UNIVERSITI PUTRA MALAYSIA

IN VITRO STUDY OF BASIL SEED OIL WITH AMOXICILLIN AGAINST *STAPHYLOCOCCUS AUREUS*

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IN VITRO STUDY OF BASIL SEED OIL WITH AMOXICILLIN AGAINST *STAPHYLOCOCCUS AUREUS*

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "*In Vitro* Study of Basil Seed Gum Oil with Amoxicillin Against *Staphylococcus Aureus*", by Muhammad Amir Asyraf bin Wahid 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
° C	=	degree Celcius
µl	=	microliter
µg	=	microgram
CFU	=	colony forming unit
CFU/ml	=	colony forming unit per milliliter
spp.	=	species
S. aureus	=	*Staphylococcus aureus*
Sig.	=	significance

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4901 - Projek

KESAN SINERGI GAM BIJI SELASIH BERSAMA *AMOXICILLIN* TERHADAP *STAPHYLOCOCCUS AUREUS* SECARA *IN VITRO*

Oleh

MUHAMMAD AMIR ASYRAF BIN WAHID

2022

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 biji selasih yang digabungkan dengan *Amoxicillin trihydrate* 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*. Ekstrak biji selasih menunjukkan aktiviti antimikrob terhadap *S. aureus* ATCC 25923. Nilai MIC ekstrak biji selasih adalah 25% manakala nilai MBC untuk ekstrak biji selasih adalah 50%. Sinergistik efek ekstrak biji selasih dikesan dalam kombinasinya dengan *Amoxicillin trihydrate*. Zon perencatan meningkat dengan ketara dalam kombinasi ekstrak biji selasih dengan *Amoxicillin trihydrate* (P <0.05). Peratusan peningkatan zon perencatan antara kombinasi ekstrak biji selasih dengan *Amoxicillin trihydrate* adalah 44.71%. Kesimpulannya, terdapat potensi antimikrob yang tinggi dari ekstrak tumbuhan ini dan ekstrak biji selasih mempunyai sinergistik efek dengan Amoxicillin trihydrate. Sinergistik efek dari gabungan ekstrak biji selasih dan *Amoxicillin trihydrate* dapat menawarkan alternatif kombinasi terapi bagi melawan jangkitan *S. aureus*.

Kata kunci: *Minimum Inhibitory Concentration* (MIC), *Minimum Bactericidal Concentration* (MBC), biji selasih, *Staphylococcus aureus*

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD 4901 – Project

IN VITRO* STUDY OF BASIL SEED GUM OIL WITH AMOXICILLIN TRIHYDRATE AGAINST *STAPHYLOCOCCUS AUREUS

By

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2022

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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 basil seed when combined with Amoxicillin trihydrate against *Staphylococcus aureus* ATCC 25923. The plant oil was screened for its antimicrobial activity using disc diffusion method. The Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) were assessed using broth microdilution method.

Basil seed oil exhibited antimicrobial activity to *S. aureus*. The MIC value of basil seed oil was 25% while MBC value was 50%. The synergistic effects of basil seed oil were observed in the combination with amoxicillin trihydrate. The inhibition zones were significantly increased in combination of plant oil with amoxicillin trihydrate ($P<0.05$). The percentage increase of inhibition zones between combination of basil seed oil with amoxicillin trihydrate was 44.71%. In conclusion, there is high antimicrobial potential of basil seed oil, and it has synergistic effect with antibiotic tested. The synergistic effect from the combination of basil seed oil and antibiotic may offer alterative of combination therapy against *S. aureus* infection.

Keywords: Minimum inhibitory concentration (MIC), Minimum bactericidal concentration (MBC), basil seed gum, *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 (Shao *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 oils which required complicated process of preparation. To my knowledge, there is no study on the combination of antibiotic with crude plant oils which can be prepared manually have been conducted. Recent studies discovered that basil seeds oil exhibited strong antibacterial activity against nine pathogenic bacteria species includes *Staphylococcus aureus* (Abraham *et. al.*, 2016). Therefore, the objective of this study was to investigate the antimicrobial activity and synergistic effect of basil seed oil when used in combination with amoxicillin trihydrate against *Staphylococcus aureus.*

1.1 Hypothesis

a) Null hypothesis: There is no significant difference between the antimicrobial activity of basil seed oil when used in combination with amoxicillin trihydrate.
b) Alternative hypothesis: There is a significant difference between the antimicrobial activity of basil seed oil when used in combination with amoxicillin trihydrate.

1.2 Objective

The objectives of this study were to investigate the antimicrobial activity and synergistic effect of basil seed extract when used in combination with amoxicillin against *Staphylococcus aureus*.

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). Then, various staphylococci species have been isolated from feline skin lesions including *S. aureus*, as well as MRSA. There are also reports of transmission of MRSA isolates between in-contact cats, dogs and humans (Woolley *et al.,* 2007). It also among the most common *Staphylococcus spp*. isolated from canine clinical samples (Hauschild and Wojcik, 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 (Kadariya *et al.*, 2014).

2.2 Basil seed (*Ocimum basilicum*)

Basil seeds, *Ocimum basilicum* Linn, belonging to family 'Lamiaceae' is white-purple flowers plant annually (Daneshian *et al.,* 2009). It has been used as traditional medicine for diarrhoea and ulcer (Hosseini-Parvar et al., 2010). It contains antibacterial, anti-oxidant and antitumor (Pietta *et al.*, 1998).

Gas chromatography analysis revealed the main components of the oil were linalool (64.35%), 1,8-cineole (12.28%), eugenol (3.21%), germacrene D (2.07%), alpha-terpineol (1.64%), and rho-cymene (1.03%) (Rattanachaikunsopon and Phumkhachorn, 2010).

2.3 Antimicrobial Activities of Basil seed

Linalool (C10H18O), is a monoterpene alcohol, which is found in the essential oils (Aprotosoaie *et al.*, 2014; Pereira *et al.*, 2018). It exhibits antibacterial activity against *Staphylococcus aureus* NCTC 10788, *Pseudomonas aeruginosa* NCTC 12924, and *Escherichia coli* NCTC 12923 (Herman *et al.*, 2016).

Linalool reduced the membrane potential (MP); caused the leakage of alkaline phosphatase (AKP); and released the DNA, RNA, and proteins of *S. putrefaciens*, thus destroying the cell structure and expelling the cytoplasmic content. It also disrupts the function of key enzymes, such as succinate dehydrogenase (SDH) and pyruvate kinase (PK) (Guo *et al.*, 2021).

According to current research, scanning electron microscopy (SEM) showed that linalool destroyed the normal cell membrane integrity, increased the membrane permeability, and cause leakage of nucleic acids (Liu *et al.*, 2020).

2.4 Amoxicillin trihydrate

Amoxicillin trihydrate is a broad-spectrum beta-lactam antibiotic, derived from penicillin. It has similar activity against gram-positive and gram-negative bacteria. It targets and kills bacteria by inhibiting the biosynthesis of the peptidoglycan layer of the bacterial cell wall. This layer makes up the outermost portion of the cell wall and is responsible for the structural integrity of the cell. Peptidoglycan synthesis involves the facilitation of DD-transpeptidases, which are a type of penicillin-binding protein (PBP). Amoxicillin trihydrate works by binding to these PBPs and inhibiting peptidoglycan synthesis, which interrupts the construction of the cell wall and ultimately leads to the destruction, or lysis, of the bacteria (Brogden *et al.*, 1981), (Benninger, 2003), (Stein & Gurwith, 1984).

3.0 MATERIALS AND METHODS

3.1 Bacteria

Staphylococcus aureus ATCC 25923 was isolated from stock culture from the Bacteriology Laboratory of Faculty of Veterinary Medicine, Universiti Putra Malaysia and it was sub-cultured onto nutrient agar plate.

3.2 Preparation of Basil Seed Oils

Basil seed was purchased from local market. 25 grams of seeds were uniformly grinded using mechanical grinder to make fine powder. The powder was serially oiled in petroleum ether, using a Soxhlet apparatus. The cycles of petroleum ether were run till complete defatting was obtained, approximately 4 hours. The crude solvent oils of *O. basilicum* seeds was then dried at room temperature, stored and considered as pure oil. The oil was dissolved in 10% aqueous dimethyl sulfoxide (DMSO) with Tween 80. The basil seed oils were prepared as 100%, 75%, 50%, 25%, 12.5% and 6.25% concentrations (Appendix 1).

3.3 Antibacterial Susceptibility Testing

The antimicrobial activities of basil seed oils 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 oils 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 CLPI for this experiment. The agar was prepared according to the manufacturer's recommendation.

3.4.2 Preparation of Basil Seed Oils Impregnated Discs

Sterile blank discs were impregnated with 20 µl of the various concentration of basil seed oils. Amoxicillin trihydrate 20µg was used as the positive control, and 10% aqueous DMSO was used as negative control.

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 2x10 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 37°C 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 Basil Seed Oils

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 6 concentrations were derived (Appendix 2). DMSO were used to dilute the oils. In the above method, the control was DMSO.

3.5.2 Preparation of Inoculum

The method used was same as the method described on page 7.

3.5.3 Broth Microdilution Testing

The 96-well plates were prepared by dispensing 100 µl of Mueller-Hinton broth (MHB), into each well. 100 µl of various oil concentrations were added to each well. Then, 100 µl of bacteria inoculum were added to each well except for negative control. 100 µl of bacteria inoculum with media was used as a positive control and DMSO with media was used as a negative control (Appendix 3). The turbidity and formation of "button" shape at the bottom of each well was determined before and after incubation period. The 96-well plates were incubated at 37°C 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).

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

10 µl 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°C for 20 hours to observe the presence of bacterial growth.

3.5.7 Interpretation of Results

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

3.6 Synergistic Test Effect

The synergistic effect of basil seed oil 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 Amoxicillin trihydrate

In synergistic test, the basil seed oils were tested with 20 μl of amoxicillin trihydrate trihydrate, 100 mg/ml.

3.6.3 Selection of Basil Seed Oils Concentration

The concentration of the basil seed oils 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, 75% concentration of basil seed oils exhibited maximum inhibition zones.

3.6.4 Preparation of Basil Seed Oils Impregnated Amoxicillin trihydrate Discs

Sterile prepared antibiotic discs were placed on sterile agar plates. 20 µl of 75% concentration of the basil seed oils were impregnated into each antibiotic disc, respectively (Appendix 4). As for negative control, sterile blank discs were impregnated with 20ul DMSO. While for positive control, the antibiotic discs were used.

3.6.5 Preparation of Inoculum and Inoculation of Test Plates

The method used was same as the method described on page 7.

3.6.6 Zone of inhibition

The method used was same as the method described on page 8. The results were subjected to One-Way Analysis of Variance (ANOVA) to compare the differences of inhibition zones with or without basil seed oils combination at 95% confidence level. Significantly different means were then elucidated using the Tukey's multiple comparison test.

4.0 RESULTS

Basil seed oils and amoxicillin trihydrate exhibited good antimicrobial activity towards *Staphylococcus aureus* ATCC 25923 (Table 1). The amoxicillin trihydrate exhibited the largest mean diameter of inhibition zones of 35.03 $\pm$ 0.26 mm. While 100% basil seed oil exhibited the smallest mean diameter of inhibition zones of 12.23 $\pm$ 1.05 mm.

The basil seed oils exhibited clear inhibition zones at various concentrations against *S. aureus*, excluding 12.5% and 6.25% concentration (Table 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 basil seed oil, with R value was 0.7997 ($p < 0.05$) based on the Pearson's correlation test (Appendix 5). The Linear Regression Analysis (Appendix 5) suggests that the increase in diameter inhibition zones were due to the increase in the concentration of basil seed oils, with R^2 values of 0.6395 ($p < 0.05$).

Based on the broth microdilution testing, the Minimal Bactericidal Concentration (MBC) for basil seed oils were 50%. While Minimal Inhibitory Concentration (MIC) was 25% (Table 3).

The basil seed oil at 75% showed synergistic effect with amoxicillin trihydrate. The inhibition zones were significantly increased ($p<0.05$) when combined with amoxicillin trihydrate (Table 4). The percentage of increased in diameter of inhibition zones when basil seed oil combined with amoxicillin trihydrate was 44.71% (Table 5).

Table 1: Diameter of inhibition zones produced by basil seed oil (100%) and amoxicillin trihydrate

Antibiotic / Basil Seed Oils	Inhibition Zones (mm)
Amoxicillin trihydrate	35.03 ± 0.26
Basil Seed 100%	12.23 ± 1.05

Values are mean ± standard deviation

Table 2: Diameter of inhibition zones produced by basil seed oil at various concentration

Concentration (%)	Inhibition zones (mm)
100	12.23 ± 1.05
75	18.56 ± 1.18
50	14.31 ± 0.59
25	8.82 ± 1.25
12.5	0
6.25	0

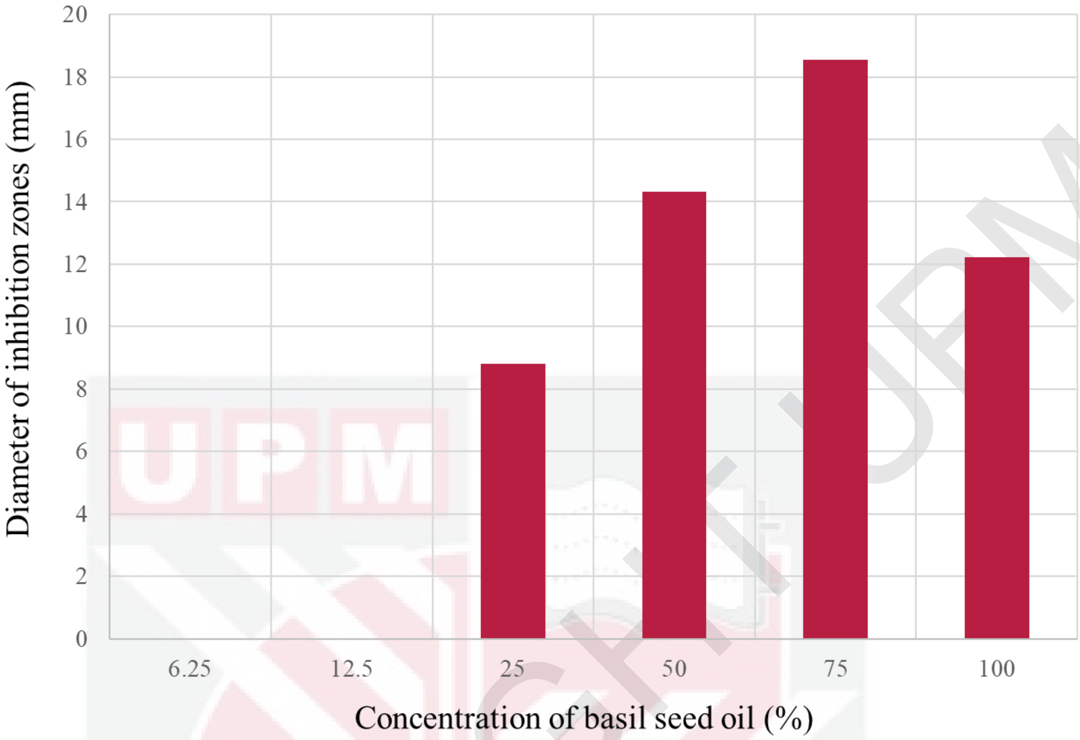

Figure 1: Mean diameter of inhibition zones of basil seed oil

Figure 2: Inhibition zones of amoxicillin trihydrate

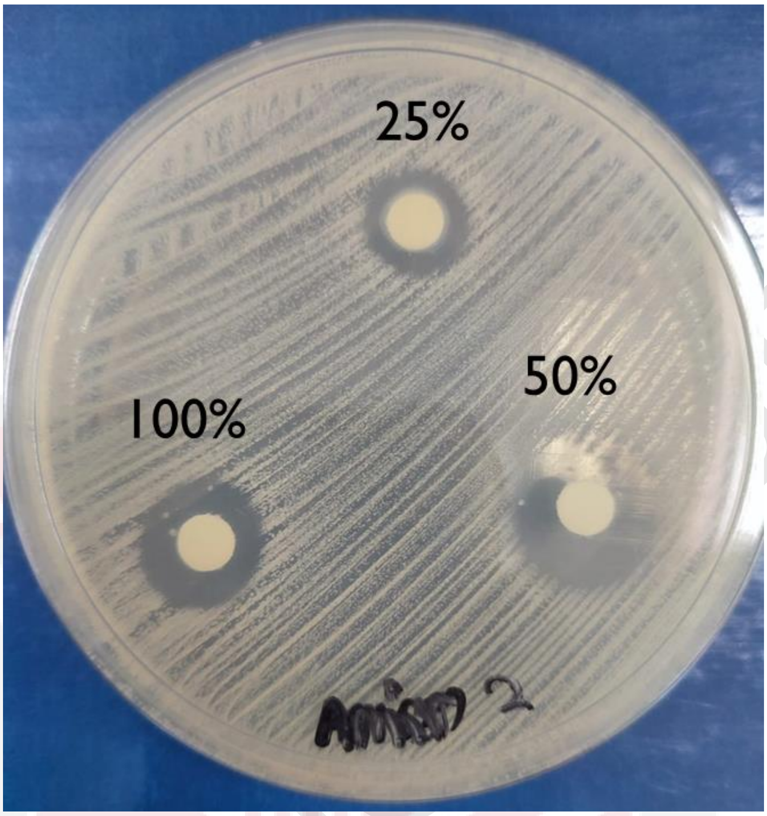

Figure 3: Inhibition zones of Basil seed oil

Table 3: The minimal inhibitory concentration and minimal bactericidal concentration of basil seed oil

Minimal Inhibitory Concentration (MIC)	Minimal Bactericidal Concentration (MBC)
25%	50%

Table 4: Diameter of inhibition zones produced by basil seed oil (75%) in combination with amoxicillin trihydrate.

10% aqueous DMSO (mm) (Negative control)	Amoxicillin trihydrate (mm)	Amoxicillin trihydrate 20 µl + Basil Seed Oil 75% (mm)
0	35.03 $\pm$ 0.26	42.14 $\pm$ 0.61

*P < 0.05 when compared to the negative control. Values are mean + std deviation.

Table 5: The percentage of increase in diameter of inhibition zones when amoxicillin trihydrate combined with basil seed oil.

Synergistic effect	Percentage of diameter of inhibition zones
Basil Seed Oil + Amoxicillin trihydrate 20 μl	44.71%

According to Singh *et al.* (2011), the formula used to calculate the percentage of increase in diameter of inhibition zone when amoxicillin trihydrate combined with basil seed oil are:

$(B^2 – A^2 / A^2) \times 100$

A = Mean diameter of inhibition zones in the absence of oil,

B = Mean diameter of inhibition zones in the presence of oil

(42.14^2 – 35.03^2) / (35.03^2) x 100 = 44.71%

5.0 DISCUSSION

In this study, *Staphylococcus aureus* was sensitive to the inhibitory activities of basil seed oil at 25%, 50%, 75% and 100% concentration using the disc diffusion method. *S. aureus* was most sensitive to 75% concentration of oil. However, at 6.25% and 12.5% concentrations, there were no inhibition zones produced towards *S. aureus*, The steepness of the graph indicates that a significant increase in the concentration of basil seed oil above 12.5% leads to greater inhibition zones. This suggests that *S. aureus* is sensitive to the changes in the oil concentration. When basil seed oil is used against *S. aureus*, care must be taken to prevent underdosing, because decrease in the oil concentration less than 25% can lead to ineffective respond towards bacteria. The high efficacy of basil seed oil against *S. aureus* indicates that high potential of the oil to treat *S. aureus* infection (Sakkas *et al.*, 2018)

S. aureus was more sensitive to basil seed oil at 75% concentration compared to 100% concentration. This indicates that the diluted basil seed oil had better diffusion rate on Mueller Hinton Agar as compared to pure basil seed oil.

In addition, the MIC and MBC values support the findings of the disc diffusion method. The antimicrobial activity of basil seed oil against *S. aureus* could be attributed to the presence of linalool in the plant oil. According to a research, significant antimicrobial activity was shown for all the tested oils that are “linalool chemotype” (Damir *et al.*, 2015). The area of concern is that MIC values of basil seed oil obtained in this study was lower than the MBC values, suggesting that basil seed oil was bacteriostatic at lower concentration and bactericidal at higher concentration.

In the present study, the basil seed oil exhibits significant increase of inhibition zones with amoxicillin trihydrate. The percentage of increase in inhibition zone when combined with amoxicillin trihydrate is 44.71%. The results of this study are comparable to the results reported by Damir *et al.*, (2015), in terms of the inhibition zones or MIC and MBC values, but no test on synergistic effect with amoxicillin trihydrate. Finally, extracts of *S. officinalis* significantly increased the activity of amoxicillin reported by Stefanovic (2018).

6.0 CONCLUSION

The overall results of the present work provide baseline information for the possible use of studied basil seed oil for *Staphylococcus aureus* infections. Basil seed oil has potential to be used as antimicrobial agent against *S. aureus*, but the effect is dose-dependent. The plant oils can easily be prepared in health or sufficient laboratory as they are widely available in local market. Basil seed oil can be used a replacement for certain antibiotics especially for topical application, as the present study showed the effectiveness of basil seed oil against *S. aureus*. In this study using paper disk diffusion assay, we showed that the antimicrobial activity of amoxicillin trihydrate was significantly increased. Thus, this study suggests the possibility of using combination therapy of antibiotic with basil seed oil 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 single type antibiotic tested. More strains or field strains need to be incorporated in future studies to determine the effectiveness of basil seed oil as an antimicrobial agent. A wider study can be done using more antibiotics to establish and understand the mechanism of synergy with plant oils. Here, it is also recommended to evaluate the exact drug-plant ratio at which the interaction is maximal between the plant oil 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 oils have been delivered. Thus, the clinical trials in experimentally infected animals can be done in future studies. More importantly, the safety of the plant oils should be determined via toxicological studies.

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APPENDICES

Appendix 1

Preparation of Basil Seed oil for the Disc Diffusion Method

Concentration (%)	Volume of pure oil (mL)	Volume of 10% DMSO (mL)	Tween 80 (drop)
100	1	0	2
75	0.75	0.25	2
50	0.5	0.5	2
25	0.25	0.75	2
12.5	0.125	0.875	2
6.25	0.0625	0.9375	2

Appendix 2

Preparation of Basil Seed Oil for Minimal Inhibitory Concentrations

Desired concentration (%)	Volume of pure basil seed oil extract (µl)	Volume of 10% aqueous DMSO (µl)
6.25	31.25	468.75
12.5	62.5	437.5
25	125	375
50	250	250
75	375	125
100	500	0

Appendix 3

Preparation of 96-well plate

Column	Mueller-Hilton Broth (µl)	Concentration of basil seed oil extracts (%) (100µl)	Bacteria inoculum (µl)
1	100	6.25	100
2	100	12.5	100
3	100	25	100
4	100	50	100
5	100	75	100
6	100	100	100
7	100	0	100
8	100	0	0

Appendix 4

Preparation of Basil Seed Oil Impregnated Antibiotic Discs

Antibiotic	Plate 1 (75% basil seed oil extract)	Plate 2 (control)
Amoxicillin trihydrate (µl)	20	0

Appendix 5

Linear regression analysis of the diameter of inhibition zones produced by basil seed oil extract.

Plant extract	R value	R^2 value	Sig.
Basil seed oil	0.7997	0.6395	0