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SYNERGISTIC EFFECT OF GARLIC AND CLOVE OIL WITH ANTIBIOTICS AGAINST STAPHYLOCOCCUS AUREUS

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SYNERGISTIC EFFECT OF GARLIC AND CLOVE OIL WITH ANTIBIOTICS AGAINST *STAPHYLOCOCCUS AUREUS*

ABDULLAH EHSAN BIN HASHIM

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Synergistic Effect of Garlic and Clove Oil with Antibiotic Against *Staphylococcus Aureus*", by Abdullah Ehsan bin Hashim and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4901 - Project.

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DEDICATION

I dedicate this project to my parents and final year project supervisor, who taught me to think, understand and express, solemnly feel that without their inspiration, able guidance and dedication, I would not be able to pass through the tiring process of this project.

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LIST OF ABBREVIATIONS

%	=	percent
ml	=	milliliter
mm	=	millimeter
g	=	gram
^{o}C	=	degree Celcius
µl	=	microliter
µg	=	microgram
CFU	=	colony forming unit
CFU/ml	=	colony forming unit per milliliter
spp.	=	species
E. coli	=	*Escherichia coli*
S. aureus	=	*Staphylococcus aureus*

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999-Projek Tahun Akhir.

SINERGISTIK EFEK BAWANG PUTIH DAN MINYAK CENGKIH BERSAMA ANTIBIOTIK TERHADAP *STAPHYLOCOCCUS AUREUS*

Oleh

Abdullah Ehsan Hashim

Penyelia: Prof. Madya Dr. Arifah Abdul Kadir

Pembantu penyelia: Dr. Sharina Omar

Kombinasi terapi antara antibiotik telah digunakan beberapa tahun terakhir ini, tetapi penggunaan kombinasi antibiotik dengan tanaman ubat belum dapat dilihat aplikasinya. Terapi gabungan antara antibiotik dan tanaman ubat-ubatan mempunyai potensi besar. Tujuan kajian ini dijalankan adalah untuk menguji potensi antimikrob dan sinergistik efek bawang putih dan minyak cengkih yang digabungkan dengan antibiotik biasa terhadap *Staphylococcus aureus*. Ekstrak tumbuhan disaring bagi menentukan aktiviti antimikrob dengan menggunakan kaedah *dics difusion*. *Minimum Inhibitory Concentration* (MIC) dan *Minimum Bactericidal Concentration* (MBC) dinilai menggunakan kaedah *broth microdilution*. Kedua-dua ekstrak tumbuhan menunjukkan aktiviti antimikrob terhadap *S. aureus*. Nilai MIC ekstrak bawang putih adalah 30% manakalanilai MBC untuk ekstrak bawang putih adalah 65%; dan nilai MBC bagi ekstrak minyak cengkih adalah 75%.

Sinergistik efek ekstrak bawang putih dikesan dalam kombinasinya dengan *neomycin* dan *gentamicin*. Zon perencatan meningkat dengan ketara dalam kombinasi ekstrak bawang putih dengan *neomycin* ($P < 0.05$) dan gentamicin ($P < 0.05$). Tidak terdapat peningkatan zon perencatan yang ketara antara kombinasi ekstrak cengkih dengan semua antibiotik yang diuji, dan juga dengan kombinasi ekstrak bawang putih dengan penicillin G. Peratusan peningkatan zon perencatan antara kombinasi ekstrak bawang putih dengan neomycin dan gentamicin adalah 104% dan 55 %, masing-masing. Kesimpulannya, terdapat potensi antimikrob yang tinggi dari kedua ekstrak tumbuhan dan hanya ekstrak bawang putih yang mempunyai sinergistik efek dengan antibiotik. Sinergistik efek dari gabungan ekstrak bawang putih dan antibiotik dapat menawarkan alternatif kombinasi terapi bagi melawan jangkitan *S. aureus.*

Kata kunci: *Minimum Inhibitory Concentration* (MIC), *Minimum Bactericidal Concentration* (MBC), bawang putih, minyak cengkih, *Staphylococcus aureus*

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4999-Final Year Project.

SYNERGISTIC EFFECT OF GARLIC AND CLOVE OIL WITH ANTIBIOTICS AGAINST *STAPHYLOCOCCUS AUREUS*

By

Abdullah Ehsan Hashim

Supervisor: Prof. Madya Dr. Arifah Abdul Kadir

Co-Supervisor: Dr. Sharina Omar

Combination therapy has been used in the recent years among antibiotics, but the use of combination of antibiotic with medicinal plants are yet to be seen. The combination therapy between antibiotic and medicinal plants has great potential. The aim of the current study was to determine the antimicrobial potential and synergistic effect of garlic and clove oil when combined with common antibiotics against *Staphylococcus aureus*. The plant extracts were screened for their antimicrobial activity using disc diffusion method. The Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) were assessed using broth microdilution method. Both plant extracts exhibited antimicrobial activity to *S. aureus.* The MIC value of garlic extract was 30% while

MBC value for garlic extract was 65%; and the MBC value for clove oil extract was 75%. The synergistic effects of garlic extract were observed in the combination with neomycin and gentamicin. The inhibition zones were significantly increased in combination of garlic extract with neomycin ($P<0.05$) and gentamicin ($P<0.05$). There was no significant increase of inhibition zone between combination of clove extract with all antibiotics tested, and also with the combination of garlic extract with penicillin G. The percentage increase of inhibition zones between combination of garlic extract with neomycin and gentamicin were 104% and 55%, respectively. In conclusion, there is high antimicrobial potential of both plant extracts and only garlic extract had synergistic effect with certain antibiotics tested. The synergistic effect from the combination of garlic extract and antibiotics may offer alternative of combination therapy against *S. aureus* infection.

Keywords: minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), garlic, clove oil, *Staphylococcus aureus*

1.0 INTRODUCTION

Antimicrobial resistance (AMR) has become a major problem in the animal industry. This has raised the alarming possibility of subsequent generation returning to the pre-antibiotic era when common infections such as *Staphylococcus* infection can cause death due to lack of effective treatments. In Malaysia, the cases of AMR infections are continuously increasing (Ministry of Health, 2017). This situation has forced scientists to search for new antimicrobial substances from various sources. A lot of studies have been done to find new potential antigen properties especially from medicinal plants. However, the use of medicinal plants as antimicrobial agents is impractical because they are expensive, produced strong flavour, and toxic at high concentration (Shoe *et. al*, 2015).

Due to the downside of medicinal plants as antimicrobial agent, new alternative approaches need to be used. Combination therapy has been used in the recent years among antibiotics, but the use of combination of antibiotic with medicinal plants are yet to be seen. The combination therapy between antibiotic and medicinal plants has high potential to reduce the dosage of effective dosage, reduce toxicity effects, produce broad spectrum activity, and suppress the emergence of AMR (Chanda *et. al.*, 2011). Only medicinal plants that have synergistic effect with antibiotics can be used in combination therapy. Thus, there is a need to study the synergistic effect of antibiotic with medicinal plants.

Most *in vitro* studies done currently towards the synergistic of antibiotics and active compound of plant extracts which required complicated process of preparation. To

my knowledge, there is no study on the combination of antibiotic with crude plant extracts which can be prepared manually have been conducted. Rawat (2015) discovered that the combination of garlic, ginger and turmeric, respectively with several antibiotics were found to be more potent. Therefore, the objective of this study was to investigate the antimicrobial activity and synergistic effect of garlic extract and clove oil when used in combination with antibiotics against *Staphylococcus aureus.*

1.1 Hypothesis

a) Null hypothesis: There is no significant difference between the antimicrobial activity of plant extracts when used in combination with antibiotics.

b) Alternative hypothesis: There is a significant difference between the antimicrobial activity of plant extracts when used in combination with antibiotics.

2.0 LITERATURE REVIEW

2.1 *Staphylococcus aureus*

Staphylococcus aureus is a non-motile gram-positive, cocci bacteria with moderate sized of white haemolytic colonies. They are facultative anaerobes and can grow on non-enriched media such as Mueller-Hinton agar. They are commonly found on skin and mucous membranes of animals and humans. Many can cause pyogenic infections as a result of trauma, immunosuppression or secondary to other infections.

S. aureus is one of the causative agents of toxic mastitis in cows (Royster and Wagner, 2015). It also among the most common *Staphylococcus spp.* isolated from canine clinical samples (Hauschild and Wójcik, 2007). It can cause deep pyoderma, wound infections, and gastroenteritis in canine. In poultry, it causes "bumble-foot" infection (Youssef *et al*., 2019). In human, *Staphylococcus spp.* are one of the most important food-borne opportunistic bacteria (Albuquenque *et al.,* 2007).

2.2 Garlic (*Allium sativum L.)*

Allium sativum commonly known as garlic, is as species of anion belongs to the *Liliaceae* family (USDA, NRCS- garlic). It is one of the oldest plants to be widely used as a medicine. The bulbs are the most frequent part of the plant to be used in medicine. Reuter *et al*. (1996), described garlic is a plant with various biological properties like antimicrobial, anti-cancer and antioxidant. They also considered having properties such as antiviral, antifungal, expectorant, antiseptic, and antihistamine (Hannan *et al.,* 2011).

The antimicrobial properties of garlic organosulfur compound is known as allicin (Ankri *et al.,* 1999). Allicin is produced from the non-protein amino acid alliin (allyl-cysteine sulfoxide) by the enzyme alliinase. In undamaged garlic cloves, alliin and alliinase are compartmented in the cytoplasm and vacuole, respectively. Allicin is produced upon tissue damage, when the enzyme and substrate mixed (Borlinghaus *et al.,* 2014). Allicin can be further decomposed upon heating into diallyl disulfides and diallyl polysulfanes with up to seven sequential sulfur atoms, which also thiols reactive (Tocmo *et al,* 2017)

2.3 Antimicrobial Activities of Garlic

Allicin and diallyl polysulfanes produced by garlic have found to exhibit a broadspectrum antimicrobial activity against various Gram-positive and Gram-negative bacteria, including multiresistance *S. aureus, Streptococcus pneumoniae, Pseudomonas aeruginosa, Helicobacter pylori, Escherichia coli, Bacillus subtilis and Klebsiella pneumoniae* (Arbach *et al.,* 2019). According to Hannan *et al.* (2011), mechanism of action for allicin is proved to be antimicrobial through RNA synthesis inhibition. Allicin has a wide range of cellular targets because it is a reactive sulfur species which inhibits protein and enzyme synthesis (Van Loi *et al*., 2019). Moreover, because of allicin volatility, the antimicrobial activity is increased as it can easily penetrate the cellular phospholipid membrane (Miron *et al.*, 2000). In *E. coli* infection, allicin had shown to cause heat shock and oxidative stress respond towards bacteria (Mueller *et al.,* 2016). Additionally, a recent study revealed that allicin leads to fragmentation of the

peptidoglycan in *S. aureus*, which inhibits cell wall synthesis and hydrolytic enzymes in bacteria (Getti *et al.*, 2019).

2.4 Clove Oil (*Syzigium aromaticum)*

Clove oil can be obtained from the flower buds of *Syzigium aromaticum,* family Myrtaceae (Chaied *et al*., 2007). Clove oil is commonly used as an anaesthetic in the relieve of toothache and antiseptic in infection (Moon *et al.*, 2011). It is also used as a carminative to increase hydrochloric acid in stomach and improve peristalsis (Phyllis and James, 2000). It is also used in herbal recipes as rubefacient and act as a preservative, and has antimicrobial properties (Odugbemi, 2006). In veterinary practice, clove oil is often used for anaesthetic agent in fishery industry.

Clove oil has biological activities, such as antimicrobial, antifungal, insecticidal and antioxidant (Gill *et al*., 2006). This essential oil has been reported to inhibit the growth of molds, yeasts, and bacteria (Matan *et al*., 2006). Amelia *et al*. (2017) found the major chemical compositions in clove oil are eugenol acetate, eugenol, and caryophyllene. The high levels of eugenol contained in clove oil are responsible for its strong biological and antimicrobial activities (Nunez *et al*., 2012).

2.5 Antimicrobial Activities of Clove Oil

Eugenol in clove oil is primarily responsible either as a bactericidal or bacteriostatic agent to fight against bacterial infections (Bankar *et al.*, 2012). Eugenol has excellent antimicrobial activity against Gram-positive and Gram-negative bacteria

such as *E. coli, S. aureus, Pseudomonas aeruginosa,* and *Listeria monocytogenes* (Mak *et al.*, 2019). It is well known that the mechanism of action of eugenol inhibits protein synthesis and changes the permeability of cell membrane (Walsh *et al*., 2003). However, in another study done by Weavers *et al.* (2001), found that the antimicrobial activities vary with time, temperature, pH and presence of organic matter. The volatility, water-insolubility and chemical instability of eugenol greatly limit its practical applications (Shao *et al.*, 2018).

2.6 Antibiotics

Antibiotics provide the primary basis for microbial infection therapy. Penicillin was discovered by Alexander Fleming in 1928 (Tan & Tatsumura, 2015). Penicillin comprises a beta-lactam ring and a thiazolidone (Rodriquez *et al.,* 2019). Penicillin bind to and inhibit the transpeptidase involved in the cross-linking of the bacterial cell wall, the final steps in cell wall synthesis in bacteria (Lee *et al.,* 2001). Rapid growing bacteria such as *S. aureus* is the most susceptible to penicillin. Penicillin G is effective against gram-positive bacteria (Lowy, 2003). Bacteria that produce beta-lactamases are resistant towards penicillin (Andersen, 1990).

Neomycin and gentamicin are classified under aminoglycoside class of antibiotic (Krause *et al.,* 2016). The aminoglycosides bind to the 30s ribosome and inhibit the rate of protein synthesis which have bactericidal effect (Wimberly *et al.,* 2000). There are effective against gram-negative bacteria (Gonzalez *et al.,* 1998).

3.0 MATERIALS AND METHODS

3.1 Bacteria

Staphylococcus aureus ATCC 29523 was isolated from stock culture of the Microbiology Laboratory of Faculty of Veterinary Medicine, Universiti Putra Malaysia and it was sub-cultured onto blood agar plate.

3.2 Preparation of Plant Extracts

Garlic and clove oil were purchased from local market. Garlic bulbs were peeled, weighed 100g, and cleaned. Clean cloves were crushed in sterile mortar and pestle and then the mixture was filtered through a sterile cheese cloth. Four ml of garlic juice was obtained from 100g of garlic cloves. The clove oil and garlic juice filtrate were considered pure plant extracts. The clove oil was dissolved in 10% aqueous dimethyl sulfoxide (DMSO) with Tween 80. The plant extracts were prepared as 100%, 80%, 50% and 25% concentrations (Appendix 1).

3.3 Antibacterial Susceptibility Testing

The antimicrobial activities of plant extracts were tested using Disc Diffusion method, following procedures stated in the CLSI, 2006. The Minimum Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of the plant extracts were determined using Broth Microdilution method (CLSI, 2010).

3.4 Disc Diffusion Method (Kirby-Bauer Method)

The method was used with reference to the CLSI, 2006.

3.4.1 Agar Medium

Mueller-Hinton Agar (MHA) was used as susceptibility test medium that has been validated by CLSI for this experiment. The agar was prepared according to the manufacturer's recommendation.

3.4.2 Preparation of Plant Extracts Impregnated Discs

Sterile blank discs were impregnated with 10µl of the various concentration of plant extracts. Ampicillin 10µg was used as the positive control, and sterile water was used as the negative control for garlic extract. While DMSO was used as negative control for clove oil.

3.4.3 Preparation of Inoculum

S. aureus was streaked onto the blood agar to obtain isolated colonies. The plate was incubated at 37°C for 24 hours. Using inoculating loop, 4 to 5 well-isolated colonies were selected and suspended into sterile distilled water, and vortexed thoroughly. The turbidity of the bacterial suspension was then compared visually to the 0.5 McFarland standard to get the suspension turbidity equivalent to approximately 1 to 2×10^8 CFU/ml. The tube was compared against a white background with contrasting black lines. The suspensions were used within 15 minutes after adjustments of the turbidity.

3.4.4 Inoculation of Test Plates

Sterile cotton swab was dipped into the suspension. The swab was rotated several times, pressing firmly against the inside wall of the test tube above the fluid level to remove excess fluid from the swab. The swab was streaked over the entire surface of the Mueller-Hilton agar three times, the plate was rotated approximately 60 degrees after each streak to ensure equal distribution of the inoculum. Finally, the edge around the agar plate was swabbed. The inoculated agar plates were allowed to dry five minutes by closing the plate top to ensure there were no excess moisture in the surface of the agar before applying the discs.

The impregnated discs and control discs were applied onto inoculated agar plates using sterile forceps. Each agar plate was allowed placement of up to four discs**.** Plates were incubated at 37ºC for 20 hours. Each test was run in triplicates.

3.4.5 Zone of inhibition

To enhance the visibility of inhibition zones, the plates were placed inverted against a dark background. Following the incubation period, the diameters of the clear zones with no colonial growth were measured using digital calliper. The zones were measured to the nearest millimetre. The results were subjected to the dose response study using Pearson's Correlation Analysis (two-tailed test of significance) and Linear Regression Analysis.

3.5 Broth Microdilution Method

The method was used with reference to the CLSI, 2010.

3.5.1 Preparation of plant extracts

The inhibition zones of the Disc Diffusion method were used as a basis for the determination of MIC and MBC. The concentration that produced 'grey area' was used as the mid-point to determine the range of concentration to test for MIC and MBC. In this range, a series of 7 concentrations were derived (Appendix 2). Sterile distilled water and DMSO were used to dilute the plant juice. In the above method, the control was sterile distilled water and DMSO.

3.5.2 Preparation of Inoculum

The method used was similar to the method described on page 8.

3.5.3 Broth Microdilution Testing

The 96-well plates were prepared by dispensing 100µl of Mueller-Hinton broth (MHB), into each well. 100µl of various extract concentrations were added to each well. Then, 100µl of bacteria inoculum were added to each well except for negative control. 100µl of bacteria inoculum with media was used as a positive control and sterile distilled water and DMSO with media was used as a negative control (Appendix 3). The turbidity of each well was determined before and after incubation period. The 96-well plates were incubated at 37ºC for 20 hours. Each test was run in triplicates.

3.5.4 Minimal Inhibitory Concentration (MIC)

Minimal Inhibitory Concentration (MIC) is the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in Broth Microdilution Susceptibility Test (CLSI, 2010). Due to natural turbidity of plant extracts, the MIC was determined as the lowest concentration showing no turbidity changes after incubation using 96-well reader with the wavelength of 450nm.

3.5.5 Minimal Bactericidal Concentration (MBC)

Minimal Bactericidal Concentration (MBC) is the lowest concentration of an antimicrobial agent that prevents bacterial growth on Mueller-Hinton Agar following the Spread Plate method.

3.5.6 Spread Plate Method

Ten microliters (10µl) of 20-hour culture from each well from 96-well plate was lawned individually onto Mueller-Hinton agar using sterile wire loops. The agar plates were then incubated at 37^{o}C for 20 hours to observe the presence of bacterial growth.

3.5.7 Interpretation of Results

As for MIC, the activity of the plant extracts towards the bacteria is bacteriostatic. While with MBC, the activity of the plant extracts towards the bacteria is bactericidal.

3.6 Synergistic Test Effect

The synergistic effect of plant extract with antibiotics were tested using Disc Diffusion method with reference to Rawat (2015).

3.6.1 Agar Medium

Mueller-Hinton Agar (MHA) was used, as it the only susceptibility test medium that has been validated by CLSI. The agar was prepared according to the manufacturer's recommendation.

3.6.2 Commercial Antibiotics

In synergistic test, the plant extracts were tested with commercial antibiotic discs. The commercial antibiotics were used namely neomycin 30µg, gentamicin 10µg, and penicillin G 10µg.

3.6.3 Selection of Plant Extracts Concentration

The concentration of the plant extracts exhibiting maximum inhibition zones in the Disc Diffusion method were used to test for synergistic effect because they have better diffusion rate on MHA. In this study, 80% concentration of both plant extracts exhibited maximum inhibition zones.

3.6.4 Preparation of Plant Extracts Impregnated Antibiotic Discs

Sterile antibiotic disc was placed on sterile agar plates. Twenty microlitre (20µl) of 80% concentration of the plant extracts were impregnated into each antibiotic disc, respectively (Appendix 4). As for negative control, sterile commercial blank discs were

impregnated with 20ul of sterile distilled water and DMSO, respectively. While for positive control, the antibiotic discs were used.

3.6.5 Preparation of Inoculum and Inoculation of Test Plates

The method used was similar to the method describe on page 8.

3.6.6 Zone of inhibition

The method used was similar to the method describe on page 9. The results were subjected to One-Way Analysis of Variance (ANOVA) to compare the differences of inhibition zones with or without plant extracts combination at 95 % confidence level. Significantly different means were then elucidated using the Tukey's multiple comparison test.

4.0 RESULTS

All extracts and commercial antibiotics exhibited good antimicrobial activity towards *Staphylococcus aureus* (Table 1). The penicillin G exhibited the largest mean diameter of inhibition zones of 34.28 ± 1.69 mm. While 100% clove oil exhibited the smallest mean diameter of inhibition zones of 15.31 ± 1.16 mm. The 100% garlic extract and gentamicin exhibited similar mean diameter of inhibition zones of 23.98 ± 1.82 mm and 23.98 ± 1.07 mm, respectively. The mean diameter of inhibition zones of neomycin (19.44 ± 3.52 mm) was smaller than that of 100% garlic extract (23.98 ± 1.82 mm).

Both extracts exhibited clear inhibition zones at various concentrations against *S. aureus*, excluding 25% concentration (Table 2). Garlic extract produced larger inhibition zones compared to clove oil extract (Figure 2). The diameters of inhibition zones were all concentration-dependent, with significant high positive correlation. The correlation between the diameter of inhibition zones and the concentration of garlic extract was 0.913 ($p < 0.05$). While the correlation between the diameter of inhibition zones and the concentration of clove oil extract was 0.900 ($p < 0.05$), based on the Pearson's correlation test. The Linear Regression Analysis (Appendix 1) suggests that the increase in diameter inhibition zones were due to the increase in the concentration of plant extracts, with R^2 values ranging from 0.833 to 0.810 ($p < 0.05$).

Based on the broth microdilution testing, the Minimal Bactericidal Concentration (MBC) for garlic and clove oil extract were 65% and 75%, respectively. While Minimal Inhibitory Concentration (MIC) was 30% (Table 3). The value of MIC

of garlic extract was determined to be lower than the lowest concentration in the Disk Diffusion method that produced inhibition zones.

The garlic extract at 80% showed synergistic effect with neomycin and gentamicin. The inhibition zones were significantly increased ($p<0.05$) when combined with neomycin and gentamicin (Table 4). While the clove oil extract at 80% showed no synergistic effect when combined with all antibiotics tested. There was no synergistic effect of both plant extracts with penicillin G (Appendix 2). The percentage of increased in diameter of inhibition zones when garlic extract combined with neomycin was 104%, while combination with gentamicin, the inhibition zones increased to 55% (Table 5).

Table 1: Diameter of inhibition zones produced by plant extracts (100%) and commercial antibiotics

Antibiotic / Plant extracts	Inhibition Zones (mm)
Garlic 100%	23.98 ± 1.82
Clove Oil 100%	15.31 ± 1.16
Neomycin 30µg	19.44 ± 3.52
Gentamicin 10µg	23.98 ± 1.07
Penicillin G 10µg	34.28 ± 1.69
Ampicillin 10µg	30.77 ± 0.55

Value are mean ± std deviation.

Table 2: Diameter of inhibition zones produced by plant extracts at various concentration

Plant extracts	100%	80%	50%	25%
Garlic	23.98 ± 1.82	22.35 ± 1.99	16.37 ± 2.71	0
Clove Oil	15.31 ± 1.16	15.93 ± 0.65	11.1 ± 0.61	0

Value are mean ± std deviation.

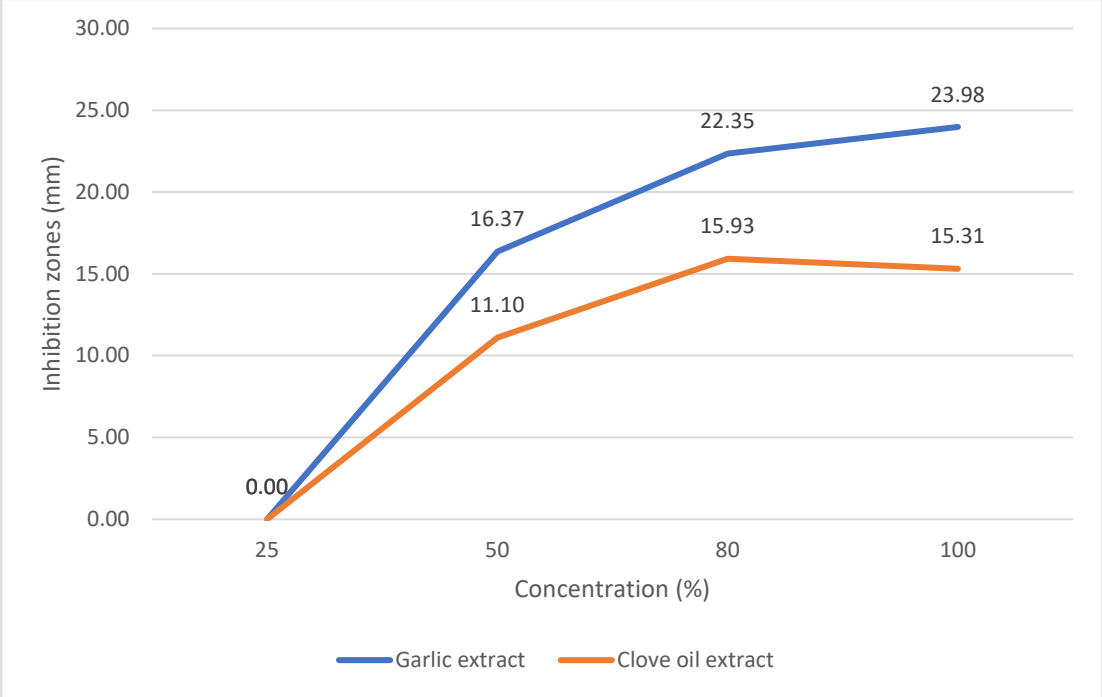

Figure 1: Mean diameter of inhibition zones of plant extracts

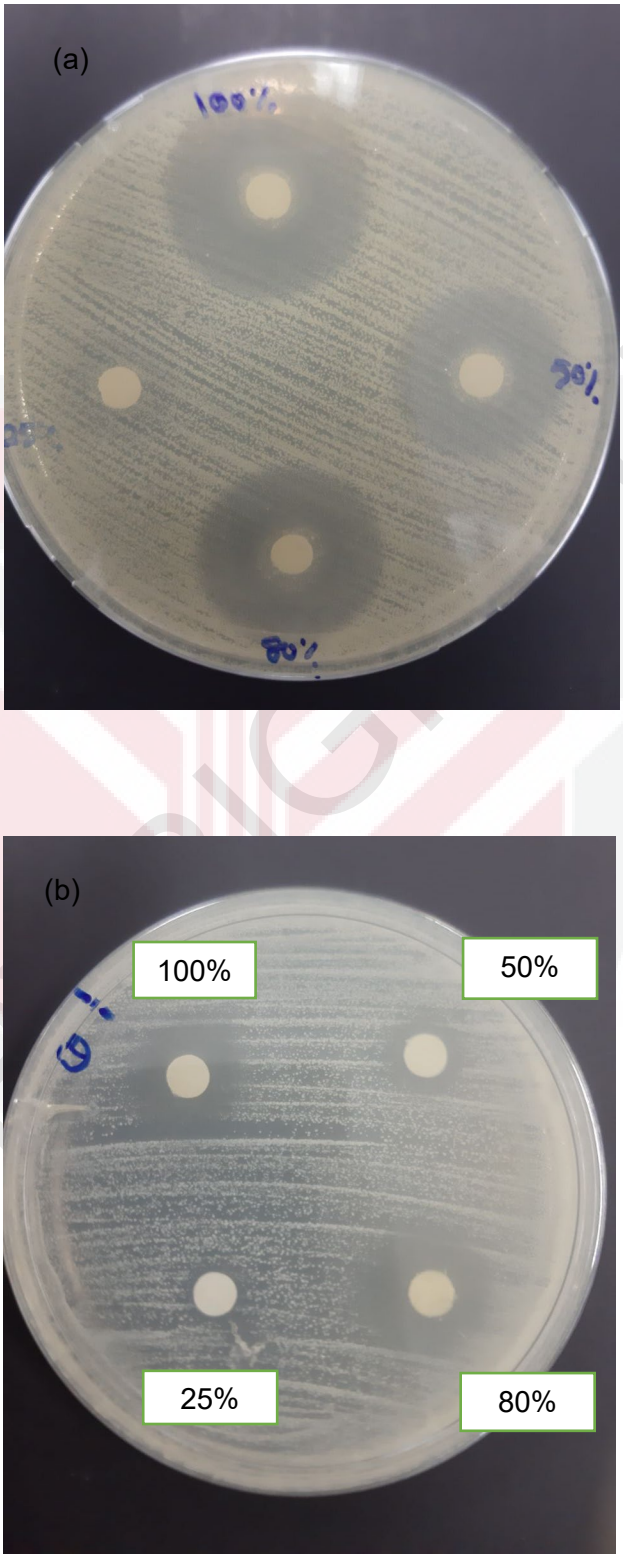

Figure 2: Inhibitions zones produced by garlic extracts (a) and clove oil extracts (b)

Table 3: The minimal inhibitory concentration and minimal bactericidal concentration of plant extracts

Plant Extracts	Minimal Inhibitory concentration (MIC)	Minimal Bactericidal Concentration (MBC)
Garlic	30%	65%
Clove Oil	-	75%

Table 4: Diameter of inhibition zones produced by plant extracts (80%) in combination with commercial antibiotics

Antibiotics	Inhibition Zones (mm)	Antibiotic + Garlic extract 80% (mm)	Antibiotic + Clove Oil extract 80% (mm)
Neomycin 30µg	19.44 ± 3.52	27.10 ± 1.18*	21.19 ± 1.48
Gentamicin 10µg	23.98 ± 1.07	29.68 ± 1.02*	23.73 ± 1.21
Penicillin G 10µg	34.28 ± 1.69	34.79 ± 1.38	34.06 ± 1.96

*$P < 0.05$ when compared to the negative control. Value are mean ± std deviation.

Table 5: The percentage of increase in diameter of inhibition zones when antibiotic combined with plant extracts

Plant extracts	Neomycin 30µg	Gentamicin 10µg	Penicillin G 10µg
Garlic	104%	55%	-7%
Clove Oil	25%	-1%	-11%

$(B^2-A^2/A^2) \times 100$, A = Mean diameter of inhibition zones in the absence of extract, B = Mean diameter of inhibition zones in the presence of extract

5.0 DISCUSSION

In this study, we found that *Staphylococcus aureus* was more sensitive to the inhibitory activities of garlic extract as compared to clove oil at 100% concentration using the disc diffusion method. *S. aureus* was most sensitive to 100% concentration of extract. However, at 25% concentration there were no inhibition zones produced towards *S. aureus*. The steepness of the graph indicates that a small increase in the concentration of garlic extract above 25% leads to greater inhibition zones. This suggests that *S. aureus* is sensitive to the changes in the extract concentration. When garlic extract is used against *S. aureus,* care must be taken to prevent underdosing, because decrease in the extract concentration can lead to ineffective respond towards bacteria. The high efficacy of garlic extract against *S. aureus* indicates that high potential of the extract to treat *S. aureus* infection such as mastitis in ruminants and 'bumble foot' in poultry.

S. aureus was more sensitive to clove oil at 80% concentration compared to the clove at 100% concentration. This indicates that the diluted clove oil had better diffusion rate on Mueller Hinton Agar as compared to pure clove oil. The steepness of the graph (slope 0.202) for concentration of clove oil is smaller than that of garlic extract (slope 0.308). This suggests that *S. aureus* was not as sensitive towards any changes in clove oil concentration as it was with garlic extract. Therefore, care must be taken to prevent under-dosing, because concentration less than 25% will make clove oil become ineffective as an antimicrobial agent. The mean diameter of

inhibition zones produced by garlic extract is higher than clove oil. This suggests that garlic extract has higher antimicrobial activity compared to clove oil. Previous study stated that garlic extract was found to be effective compared to clove extract against pathogenic bacteria (Kumar *et al.,* 2014). These suggest that garlic extract was more efficacious as compared to clove oil extract.

In addition, the MIC and MBC values support the findings of the disc diffusion method. The antimicrobial activity of garlic extract and clove oil against *S. aureus* could be attributed to the presence of allicin and eugenol in the plant extracts, respectively. Allicin is proven to be antimicrobial by inhibiting RNA synthesis (Hannan *et al*., 2011). This also supported by another study which proposed that eugenol inhibits the production of an essential enzyme in bacteria and/or cause damage to the cell wall (Burt, 2004). The area of concern is that MIC values of garlic extract obtained in this study was lower than the MBC values, suggesting that garlic extract was bacteriostatic at lower concentration and bactericidal at higher concentration. However, we are unable to determine the MIC value for clove oil. We speculate that this might be due to insolubility of clove oil extract with water in Mueller Hinton Broth and bacteria suspension in 96-well. According to Jang *et al*. (2019), small oil particles have the tendency to become large particles due to relatively higher insolubility and permeability from oil to water phase, resulting in instability of eugenol and form precipitation. Thus, the condition limits the ability of the 96-well reader to read the turbidity changes in 96-well which later gave inconsistent value of MIC for clove oil.

In the present study, the garlic extract exhibits significant increase of inhibition zones with aminoglycoside antibiotics (neomycin and gentamicin). The percentage of increase in inhibition zone when combined with neomycin is higher (104%) compared to gentamicin (55%). However, the antimicrobial activity of the combination of garlic extract with gentamicin is higher than neomycin. The results of this study are comparable to the results reported by Ivana *et al.* (2012), in which there was synergistic activity between garlic extract with gentamicin, but no synergistic effect with penicillin. The activity can be explained as allicin is able to cause pore formation in membranes and lipid bilayers, which promote the uptake and antimicrobial action (Gruhlke *et al.,* 2015).

In general, there was no significant increase of inhibition zones of clove oil extract with antibiotics tested. The results were in agreement with Monika *et. al* (2017), that there were no significant increases in inhibition zones exhibited by the combination of clove oil with antibiotics when compared to clove oil alone. However, a study done by Sang-Eun *et. al* (2011) using different synergistic test methods found that clove oil had synergistic effect with ampicillin and gentamicin when tested against oral bacteria.

6.0 CONCLUSION

The overall results of the present work provide baseline information for the possible use of studied plant extracts for *Staphylococcus aureus* infections. Garlic and clove oil extract have potential to be used as antimicrobial agent against *S. aureus*, but the effect is dose-dependent. The plant extracts can easily be prepared as they are widely available in local market. Garlic extract can be used a replacement for certain antibiotics especially for topical application, as the present study showed the effectiveness of garlic extract against *S. aureus*. In this study using paper disk diffusion assay, we showed that the antimicrobial activity of gentamicin and neomycin were significantly increased. However, there were no significant increase of antimicrobial activity when antibiotic is used in combination with clove oil. Thus, this study suggests the possibility of using combination therapy of antibiotic with garlic in treating *S. aureus* infections.

7.0 RECOMMENDATION

More research needs to be performed to further support the findings of this study. The present study is limited by the use of only *S. aureus* ATCC 25923 and few antibiotics tested. More strains or field strains need to be incorporated in future studies to determine the effectiveness of both plant extracts as an antimicrobial agent. A wider study can be done using more antibiotics to establish and understand the mechanism of synergy with plant extracts. Here, it is also recommended to evaluate the exact drug-plant ratio at which the interaction is maximal between the plant extract and antimicrobial drug.

Furthermore, it is hard to predict the synergistic effects *in vivo* with the evidence of *in vitro* study alone because it is difficult to estimate the availability of concentration of active ingredient after the plant extracts have been delivered. Thus, the clinical trials in experimentally infected animals can be done in future studies. More importantly, the safety of the plant extracts should be determined via toxicological studies.

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APPENDICES

Appendix 1

Preparation of Plant Extracts for Disc Diffusion Method

Preparation of Garlic Extract of Various Concentrations

Desired concentration (%)	Volume of pure garlic extract (ml)	Volume of sterile distilled water (ml)
100	1	0
80	0.8	0.2
50	0.5	0.5
25	0.25	0.75

Preparation of Clove Oil Extract of Various Concentrations

Desired concentration (%)	Volume of pure clove oil extract (ml)	Volume of 10% DMSO (ml)	Tween 80 (drop)
100	1	0	2
80	0.8	0.2	2
50	0.5	0.5	2
25	0.25	0.75	2

Appendix 2

Preparation of Plant Extracts for Minimal Inhibitory Concentration

Preparation of Garlic Extract of Various Concentrations

Desired concentration (%)	Volume of pure garlic extract (µl)	Volume of sterile distilled water (µl)
80	400	100
75	375	125
65	325	175
55	275	225
45	225	275
35	175	325
25	125	375

Preparation of Clove Oil Extract of Various Concentrations

Desired concentration (%)	Volume of pure clove oil extract (µl)	Volume of 10% DMSO (µl)
80	400	100
75	375	125
65	325	175
55	275	225
45	225	275
35	175	325
25	125	375

Appendix 3

Preparation of 96-well

Column	Mueller-Hilton Broth (µl)	Concentration of plant extracts (%) (100µl)	Bacteria inoculum (µl)
1	100	80	100
2	100	75	100
3	100	65	100
4	100	55	100
5	100	45	100
6	100	35	100
7	100	25	100
8	100	0	100
9	100	0	0

Appendix 4

Preparation of Plant Extracts Impregnated Antibiotic Discs

Antibiotics	Plate 1 (80% garlic extract)	Plate 2 (80% clove oil extract)	Plate 3 (control)
Neomycin 30µg	20µl	20µl	0µl
Gentamicin 10µg	20µl	20µl	0µl
Penicillin G 10µg	20µl	20µl	0µl

Appendix 5

Linear regression analysis of the diameter of inhibition zones produced by plant extracts

Plant Extracts	R value	R^2 value	Sig.
Garlic	0.913	0.833	0
Clove Oil	0.9	0.81	0

Appendix 6

One-way ANOVA analysis of diameter of inhibition zones produced by commercial antibiotic alone with combination of antibiotic and plant extracts

Antibiotics	Garlic extract, P value	Clove Oil, P value
Neomycin 30µg	0	0.272
Gentamicin 10µg	0	0.916
Penicillin G 10µg	0.851	0.971