UNIVERSITI PUTRA MALAYSIA

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SERDANG, SELANGOR

2017

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NUR SHAZA ABDULLAH

A project paper submitted to the

Faculty of Veterinary Medicine, Universiti Putra Malaysia

In partial fulfilment of the requirement for the

DEGREE OF DOCTOR OF VETERINARY MEDICINE

Universiti Putra Malaysia

Serdang, Selangor Darul Ehsan

MARCH, 2017

CERTIFICATION

It is hereby certified that we have read this project paper entitled "Antibacterial Effect of *Melastoma Malabathricum*, *Tetracera Indica* and *Ardisia Crispa* against Bacterial Isolates of Subclinical Mastitis in Dairy Cattle", by Nur Shaza Abdullah and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4999 – Final Year Project.

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DEDICATIONS

In the name of Allah, The Most Benevolent, The Most Merciful

Mostly dedicated to:

Zaliha Japar, Peter Kong and friends who helped me along the way.

Love you guys so much.

ACKNOWLEDGEMENTS

Alhamdulillah, first and foremost I am very thankful to Allah SWT for giving me strength to carry out this study. I wish to express my deepest appreciation to my project supervisor, Prof. Madya Dr. Arifah Abdul Kadir for her endless guidance, support and supervision throughout this project.

I would also like to thank Professor Dr. Saleha Abdul Aziz for her guidance and valueble insights to make this project successful. A special mention to Professor Mohammad Ariff Omar for his patience and guidance in statistical analysis for my project.

Thank you to all the staff at Taman Pertanian Universiti, Universiti Putra Malaysia in assisting me for milk sample collection. I am also very thankful to Dr. Sabri, Mr. Azri and all the personnel at Bacteriology Laboratory, Veterinary Lab Service Unit, UPM for helping me in laboratory analysis.

Big thanks also directed to my beloved family for their endless encouragement and unconditional love towards me.

Last but not definitely not least, a big thank you to all my friends and course mates, thank you very much for all the supports and I would cherish every moment.

CONTENTS

LIST OF ABBREVIATIONS

UPM	=	Universiti Putra Malaysia
FPV	=	Faculty of Veterinary Medicine
UVH	=	University Veterinary Hospital
TPU	=	Taman Pertanian Universiti
IMI	=	Intramammary Infections
CMT	=	California Mastitis Test
SCC	=	Somatic Cell Counts
SNF	=	Soli non-fat
FFA	=	Free fatty acids
MIC	=	Minimum Inhibitory Concentration
MBC	=	Minimum Bactericidal Concentration
DMSO	=	Dimethyl sulfoxide
%	=	Percentage
mm	=	Millimetre
mg/mL	=	Milligram/Millilitre
cfu/mL	=	colony forming unit/millilitre
µg	=	microgram
et al.	=	et al. (abbr. Latin) et alii (and others)

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ABSTRAK

Abstrak daripada projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek

KOMPOSISI-KOMPOSISI SUSU LEMBU TENUSU DENGAN JANGKITAN INTRAMAMMARI KLINIKAL DAN SUBKLINIKAL

Oleh

Nur Shaza Abdullah

2017

Penyelia: Prof. Madya Dr. Arifah Abdul Kadir

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Pengeluaran dan kualiti susu boleh merosot dengan jangkitan intramammari (IMI) yang menyebabkan perubahan komposisi-komposisi susu. Kajian ini dijalankan untuk menentukan perubahan dalam parameter komposisi-komposisi susu dan untuk mengaitkan hubungan antara bilangan sel somatik (SCC) dan parameter komposisi-komposisi susu lembu tenusu dengan jangkitan intramammari dan tanpa jangkitan intramammari. Sebanyak 20 ekor lembu tenusu dari Taman Pertanian Universiti (TPU) dan Ladang Angkat (*Foster Farms*) dimasukkan di dalam kajian ini. Ujian California Mastitis (CMT) telah digunakan untuk mengenal pasti jangkitan

inramammari subklinikal dalam lembu tenusu (n=10) manakala lembu tenusu yang sihat (n=10) telah digunakan sebagai kawalan negatif berdasarkan keputusan CMT (negatif, surih) tanpa tanda-tanda jangkitan intramammari klinikal. Malangnya, tidak ada kes jangkitan intramammari klinikal ditemui dalam kajian ini. SCC dan parameter komposisi-komposisi susu (susu lemak, protein, kasein, laktosa, jumlah pepejal, pepejal bukan lemak (SNF), asid lemak bebas (FFA), dan keasidan) telah ditentukan dan kasein, laktosa, SNF dan SCC ditemui akan berbeza dengan ketara antara kawalan dan kumpulan jangkitan intramammari subklinikal. Kasein, laktosa dan SNF juga didapati negatif dikaitkan dengan SCC. Kesimpulannya, perubahan ketara dalam parameter komposisi-komposisi susu boleh ditemui di dalam susu lembu tenusu dengan jangkitan intrammari subklinikal yang seterusnya menjejaskan kualiti susu.

Kata kunci: Jangkitan Intramammari (IMI), parameter komposisi susu, bilangan sel somatik (SCC), Ujian California Mastitis (CMT), kualiti susu.

ABSTRACT

An abstract from the project paper presented to the Faculty of Veterinary Medicine in the partial fulfilment of course VPD 4999 –Final Year Project

ANTIBACTERIAL EFFECT OF *MELASTOMA MALABATHRICUM*, *TETRACERA INDICA* AND *ARDISIA CRISPA* AGAINST BACTERIAL ISOLATES OF SUBCLINICAL MASTITIS IN DAIRY CATTLE

By

Nur Shaza Abdullah

2017

Supervisor: Assoc. Prof. Dr. Arifah Abdul Kadir

Co-Supervisor: Prof. Dr. Saleha Abdul Aziz

Subclinical mastitis is a form of mastitis that does not exhibit any obvious signs on the animal itself. However, it impacts greatly on the economical lost in the dairy industry. To make matters worse, antimicrobial resistance has become a serious threat and herbal alternatives are greatly sought after. In this project, the leaf extracts of *Melastoma malabathricum* (Senduduk), *Ardisia crispa* (Mata Ayam) and *Tetracera indica* (Mempelas) were tested against bacterial isolates *Staphylococcus aureus*, *Staphylococcus hyicus* and *Staphylococcus intermediate*

obtained from milk samples from a total of 14 subclinical mastitis positive cows in Taman Pertanian Universiti, UPM. The Kirby-Bauer method or disc diffusion method shows that all plant extract possess antimicrobial activity with *M. malabathricum* (inihibtion zone= 22.045± 0.828mm) as the most potent extract against all three strains of *Staphyloccocus* spp. followed by *A. crispa* (inhibition zone= 13.303± 0.828mm) and *T. inidica* (inhibition zone= 9.727± 0.828mm). Minimum inhibitory concentration and minimum bactericidal concentration was also recorded in this study; however, the values were found to be not significant. In conclusion, all the extracts exhibit antimicrobial properties against the common bacterial isolates of subclinical mastitis.

Keywords: Subclinical mastitis, *Melastoma malabathricum*, *Ardisia crispa*, *Tetracera indica*

1.0 INTRODUCTION

Mastitis refers to the inflammation of the mammary gland commonly caused by bacterial and mycotic pathogens. The inflammation causaed by the pathogens resulted in decrease of functional capacity, thus resulting in reduced milk yield, unwanted changes in the milk's composition, increased cost of veterinary services and medicine (Ogola *et al*., 2007; Abrahmsén et al., 2014; Ayano et al 2013).

The clinical form of mastitis exhibits abnormalities in the milk, udder and even the animals in severe cases, while the subclinical form will show no apparent signs of local infection or systemic involvement but milk production declined by 10 to 20% with inadmissible effect on its constituents and nutritional value rendering it of deficit quality. (Holdway, 1992). For the subclinical form of mastitis in cows, it can be detected by using the Californian Mastitis Test (CMT) which will react according to the somatic cell count in the milk. This is due to the distinctive characteristic of mastitis where there is an increase in somatic cells, especially leukocytes in the milk (Ranjan *et al.*, 2010).

It is more economical to treat an entire herd with antimicrobial in order to reduce prevalence of mastitis pathogen such as *S. agalactiae*. However, this practice promotes antimicrobial resistance among the pathogen, rendering the organisms to adapt to the antibiotics that are designed to kill them. (Centre for Disease Control and Prevention, 2017).

Thus, treatment using medicinal herbs is sought after as an alternative. The herbs provide a rich source of bioactive molecules that have evolved as a defense mechanism against infection and therefore, has great therapeutic potential (Modi *et*

al., 2012). In addition, according to Sengul *et al*. (2009), medicinal plants are also known to produce certain bioactive molecules which react with other organisms in the environment, inhibiting fungal or bacterial growth.

In this project, three plants with a long history of traditional medicinal usage were chosen. The plants are *Melastoma malabathricum* (Senduduk)*, Tetracera indica* (Mempelas) and *Ardisia crispa* (Mata Ayam). The roots of the plants were used to treat various ailments such as fever, rheumatoids and hypertension (Chong, 2009). Besides *Melastoma malabathricum*, there is no study published to show the antimicrobial properties of the Ardisia cripa and Tetracera indica. (Hamsin *et al*., 2014). *Melastoma malabathricum* was proven to have antimicrobial properties against *S. aureus, S. cerevisiae*, *and F. Oxysporum* (Grosvenor, 1995). Therefore, this study aims to determine the existence of antibacterial activity of selected plants against to the most common bacterial isolates from subclinical mastitis in cows and to calculate the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the selected plants.

2.0 LITERATURE REVIEW

2.1 Subclinical Mastitis

In Malaysia, the prevalence of subclinical mastitis is very high as shown by Marimuthu *et al*. (2014), which is about 70 -100% around Selangor area. Earlier studies done by Othman and Bahaman (2005) revealed that the total prevalence of subclinical mastitis of farms in Selangor and Johor area was 81.7%. According to Wilson *et al.* (1997), subclinical mastitis leads to significant proportion (20–25%) of the load of mastitis in modern dairy management. The fact that sub clinically infected mammary gland quarters can form clinical mastitis is also a concern (Zdunczyk *et al*., 2003).

The existing treatment regime for mastitis includes intramammary infusions that consist of amoxicillin, penicillin and erythromycin and an option of systemic administration of tetracycline for a period of 7-10 days to allow effective killing of the pathogen (Merck Veterinary Manual, 2010). Although antimicrobial therapy improves animal health and well-being, the economic losses associated with additional labour costs and discarded milk are significant (Erskine *et al*., 2003). Specifically, economic losses are due to (a) fall in milk production, (b) degrading of milk quality and price due to increase somatic cell count (SCC) or bacteria, (c) discarding atypical milk and milk withheld from cows treated with antibiotics, (d) cost of drugs, (e) veterinary services and increased labour price, (f) increased risk of developing mastitis, (g) herd replacement, and (h) problems related to antibiotics remnant in milk and its commodity (Harmon, 1994).

2.2 California Mastitis Test (CMT)

CMT is used as a reliable and rapid screening test for subclinical mastitis that can be used to accurately forecast the somatic cell count of milk from individual quarters or on composite milk samples (Leslie *et al.,* 2002). However, the bacteriological culture of milk samples remains as the standard method for analysing intramammary infection (Mellenberger, 2001). Other various diagnostic methods can be done to diagnose subclinical mastitis such as Somatic Cell Count (SCC), Modified White Side Test (MWT), chlorine, pH, and catalase tests.

Mainly, CMT was selected in many investigations and researches because it is more efficient and decisive than other field and chemical tests for diagnosis of subclinical mastitis (Behera and Dwivedi, 1992). El-Attar *et al.* (2002) noticed that the CMT is able to detect higher cases of subclinical mastitis rather than MWT while Park *et al*. (1982) reported that CMT was in a better agreement with bacteriological results. Based on a study by Kaşikçi *et al.* (2012), there is a high similarity between CMT and SCC in the detection of subclinical mastitis. As mentioned by Dingwell (2003), the sensitivity (82.4%) and specificity (80.6%) of a positive CMT were optimum on the 4th day of lactation by comparing with the bacteriological culture of individual milk samples.

According to Radostits *et al*. (2005), when CMT reagent and the milk are mixed in equal amount (2-3mL), the reagent will disrupt or dissolve the outer cell wall, which consist primarily of fat and the nuclear cell wall of any leucocyte. Then, the DNA is immediately released from the nuclei and will string or gel together to form a stringy mass. As the number of leucocytes increases in a quarter, the amount

of gel formation will increase with a linear relation.

Leukocytes present in the milk are considered somatic cells and the consolidation of somatic cells in milk is referred as somatic cell count (SCC). A higher SCC indicates a higher level of inflammation. Since diapedesis of leukocytes is localized, only the udder quarter that is contaminated with pathogens will have a significant increase in the concentration of leukocytes.

CMT has also been investigated as a tool to detect cows for selective dry cow therapy due to its ability to correctly identify 75% - 80% of infected cows depending on type of pathogens infecting the udder and teats (Ruegg and Reineman, 2002). Furthermore, CMT could also be a fast and cost effective side test to indentify intramammary infection before the cow reaches high production phase as studied by Dingwell *et al*. (2004).

2.3 Bacterial Isolates

Mastitis is a complicated disease that depends on multiple factors and variables related to the animal, environment and pathogen (Radostits *et al*., 2006). Although mycotic pathogen is possible, bacterial pathogen remains the most common cause of mastitis, which is broadly distributed in the environment of dairy cows, hence is a common threat to the mammary gland (Bradley, 2002). Table 1 illustrates the different bacterial isolates in clinical and subclinical mastitis:

Mastitis isolates	Subclinical mastitis isolates
1. *Staphylococcus aureus*	1. *Staphylococcus aureus*
2. *Streptococcus agalactiae*	2. *Escherichia coli*
3. *Escherichia coli*	3. *Streptococcus agalactiae*
4. Klebsiella pneumonia	4. *Staphylococcus uberis*
5. Coagulase-negative staphylococci (CNS)	5. *Bacillus sp.*
6. *Streptococcus dysagalactiae*	6. *Corynebacterium sp.*

Table 1: Common bacterial isolates from mastitis and subclinical mastitis in dairy cattle. (Demme & Abegaz, 2015; Marimuthu *et al.,* 2014; Ragi *et al.,* 2014; Zaryehun *et al.,* 2013; Mubarack *et al.,* 2011)

Although there is a slight difference of the bacterial isolates in clinical and subclinical mastitis, the most common bacteria isolated from both clinical and subclinical mastitis is *Staphylococcus aureus* (Hussein, 2012; Riekerink *et al*., 2008; Ferguson *et al*., 2007; Pitkala *et al*., 2004). The organism has been reported as a causative agent of more than 80% intramammary infection within the US dairy herds (Roberson *et al*., 1998). Heifers were also reported to be susceptible to intramammary infection by *Staphylococcus aureus* (Oliver *et al*., 2005).

Besides the ability to produce various toxins and enzymes to destroy mammary tissue, *Staphylococcus aureus* has proven to be a successful pathogen by evading host immune system by resisting phagocytic cells such as macrophages. It is also able to survive in keratin of the teat canal of healthy cows, which is supposed to inhibit bacterial growth (Green and Bradley, 2004).

2.4 Antimicrobial Resistance in Mastitis

As mentioned earlier, antibiotic is commonly used in treating subclinical mastitis. However, unsupervised administration of antibiotics within dairy herd resulted in presence of prohibited antibacterial residues in marketed milk due to failure to adhere to the withdrawal period as reported by Erskined (1996). This situation greatly promotes antimicrobial resistance (AMR) within milk pathogen (White, 1999). According to Berkema *et al*. (2006), low cure rates are due to AMR towards *S. aureus*, which is further supported by studies done by Marimuthu *et al.* (2014) and Kalmus *et al*. (2011), where penicillin and ampicillin have high MIC value towards Staphylococcus aureus indicating resistance.

This is due to the production of beta-lactamase by the evolved bacteria. It considered as a major concern due to the fact that foodborne disease outbreak in humans could occur as the result of animal pathogens increasingly developing resistance towards existing antibiotics (Landers *et al.,* 2012). Practices that promote AMR include using the wrong dose, drug or duration as well as continuous usage of antimicrobials towards resistant pathogens (Williams, 2000). Hence, several studies conducted to use herbal plants as an alternative treatment in reducing antibiotic or antimicrobial resistance (Xu *et al.,* 2015; Mussarat *et al.,* 2014).

2.5 Senduduk (*Melastoma malabathricum*)

M. Malabathricum is a shrub that grows in the wild of tropical climate countries with the height of 2.5 meters and is traditionally used as astringent, stomachache, compress wounds, post-partum care, toothache. It's most distinguish features is it's white or purple flower (Meyer, 2001). As reported by Joffry *et al*. (2011), numerous studies have been done regarding the phytochemical contents of different extracts and different part of the plant. In a study by Grosvenor *et al*. (1995), it was found that 70% methanol extract of combined *M. malabathricum* leaf, stem, and flower is effective against *S. aureus*, *S. cerevisiae*, and *F. oxysporum*.

2.6 Mata Ayam (*Ardisia crispa*)

A. crispa is woody a shrub that can reach the height of 3 meters with the distinguishing red, glossy round fruits around 5mm diameter. It is used traditionally among the Malay and Chinese to improve blood circulation, as an anti-poison and anti-diarrhoea (Meyer, 2011). A unique dimeric benzoquinone compound known as ardisiaquinone is known to inhibit bacterial peptidoglycan synthesis *in vitro* (Yang K.L *et al*., 2011).

2.7 Mempelas (*Tetracera indica*)

T. indica is a woody climber that can grow up to 5 meters long. It's distinguishing feature is a white and pink fragrant flower. Traditionally it used to relief itchy skin, reduce blood pressure, cure high fever as well as headaches (Meyer, 2011). According to Do *et al*. (1987), betulinic acid, which is a naturally occurring

pentacyclic triterpenoid was found in high yield from this plant. Betulinic acid has been shown to exhibit a variety of biological activities including inhibition of human immunodeficiency virus (HIV), antibacterial, antimalarial, antiinflammatory, anthelmintic and antioxidant properties (Yogeeswari *et al*., 2005).

3.0 MATERIALS AND METHODS

3.1 Milk Screening

At the time of this study, a total number of 14 cows were milked at Taman Pertanian Universiti (TPU) in Universiti Putra Malaysia, Serdang. The cows were examined for general health and abnormalities of the udder. The milk was then screened using CMT to diagnose presence of subclinical mastitis before the collection of milk. Approximately 3mL of milk was taken directly from each quarter of the udder and was placed in 4 different shallow cups in the CMT paddle along with equal volume of CMT was added as well. The milk and the reagent were mixed together by swirling the paddle in a gentle circular motion in a horizontal plane for a few seconds and gel formation was observed (Quinn *et al*. 1994). The scoring is recorded according to Table 2 below and only CMT scoring of 2+ and 3+ are considered as positive samples (Antunes *et al.,* 2015).

Score	Interpretation	Somatic Cell Count (SCC)
Negative	No gel formation	0 – 200,000
Trace	A slight slime formation	150,000 - 500,000
1+	Distinct slime formation immediately	400,000 - 1,500,000
2+	Formed slime settles at the bottom and side	800,000 - 5,000,000
3+	Formed slime is convex and domed up	>5,000,000

Table 2. CMT reaction and equivalent milk somatic cell counts (SCC) in cattle (Radostits *et al*., 2005).

3.2 Milk Sample Collection

Milk samples were collected in accordance to the National Mastitis Council NMC (1990). Each quarter was first washed with tap water and dried in cases where considerable amount of dirt needs to be removed. Then, each teat end was swabbed with cotton soaked in 70% ethyl alcohol. Approximately 5mL of milk was then collected from subclinical mastitis positive (CMT positive) teats into sterile collection tubes after discarding the first three streams of milk. Milk samples were then transported on ice to Bacteriology and Microbiology Laboratory of the Faculty of Veterinary Medicine, University Putra Malaysia.

3.3 Laboratory Analysis

3.3.1 Isolation and Identification

Primary cultures were obtained by growing sample cultures on blood agar supplemented with 5% equine blood following incubation of the agar at 37°C for 24 – 48 hours in aerobic condition (Quinn et al., 1999). The colonies obtained on the primary culture were further subcultured onto individual blood agar to obtain a pure colony. Gram staining was done to identify bacteria morphology in each colony in order to determine the appropriate biochemical test to be carried out. The result of biochemical tests was then referred to Bacteria Identification Manual.

3.3.2 Extracts Dilution

Methanolic leaf extract of Senduduk (*Melastoma malabathricum*) and hydormethanolic leaf extract of both Mata Ayam (*Ardisia crispa*) and Mempelas (*Tetracera indica*) were obtained from Pharmacology and Toxicology Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia. All the extracts were in paste form and needed to be diluted before use. Dimethyl sulfoxide (DMSO) as a universal solvent combined with sterile distilled water is used to dilute the paste. According to Wadhwani *et al*. (2009), the percentage of DMSO used as the solvent cannot be more than 3% as DMSO may also exhibit antibacterial properties. Thus, a fixed concentration of 2% DMSO is used to dilute the extract to avoid the inhibition of bacteria influenced by DMSO. A stock dilution of 1000mg/mL of *Melastoma malabathricum* and *Ardisia crispa* were prepared while *Tetracera indica* extract was prepared at 500mg/mL as a stock solution due to the difficulty in handling the extract at 1000mg/mL concentration. A blank antimicrobial disc is then soaked with the respective stock solution for 24 hours before subjecting it for disc diffusion test.

3.3.3 Standardization of Isolates

A standard stock of pure culture of selected bacteria was prepared by suspending a full loop of bacteria into 2mL sterile distilled water. It then was compared to 0.5 McFarland's standard, which correspond to bacteria load of approximately 1.0×10^8 cfu/mL. The bacteria must be cultured onto Mueller-Hinton (MH) agar within 15 minutes after it has been standardized to maintain uniform bacterial load.

3.3.4 Kirby-Bauer Method

Kirby-Bauer method or also known as the disc diffusion method is used to test the susceptibility of the bacteria towards the extract. The bacteria which has been standardized to 0.5 McFarland standard to obtain 1.0 x 10^8 cfu/mL was spread on Mueller Hilton (MH) agar by means of sterile swab dipped in the suspension and streaked on the agar plate surface, and the plates were left on the bench for excess fluid to be absorbed (Owhe-Ureghe *et al.,* 2012). Then, the blank antimicrobial discs that have been soaked overnight with the extract were placed on the bacterial seeded MH agar.

For the positive control, a combination of 30µg amoxicillin (20µg) and clavulanic acid (10µg) was used while a blank antimicrobial disc soaked overnight in DMSO 2% was used as the negative control. According to Aibinu (2007), the plate was allowed to stabilize for three hours before being incubated at 37°C for 24 hours. The mean zone of inhibition was measured in mm, for all the individual isolates. The mean of quadruplicate results was calculated.

3.3.5 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The minimum inhibitory concentration (MIC) was determined by using broth dilution method. 100µL of active inoculum of standardized bacterial isolates was mixed with equal volume of a plant extract with a concentration of 100%, 50%, 25%, and 12.5% from the stock extract solution in a 96-well plate. The actual concentration corresponding to each percentage concentration is as follow.

	Percentage concentration	**100%**	**50%**	**25%**	**12.5%**
Actual concentration (mg/mL)	Senduduk	1000	500	250	125
	Mata Ayam	1000	500	250	125
	Mempelas	500	250	125	62.5

Table 3: Actual concentration (mg/mL) of each extract with respect to the percentage concentration.

The 96-well plate is then incubated at 37°C for 24 hours and observed for turbidity as sign of bacterial growth. The lowest dilution with no detectable growth by visual inspection was considered the MIC (Abubakar, 2010). The mean of 4 replicates was then calculated to obtain the average MIC.

As a supplementary test to MIC, the MBC test is done after incubation for

determining the MIC by streaking all the samples out of the 96-well plate onto blood agar to determine the minimum concentration of the extract to kill the bacteria. The blood agar was then incubated at 37°C for 24 hours. After incubation, the concentration at which no visible growth was seen was recorded as MBC (Abubakar, 2010).

3.4 Data Analysis

Data analysis was done using IBM SPSS version 22.0. The mean of SCC and milk compositions parameter between group with and without IMI was compared using Independent sample T-test. The correlation between SCC and milk compositions parameter was determined using Pearson correlation and $P < 0.05$ was considered significant.

4.0 RESULTS

A total of 14 cows were successfully examined and a total of 56 functional teats with no signs of inflammation were screened with California Mastitis Test (CMT). The samples were scored as in Table 4. The prevalence of CMT positive was 30.36% and all 17 milk samples were cultured on blood agar. Out of 17 samples, only 14 milk samples yielded bacterial growth.

CMT score	Negative	Trace	1	2	3
Number of teats	18	4	17	11	6
Total Positive				17	
Prevalence				30.36%	

Table 4: Milk scores using CMT.

4.1 Bacterial Identification

From the 17 primary cultures from the milk sample, a total of 23 pure colony cultures were grown. All the isolates obtained were gram-positives inclusive of 18 catalase positive cocci, 3 catalase positive short rods, a catalase positive rod and a yeast species.

The biochemical test reagents used to determine the bacteria changes depending on the bacterial morphology, catalase test as well as the coagulation test. Table 5 and Table 6 below show the list of biochemical test for catalase positive gram-positive cocci and rods that were carried out in this study, respectively.

However, the *Yeast sp.* was not identified in this study.

Cat	Coa	BB	VP	Mal	Man	ADH	Bacteria identification
+	-	-	-	-	-	+	*S. hyicus*
+	+	+	+	+	+	-	*S. aureus*
+	+	+	-	-	-	-	*S. intermedius*

Table 5: Biochemical characteristic for catalase positive gram-positive cocci

Cat	Urea	Glu	Nit	Suc	BB	Tre	SIM (Motility)	Bacteria identification
+	-	-	-	-	-	-	+	*Actinomyces* sp.
+	+	+	-	-	-	-	-	*Corynebacterium renale*
+	-	+	-	-	+	+	+	*Bacillus* sp.

Table 6: Biochemical characteristic for catalase positive gram-positive rods.

Cat-catalase; Coa-coagulase; BB-blood broth; VP-Voges-Proskauer; Mal-maltose; Man-mannitol; ADH-Arginine DiHydrolase; Glu-glucose; Nit-nitrate; Suc-sucrose; Tre-trehalose; SIM-Sulfur Indole Motility agar; +ve-positive; -ve-negative.

From these various biochemical tests that have been used to identify the species of the isolates, it was found that the *Staphylococcus hyicus* was the most common bacteria isolated with a total of 9 out of 23 isolates as in Table 7. This followed by *Staphylococcus aureus* and *Staphylococcus intermedius* at both 5 and 4 isolates, respectively.

Microorganism	No. of isolates	%
Staphylococcus hyicus	9	39.13%
Staphylococcus aureus	5	21.74%
Staphylococcus intermedius	4	17.39%
Actinomyces sp.	2	8.69%
Corynebacterium renale	1	4.35%
Bacillus sp.	1	4.35%
Yeast species	1	4.35%
Total	**23**	**100%**

Table 7: Frequency of bacterial isolates from CMT positive milk samples.

The colony morphology of *Staphylococcus hyicus* is circular shape with flat surface at about 2mm diameter. The colony has a whitish opaque grey with smooth edge and shiny appearance. *Staphylococcus aureus* has the same characteristic with *Staphylococcus hyicus* but the colony is yellowish and hemolytic. *Staphylococcus intermedius* also seems to be opaque white with low convexity and smooth edges. The colony size is about 2-4mm in diameter and may be larger than *Staphylococcus aureus.*

4.2 Kirby-Bauer method

Three most common bacterial isolates; *Staphylococcus hyicus, Staphylococcus aureus* and *Staphylococcus intermedius* were tested against the plant extracts for antimicrobial properties. Table 8 shows the mean diameter of inhibition zone produced by combination of plant extracts against the three respective bacteria that were selected.

Plant extract	**Inhibition Zone Diameter (mm)**			**Average inhibition Diameter (mm)**
	S. aureus	*S. intermedius*	*S. hyicus*	
Senduduk (1000mg/mL)	19.47 ± 2.60^{a}	23.13 ± 0.70^{e}	23.54 ± 3.53^{i}	22.05
Mata Ayam (1000mg/mL)	12.19 ± 0.80^{b}	12.82 ± 0.60^{f}	14.91 ± 0.71^{j}	13.31
Mempelas (500mg/mL)	8.78 ± 1.54^{c}	10.45 ± 0.54^{g}	9.95 ± 1.20^{k}	9.73
Positive Control (Clavamox)	47.58 ± 3.28^{d}	41.16 ± 1.62^{h}	39.28 ± 3.28^{l}	42.67

Table 8: Mean diameter zone of inhibition produced by plant extracts against respective bacteria and the average inhibition diameter.

Value represent mean ± SEM (n=4). Means with different alphabets (a to i) indicate significant differences among each plant extract towards respective bacteria.

Regardless of the bacteria, the largest mean inhibition zone is produced by the positive control, which is amoxicillin-clavulanic with the mean value of 42.67mm followed by *M. malabathricum* (22.05mm), *A. crispa* (13.31mm) and *T. indica* (9.73mm). On the other hand, 2% DMSO which was used as negative control did not inhibit any bacteria. When analysis of variance (ANOVA) by univariate

approach was done, the mean zone of inhibition of all the plant extracts and the positive control was significantly different ($p<0.05$) against all the bacteria tested.

4.3 MIC and MBC

Bacteria / Plant Extract	*S. aureus*		*S. intermedius*		*S. hyicus*	
	MIC	MBC	MIC	MBC	MIC	MBC
Senduduk (mg/mL)	375.0	218.8	562.5	875.0	250.0	343.8
Mata ayam (mg/mL)	437.5	250.0	343.8	406.3	218.8	781.3
Mempelas (mg/mL)	187.5	78.13	281.3	265.6	109.4	255.2

Table 9: Mean concentration of MIC and MBC for each plant extract against respective bacteria isolated.

As shown in Table 9, the mean concentration for the MIC and MBC was calculated and was subjected to ANOVA statistical test. However, it was found that the result was not significantly different ($P>0.05$) between the extracts against different bacterial isolates.

5.0 DISCUSSION

Out of a total 23 bacterial colonies successfully grown, isolated, and identified from CMT positive samples, *Staphylococcus hyicus* is the most common isolated bacteria in this study with the frequency of 39. 13% followed by *Staphylococcus aureus* with the frequency of 21.74%. This varies slightly with several previous study done by Ayano *et al.* (2013), Saidi *et al.* (2013), and Zeryehun *et al.* (2013), which states that *Staphylococcus aureus* was the most common bacteria isolated from subclinical mastitis in dairy cattle. Although difference in sample size and geographical area could be factors in the variation, Pyöräla *et al*. (2009) stated that coagulase negative streptococci are currently the most commonly isolated microorganisms in cows and heifers in herds.

According to Takeuchi *et al.* (1985), *S. hyicus* commonly found on cow skin and udder. In a study by Roberson *et al.* (1996), *S. hyicus* infections are usually mild and will be persist as subclinical mastitis. On the other hand, *S. aureus* is classified as a chief aetiological agent of mastitis (Sharma and Maiti, 2010; Rahman *et al*., 2010). *S. aureus* do not persist on healthy teat skin but may colonize damaged skin and teat lesions cause damage to the milk producing tissues (Petersson-Wolfe *et al*. 2010). *Staphylococcus intermedius* do not appear to be an important mastitis pathogen but still can be found in milk (Roberson *et al.,* 1996).

Kirby-Bauer method or disc diffusion method has been widely adopted by clinical laboratories six years after its introduction starting from 1972 (Biemer, 1973). This study also agrees with Grosvner *et. al*.(1995) where *M. malabathricum*

extract is effective against *S. aureus* and it can be assumed that all three plants have inhibitory effect against the bacteria tested as all extracts produce significant inhibition zones. However, factors such as precipitation of water-insoluble substances in the disc may influence the inhibition diameter by preventing any diffusion of antimicrobial substances into the agar (Valgas, 2007). Thus, is best to filter the extracts beforehand and complement the agar dilution test with MIC and MBC test.

MIC tests are used by diagnostic laboratories, mainly to confirm antimicrobial resistance and also often used as a research tool to determine the *in vitro* activity of new antimicrobials by determining the MIC breakpoints (Andrews, 2002). MBC determinations are undertaken less frequently and their major use has been reserved for isolates from the blood of patients with endocarditis. Antibacterial agents are usually regarded as bactericidal if the MBC is no more than four times the MIC (French, 2006).

In a previous study done by Abubakar (2010) and Valgas (2007), MIC and MBC test is solely dependent on visual inspection. However, the dark color of the extracts proved to be a challenge to observe for turbidity and bacterial growth particularly in the broth dilution method of the MIC test. A study by Perilla *et al*. (2003) stated that the turbidity of the well might be affected by the presence of sediment; for example, dust from surrounding, ashes from the loop during the procedure or the composition of the extract. MBC results may also be affected by phase of bacterial growth and mode of bacterial inoculation (Taylor *et al*., 1983).

6.0 CONCLUSION AND RECOMMENDATIONS

From this study, the common bacterial isolates from subclinical mastitis of cows in TPU, UPM were *Staphylococcus hyicus, Staphylococcus aureus,* and *Staphylococcus intermedius*. All plant extracts were able to inhibit bacterial growth of the common isolates. M. malabathricum produced the largest inhibition zone between the extracts regardless of bacteria at 22.05mm followed by A. crispa at 13.31mm and lastly is T. indica at 9.73mm. The results of the MIC and MBC test were not significant and should be improved in the future. Thus, it can be concluded that Mempelas, Mata Ayam and Senduduk have potential as new antimicrobial agents to combat the emergence antimicrobial resistance towards the existing antimicrobials.

For future studies, increasing the number of milk sample can be considered to obtain a more accurate bacterial result. The extracts can be filtered to ease handling and reduce experiment disturbances. Furthermore, The active substances in the plant that act as the antibacterial agent can be determined and isolated to produce a more accurate result. For determination of MIC, spectrophotometer can be used to analyse the results rather than visual inspection. Besides that, further studies on toxicity tests are recommended once the exact effective concentration of plant extract has been identified.

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