainil *et al*., 2021).

Staphylococcus, such as SA, SC and SH are common mastitis pathogens. SA is well known as toxin-producing mastitis pathogens that destroys the cell membranes and has the ability to destroy milk-producing tissue. Besides, SA mediate the antioxidant enzyme, such as superoxide dismutase, pigment and catalase to neutralize reactive oxygen species (ROS) and reactive nitrogen species (RNS) from host body (Foster, 2005), as well as to degrade the antimicrobial protein (aureolysin and staphylokinase) (Sieprawska-Lupa *et al*., 2004; Jin *et al*., 2004). Alwash *et al.* (2013) reported similar research finding with MIC 1.56 mg/mL of methanol crude extract. This was in line with our study, which reported the MIC value of 1.56±0.00 mg/mL for ethanol MM extract. However, two other studies reported higher MIC value

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using methanol extracts, which were 3.0-7.0 mg/mL (Sunilson *et al*., 2008) and 6.25 mg/mL (Das *et al*., 2021) when compared to our hot aqueous MM extract (3.12±0.00 mg/mL). Besides, *S. agalactiae* was inhibited with 1.25 mg/mL of ethanol MM extract (Alnajar *et al.,* 2012). The hot aqueous and 80% ethanol extraction used in this study also showed potent killing towards the RK3C with the MIC value of 1.56±0.00 mg/mL. Past studies showed that methanol and ethanol have been extensively used as extraction solvents for various plants and plant-based foods. In our current study, hot aqueous MM extract showed the same inhibition effect at the similar or lower concentration against pathogenic microbes.

Other Gram negative pathogens, such as *Pseudomonas* and *Escherichia*, are also commonly found in mastitis. Gram negative bacteria are hardly to be killed by antibacterial agents due to the presence of their outer membrane that consists of lipopolysaccharides compounds (Taveres *et al*., 2020). In this study, the hot aqueous and ethanol MM extract showed a MIC value of 6.25±0.00 mg/mL against PA. However, methanol crude extract from Alwash *et al*. (2013)'s study showed inhibition effect at a low MIC value of 1.56 mg/mL, While Sunilson *et al.* (2008) reported higher MIC value of methanol extract (8.0±0.0 mg/mL) as opposed to this study. *Escherichia,* especially *E. coli* is responsible for a high proportion in clinical mastitis cases (Verbeke *et al*., 2014). It was found highly in organic material such as bedding and manure, which indicated *E. coli* is one of the most commonly environmental pathogens in mastitis (Rangel, 2009; Liu *et al*., 2018). On the other hand, our study showed higher MIC value (12.50±0.00 mg/mL) of GN9B using hot aqueous MM extract when compared with the experiment done by Das *et al*. (2021) using methanol extract (MIC: 3.125 mg/mL) in inhibiting the growth of *E. coli*. Nevertheless, the MM crude extract still proven comparable effective and some showed better in MIC value when compared with other similar studies.

Our MM crude extract showed potential bactericidal effect for all tested pathogens. MM using aqueous extraction was less effective and recorded the highest value of MIC/MBC for SA (MIC/MBC: 6.25/12.50 mg/mL), SC (MIC/MBC: 50.00/100.00 mg/mL), PA (MIC/MBC: 12.50/25.00 mg/mL), RK3C (MIC/MBC: 3.12/6.25 mg/mL), HS09A (MIC/MBC: 25.00/50.00 mg/mL), and GN9B (MIC/MBC: 25.00/50.00 mg/mL). MM crude extract using 80% ethanol was more effective in killing SA (MIC/MBC: 1.56/3.12 mg/mL) compared to the other extracts. In contrast, SH and HS09A (MIC/MBC: 3.12/6.25 mg/mL and 12.5/25.00 mg/mL, respectively) was more susceptible to hot aqueous MM extract. Overall, hot aqueous and 80% ethanol MM extracts have similar MIC and MBC reading to cause potent killing effect on SC (MIC/MBC: 25.00/50.00 mg/mL), SU (MIC/MBC: 1.56/3.12 mg/mL), PA (MIC/MBC: 6.25/12.50 mg/mL), RK3C (MIC/MBC: 1.56/3.12 mg/mL), and GN9B (MIC/MBC: 12.50/25.00 mg/mL). This study finding has suggested that both hot aqueous and 80% ethanol MM extract were comparable effective and showed better inhibition effect than aqueous MM extract. Therefore, crude extract from hot aqueous extraction was chosen to be proceeded to time kill assay since it was the best choice as non-toxic extraction when compared to other organic solvents.

4.3 Time kill study

In this time kill study, hot aqueous MM extract was chosen to determine the optimal time of causing 100% inhibition among all mastitis pathogens tested. Generally, seven of the eight pathogens (except SC), were killed within 24 hrs at the dosage of 25 mg/mL of hot aqueous MM extract (Figure 2). However, a higher concentration is needed to inhibit all the pathogens that might exist in the intramammary glands. Therefore, 50 mg/mL of hot aqueous MM extract was suggested to be used in a time kill test to check on its efficacy level. As a comparison, pathogens such as SA, SH, SU, PA, and RK3C were killed almost immediately within the first 30 minutes after exposed to hot aqueous MM extract. However, HS09A was only killed after eight hours of exposure, while none of SC and GN9B grew after 12 h of incubation. In general, the time kill assay concluded that all mastitis pathogens can be killed within 12 h using 50 mg/mL of hot aqueous MM extract. In other words, hot aqueous MM extract exhibits great potential as a plant-based substitute for mastitis drugs with the recommendation dosage of 50 mg/mL at the frequency of 12 to 24 hourly treatments.

4.4 Bacteriostatic and bactericidal

All this while, scientists believed bactericidal agents are more effective in controlling pathogens as opposed to bacteriostatic agents, stopping bacteria from reproducing instead of killing them. However, almost all antibacterial agents are potential to be both bacteriostatic and bactericidal. A report by Wald-Dickler *et al.* (2018) indicated that bacteriostatic agents can play a role in bactericidal at a higher concentration. Therefore, the evidence showed that both agents are similar in terms of efficacy in human medicine (Wald-Dickler *et al*., 2018). In the case when bactericidal are more effective, it is more likely that bacteriostatic agents are inadequate dosing for the infection. On the other hand, if the bacteriostatic was chosen, that would usually be more cost effective than the bactericidal agent.

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5. Conclusion

Extracts of MM, a traditional medical plant can be easily grown at biodiversity rich areas in Malaysia, provide a potential source of natural antibacterial agents for bovine mastitis treatment. At the concentration of 50 mg/mL, MM extract has been shown to effectively inhibit a wide range of common mastitis pathogens within 12 hrs in-vitro. Furthermore, the extract can be obtained through hot water extraction, make it more compatible to be applied on dairy cows. Therefore, it was suggested that this effective concentration can be used for field testing on real mastitis-infected dairy cow models.

Conflict of interest

The authors declare no conflict of interests.

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References

Alnajar, Z.A.A., Abdulla, M.A., Ali, H.M., Alshawsh, M.A. and Hadi, A.H.A. (2012). Acute toxicity evaluation, antibacterial, antioxidant and immunomudulatory effects of *Melastoma malabathricum*. *Molecules*, 17, 3547-3559. https://doi.org/10.3390/molecules17033547

Ahmed, S.R., Roy, R., Romi, I.J., Hasan, M., Bhuiyan M.K.H. and Khan, M.M.M. (2019). Phytochemical Screening, Antioxidant and Antibacterial Activity of Some Medicinal Plants Grown in Sylhet Region. *Journal of Pharmacy and Biological Sciences*, 14(1), 26-37.

Alwash, M.S., Ibrahim, N. and Ahmad, W.Y. (2013). Identification and mode of action of antibacterial components from *Melastoma malabathricum* Linn Leaves. *American Journal of Infectious Diseases*, 9 (2), 46-58. https://doi.org/10.3844/ajidsp.2013.46.58

Ameen, F., Reda, S.A., El-Shatoury, S., Riad, E.M., Enany, M.E. and Alarfaj, A.A. (2019). Prevalence of antibiotic resistant mastitis pathogens in dairy cows in Egypt and potential biological control agents produced from plant endophytic actinobacteria. *Saudi Journal of Biological Sciences*, 26(7), 1492-1498. https://doi.org/10.1016/j.sjbs.2019.09.008

Apridamayanti, P., Sari, R., Rachmaningtyas, A. and Aranthi, V. (2021). Antioxidant, antibacterial activity and FICI (Fractional Inhibitory Concentration Index) of ethanolic extract of *Melastoma malabathricum* leaves with amoxicillin against pathogenic bacteria. *Nusantara Bioscience*, 13(2), 140-147. https://doi.org/10.13057/nusbiosci/n130202

Arabski, M., Wasik, S., Dworecki, K. and Kaca, W. (2009). Laser interferometric and cultivation methods for measurement of colistin/ampicilin and saponin interactions with smooth and rough of Proteus mirabilis lipopolysaccharides and cells. *Journal of Microbiological Methods*, 77(2), 178-183. https://doi.org/10.1016/j.mimet.2009.01.020

Bilal, M., Rasheed, T., Iqbal, H.M.N., Hu, H., Zhang, X. and Wang, W. (2017). Macromolecular agents with antimicrobial potentialities: A drive to combat antimicrobial resistance. *International Journal of Biological Macromolecules*, 103, 554-574. https://doi.org/10.1016/j.ijbiomac.2017.05.071

Cheng, J., Qu, W., Barkema, H.W., Norbrega, D.B., Gao, J., Liu, G., Buck, J.D., Kastelic, J.P., Sun, H. and Han, B. (2019). Antimicrobial resistance profiles of 5 common bovine mastitis pathogens in large Chinese dairy herds. *Journal of Dairy Science*, 102 (3), 2416-2426. https://doi.org/10.3168/jds.2018-15135

Cheng, Y., Xua, F., Yu, S., Du, S. and Yang, Y. (2021). Subcritical water extraction of natural products. *Molecules*, 26, 4004. https://doi.org/10.3390/molecules26134004

Choudhury, M.D., Nath, D. and Talukdar, A.D. (2011). Antimicrobial Activity of *Melastoma malabathricum* L. *Biological and Environmental Sciences*, 7(1), 76-78.

Cobirka, M., Tancin, V. and Slama, P. (2020). Epidemiology and classification of mastitis. *Animals,* 10, 2212. https://doi.org/10.3390/ani10122212

Cos, P., Vlietinck, A.J., Berghe, D.V. and Maes, L. (2006). Anti-infective potential of natural products: how to develop a stronger in vitro 'proof-of-concept'. *Journal of Ethnopharmacology*, 106(3), 290-302. https://doi.org/10.1016/j.jep.2006.04.003

Das, H., Samanta, A.K., Kumar, S., Roychoudhury, P., Sarma, K., Akter, F., Sabudhi, P.K. and Dutta, T.K. (2021). *In vitro* antimicrobial, antibiofilm and antiquorum sensing activity of Indian Rhododendron (*Melastoma malabathricum*) against clinical isolates

of *Escherichia coli* and *Staphylococcus aureus*. *Indian Journal of Animal Research*, 2021, B-4415. https://doi.org/10.18805/IJAR.B-4415

Dingwell, R.T., Kelton, D.F. and Leslie, K.E. (2003). Management of the dry cow in control of peripartum disease and mastitis. *Veterinary Clinics of North America: Food Animal Practice*, 19(1), 235-265. https://doi.org/10.1016/S0749-0720(02)00072-5

Dong, S., Yang, X., Zhao, L., Zhang, F., Hou, Z. and Xue, P. (2020). Antibacterial activity and mechanism of action saponins from *Chenopodium quinoa* wild husks against foodborne pathogenic bacteria. *Industrial Crops and Products*, 149, 112350. https://doi.org/10.1016/j.indcrop.2020.112350.

Erskine, R.J., Wagner, S. and DeGraves, F.J. (2003). Mastitis therapy and pharmacology. *Veterinary Clinics of North America: Food Animal Practice,* 19, 109-138. https://doi.org/10.1016/S0749-0720(02)00067-1

Foster, T.J. (2005). Immune evasion by *staphylococci. Nature Reviews Microbiology*, 3, 948-958. https://doi.org/10.1038/nrmicro1289

Gani, S.S.A., Azahar, N.F., Zaidan, U.H., Bawon, P. and Halmi, M.I.E. (2020). Antioxidant and antibacterial properties of *Melastomataceae* species (*M. Malabathricum*, *M. Hirta*, and *M. Decemfidum*). *Malaysian Journal of Chemistry*, 22(4), 117-124.

Gupta, R., Kumar, S. and Khurana, R. (2020). Essential oils and mastitis in dairy animals: A review. *Haryana Veterinarian*, 59(S1), 1-9.

Hainil, S., Rachdiati, H. and Prawita, D. (2021). Phytochemical Screening and Antibacterial Activity of Senduduk Leaves (*Melastoma malabathricum* L.). In Proceedings of the 2nd International Conference on Contemporary Science and Clinical Pharmacy 2021 (ICCSCP 2021), p 211-213. Amsterdam, Netherland: Atlantis Press International B.V. https://doi.org/10.2991/ahsr.k.211105.031

Hanafiah, R.M., Aqma, W.S., Yaacob, W.A. and Ibrahim, N. (2015). Antibacterial and biofilm inhibition activities of *Melastoma malabathricum* stem bark extract against *Streptococcus mutans*. *Malaysian Journal of Microbiology*, 11(2), 199-206. https://doi.org/10.21161/mjm.13314

Idriss, S.E., Foltys, V., Tančin, V., Kirchnerová, K. and Zaujec, K. (2013). Mastitis pathogens in milk of dairy cows in Slovakia. *Slovak Journal of Animal Science,* 46, 115-119.

Isnaini, I., Budarti, L., Muthmainah, N., Baringgo, D.S., Frisilia, R., Sulistyaningrum, N., Batubara, I.R., Sofiratmi, W. and Renalta, W.D. (2021). Antibacterial activities of ethanol extract of Karamunting (*Melastoma malabathricum* L.) leaf and flowers on *Salmonella typhi*, *Escherichia coli*, *Staphylococcus aureus*. In Proceedings of BROMO Conference (BROMO 2018). Surabaya, Indonesia: The Indonesian Association of Natural Drug Researchers (PERHIPBA).

Jacob, M.C., Favre, M. and Bensa, J.C. (1991). Membrane cell permeabilisation with saponin and multiparametric analysis by flow cytometry. *Cytometry,* 12(6), 550-558. https://doi.org/10.1002/cyto.990120612

Jin, T., Bokarewa, M., Foster, T., Mitchell, J., Higgins, J. and Tarkowski, A. (2004). *Staphylococcus aureus* resists human defensins by production of staphylokinase, a novel bacterial evasion mechanism. *The Journal of Immunology*, 172, 1169-1176. https://doi.org/10.4049/jimmunol.172.2.1169

Jones, G.M., Bailey, T.L. and Roberson, J.R. (1998). *Staphylococcus aureus* mastitis: cause, detection, and control. Retrieved on 22 February 2023 from website: https://www.thecattlesite.com/articles/679/staphylococcus-aureus-mastitis-cause-detection-and-control/

Klaas, I.C. and Zadoks, R.N. (2017). An update of environmental mastitis: Challenging perceptions. *Transboundary and Emerging Diseases*, 65(Suppl. 1), 166-185. https://doi.org/10.1111/tbed.12704

Koehn, F.E. and Carter, G.T. (2005). The evolving role of natural products in drug discovery. *Nature Review Drug Discovery,* 4, 206-220. https://doi.org/10.1038/nrd1657

Kovačević, Z., Radinovi´c, M., Čabarkapa, I., Kladar, N. and Božin, B. (2014). Natural Agents against Bovine Mastitis Pathogens. *Antibiotics*, 10, 205. https://doi.org/10.3390/antibiotics10020205

Krömker, V. and Leimbach, S. (2017). Mastitis treatment -reduction in antibiotic usage in dairy cows. *Reproduction in Domestic Animals*, 52(S3), 21-29. https://doi.org/10.1111/rda.13032

Kumar, V., Ahmed, D.M., Gupta, D.A., Anwar, F. and Mujeeb, M. (2013). Anti-diabetic, anti-oxidant and anti-hyperlipidemic activities of *Melastoma malabathricum* Linn. Leaves in streptozotocin induced diabetic rats. *Complementary and Alternative Medicine*, 13, 222. https://doi.org/10.1186/1472-6882-13-222.

Langeveld, W.T, Veldhuizen, E.J. and Burt, S.A. (2014). Synergy between essential oil components and antibiotics: A review. *Critical Reviews in Microbiology,* 40(1), 76-94. https://doi.org/10.3109/1040841X.2013.763219

Lestari, O.A., Palupi, N.S., Setiyono, A., Kusnandar, F.

and Yuliana, N.D. (2021). In vitro antioxidant potential and phytochemical profiling of *Melastoma malabathricum* leaf water extract. *Food Science and Technology*, *Campinas*, 42, e92021. https://doi.org/10.1590/fst.92021

Liu, G., Ding, L., Han, B., Plepers, S., Naqvi, S.A., Barkema, H.W., Ali, T., Vliegher, S.D., Xu, S. and Gao, J. (2018). Characteristics of *Escherichia coli* Isolated from Bovine Mastitis Exposed to Subminimum Inhibitory Concentrations of Cefalotin or Ceftazidime. *BioMed Research International*, 2018, 4301628. https://doi.org/10.1155/2018/4301628

Maftuch, Kurniawati, I., Adam, A. and Zamzami, I. (2016). Antibacterial Effect of *Gracilaria verucosa* bioactive on Fish Pathogenic Bacteria. *Egyptian Journal of Aquatic Research,* 42(4), 405-410. https://doi.org/10.1016/j.ejar.2016.10.005

Mogana, R., Adhikari, A., Tzar, M.N., Ramliza, R. and Wiart, C. (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. https://doi.org/10.1186/s12906-020-2837-5

Martins, T., Rosa, A.F., Castelani, L., de Miranda, M.S., Arcaro, J.R.P. and Pozzi, C.R. (2016). Intramammary treatment with gentamicin in lactating cows with clinical and subclinical mastitis. *Pesquisa Veterinaria Brasileira*, 36(4), 283-289. https://doi.org/10.1590/S0100-736X2016000400006

Mayasari, D., Murti, Y.B., Sudarsono, S. and Pratiwi, S.U.T. (2021). Phytochemical, antioxidant and antibacterial evaluation of *Melastoma malabathricum* L.: An Indonesian traditional medicinal plant. *Tropical Journal of Natural Product Research*, 5(5), 819-824. https://doi.org/10.26538/tjnpr/v5i5.5

Mazura, M.P., Susanti, D. and Rasadah, M.A. (2007). Anti-inflammatory action of components from *Melastoma malabathricum*. *Pharmaceutical Biology*, 45(5), 372-375. https://doi.org/10.1080/13880200701214797

Mushtaq, S., Shah, A.M., Shah, A., Lone, A.S., Hussain, A., Hassan, Q.P. and Ali, M.N. (2018). Bovine mastitis: an appraisal of its alternative herbal cure. *Microbial Pathogenesis*, 114, 357-361. https://doi.org/10.1016/j.micpath.2017.12.024

Pandey, A. and Negi, P.S. (2017). Phytochemical composition, in vitro antioxidant activity and antibacterial mechanisms of *Neolamarckia cadamba* fruits extracts. *Natural Product Research*, 32(10), 1189-1192. https://doi.org/10.1080/14786419.2017.1323209

Pandey, A.K. and Kumar, S. (2013). Perspective on plant products as antimicrobials agents: A Review. *Pharmacologia*, 4(7), 469-480. https://doi.org/10.5567/pharmacologia.2013.469.480

Petersson-Wolfe, C.S., Mullarky, I.K. and Jones, G.M. (2010). *Staphylococcus aureus* Mastitis: Cause, Detection, and Control. Blacksburg, Virginia, USA: Virginia Cooperative Extension.

Rangel, P. (2009). Analysis of *Escherichia coli* isolated from bovine mastitic milk. *Pesquisa Veterinaria Brasileira*, 29(5), 363-368. https://doi.org/10.1590/S0100-736X2009000500001

Ruegg, P.L. (2009). Management of mastitis on organic and conventional dairy farms. *Journal of Animal Science,* 87(13), 43-55. https://doi.org/10.2527/jas.2008-1217

SAS Institute Inc. (2011). SAS/STAT® 9.3 User's Guide. Cary, North Carolina, USA: SAS Institute Inc.

Sarbadhikary, S.B., Bhowmik, S., Datta, B.K. and Mandal, N.C. (2015). Antimicrobial and Antioxidant Activity of Leaf Extracts of Two Indigenous Angiosperm Species of Tripura. *International Journal of Current Microbiology and Applied Sciences*, 4(8), 643-655.

Scheetz, M.H., Qi, C., Warren, J.R., Postelnick, M.J., Zembower, T., Obias, A. and Noskin, G.A. (2007). *In vitro* activities of various antimicrobials alone and in combination with tigecycline against carbapenem-intermediate or -resistant *Acinetobacter baumannii. Antimicrobial Agents and Chemotherapy*, 51(5), 1621-1626. https://doi.org/10.1128/AAC.01099-06

Sharif, A., Umer, M. and Muhammad, G. (2009). Mastitis control in dairy production. *Journal of Agriculture and Social Sciences*, 5, 102-105.

Sieprawska-Lupa, M., Mydel, P., Krawczyk, K., Wojcik, K., Puklo, M., Lupa, B., Suder, P., Silberring, J., Reed, M., Pohl, J., Shafer, W., McAleese, F., Foster, T., Travis, J. and Potempa, J. (2004). Degradation of human antimicrobial peptide LL-37 by *Staphylococcus aureus*-derived proteinases. *Antimicrobial Agents and Chemotherapy*, 48, 4673-4679. https://doi.org/10.1128/AAC.48.12.4673-4679.2004

Sunilson, A.J., James, J., Thomas, J., Jayaraj, P., Varatharajan, R. and Muthappan, M. (2008). Antibacterial and wound healing activities of *Melastoma malabathricum* Linn. *African Journal of Infectious Diseases*, 2(2), 68-73.

Supre, K., Lommelen, K. and De Meulemeester, L. (2014). Antimicrobial susceptibility profiling of

bovine mastitis pathogens in Flanders, Belgium. *Veterinary Microbiology*, 171(3-4), 374-381. https://doi.org/10.1016/j.vetmic.2014.02.045

Tavares, T.D., Antunesm J.C., Padrão, J., Ribeiro, A.I., Zille, A., Amorim, M.T., Ferreira, F. and Felgueiras, H.P. (2020). Activity of specialized biomolecules against gram-positive and gram-negative bacteria. *Antibiotics,* 9(6), 314. https://doi.org/10.3390/antibiotics9060314

Tsuchiya, H. and Iinuma, M. (2000). Reduction of membrane fluidity by antibacterial sophoraflavanone G isolated from *Sophora exigua*. *Phytomedicine*, 7 (2), 161-165. https://doi.org/10.1016/S0944-7113(00)80089-6

Ulrih, N.P., Ota, A., Šentjurc, M., Kure, S. and Abram, V. (2010). Flavonoids and cell membrane fluidity *Food Chemistry*, 121, 78-84. https://doi.org/10.1016/j.foodchem.2009.12.006

Vance, S.H., Tucci, M. and Benghuzzi, H. (2011). Evaluation of the antimicrobial efficacy of green tea extract (egcg) against *streptococcus pyogenes in vitro. Biomedical Sciences Instrumentation*, 47, 177-182.

Verbeke, J., Piepers, S., Supre, K. and De Vliegher, S. (2014). Pathogenspecific incidence rate of clinical mastitis in Flemish dairy herds, severity, and association with herd hygiene. *Journal of Dairy Science*, 97(11), 6926-6934. https://doi.org/10.3168/jds.2014-8173

Vergara-Salinas, J.R., Cuevas-Valenzuela, J. and Perez-Correa, J.R. (2015). Chapter 3: Pressurized hot water extraction of polyphenols from plant material. In Gupta, V.J. and Tuohy, M.G. (Ed.) Biotechnology of bioactive compounds: Sources and applications, p. 64-101. United Kingdom: Wiley Online Library. https://doi.org/10.1002/9781118733103.ch3

Virto, M., Santamarina-Carcia, G., Amores, G. and Hernandez, I. (2022). Antibiotics in dairy production: Where Is the problem? *Dairy*, 3(3), 541-564. https://doi.org/10.3390/dairy3030039

Wald-Dickler, N.W., Holtom, P. and Spellberg, B. (2018). Busting the myth of "static vs cidal": a systematic literature review. *Clinical Infections Diseases*, 66(9), 1470-1474. https://doi.org/10.1093/cid/cix1127

World Health Organization (WHO). (2015). Global Action Plan on Antimicrobial Resistance. Retrieved from WHO website: https://iris.who.int/bitstream/handle/10665/193736/9789241509763_eng.pdf?sequence=1

Zhang, J., Wen, C., Zhang, H., Duan, Y. and Ma, H. (2020). Recent advances in the extraction of bioactive compounds with subcritical water: A review. *Trends in Food Science and Technology*, 95, 183-195. https://doi.org/10.1016/j.tifs.2019.11.018

