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Antibacterial activity of Sireh (*Piper betle* L.) leaf extracts for controlling bacterial leaf blight diseases in rice plant

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ABSTRACT

Aims: This study aimed to determine the antibacterial activity of *Piper betle* L. leaf extract against *Xanthomonas oryzae* pv. *oryzae* that causes bacterial leaf blight in rice plant.
Methodology and results: The antibacterial activity of the *P. betle* leaf extract (100, 50, 25 and 12.25 mg/mL) with four different solvents (methanol, ethyl acetate, hexane and acetone) was evaluated using a disc diffusion assay, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values. The inhibition zone of methanolic extract appeared to have the maximum diameter compared to those of other extracts, which is 32.67 mm at a concentration of 100 mg/mL, followed by 30.33 mm, 22.00 mm and 20.30 mm for the concentrations of 50 mg/mL, 25 mg/mL and 12.5 mg/mL, respectively. The MIC and MBC values of the methanolic extract were 0.625 mg/mL suggesting that the extract has a bactericidal effect on *X. oryzae* pv. *oryzae* (*Xoo*). The time-kill curve studies revealed that the 1× MIC (0.625 mg/mL) concentration of methanolic extract had a time and concentration-dependent killing effect on *Xoo*. Gas chromatography-mass spectrometry (GC-MS) analysis of methanol extract revealed the presence of eugenol acetate (29.53%), 4-allyl-1,2-diacetoxybenzene (29.51%) and 2,3-dimethyl benzoic acid (22.82%) as major compounds.
Conclusion, significance and impact of study: The methanolic leaf extract of *P. betle* was proven to have an effective inhibitory effect on *Xoo* and may have the potential to be used as an alternative management strategy for controlling rice diseases. In the future study, the methanolic leaf extract of *P. betle* is one of the recommendations to be applied in glasshouse and field trials.

Keywords: Sireh, *Piper betle* L., bacterial leaf blight, rice

INTRODUCTION

Rice is one of the primary food crops for the global population and plays a vital role in nutrition (Khan *et al*., 2015). In terms of consumers and producers in Asia, it ranks second after wheat (Rajamoorthy *et al*., 2015). The most common rice disease is bacterial leaf blight (BLB), which is caused by the Gram-negative bacteria, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) has a significant impact on rice productivity (Wang *et al*., 2017). Plant extracts possess potent activity against rice pathogenic microorganisms (Chanprapai and Chavasiri, 2017). *Piper betle*, often known as Sireh in Malaysia and betel in English, belongs to the Piperaceae family of plants (Venkateswarlu *et al*., 2014). The extract of *P. betle* leaf also had the best antibacterial effect on both Gram-negative and Gram-positive bacteria (Nguyen *et al*., 2020). The therapeutic profile reveals *P. betle* to have a high potential for treating many diseases (Umar *et al*., 2018). Because of their potential, the extracts or compounds of *P. betle* leaf could be highly promising to be effective antibacterial agent for treating diseases (Syahidah *et al*., 2017). Researchers have discovered that *P. betle* leaf has a variety of active chemicals that make it an effective healer. The bioactive compounds in a plant are referred to as phytochemicals and they function to protect the plant from diseases and pests (Doughari, 2012). *Piper betle* leaf also has an antimicrobial effect on

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a wide range of microbes (Gram-negative and Gram-positive bacteria) (Jesonbabu *et al*., 2011). *Piper betle* leaf extract also inhibits the growth of *Staphylococcus aureus* in conjunctivitis patients and has potential use as an antibacterial agent (Lubis *et al*., 2020). The methanol extract was considered the most effective, with a maximum inhibition zone up to 40 mm (Jayalakshmi *et al*., 2013). *Piper betle* leaf extract has an antibacterial effect on bacteria by damaging the plasma cell membrane and producing nucleoid coagulation (Jesonbabu *et al*., 2011). From the previous study revealed by Syahidah *et al*. (2017), eugenol and hydroxychavicol were the active compounds in charge for the antibacterial activity of the methanolic extract of *P. betle* leaf.

Piper betle leaf research has exploded in popularity over the past, with numerous studies demonstrating plants' antimicrobial potential. Plant-based antibiotics, bactericides and biopesticides have been the focus of recent studies and research. Plant extracts, which contain a variety of phytochemicals, have been shown to have antimicrobial effects. Previous study by Jayalakshmi *et al*. (2013), the extract of *P. betle* leaf showed significant activities against a broad array of plant pathogenic bacteria. However, there are few reports *in vitro* and *in vivo* on the antibacterial effect of *P. betle* leaf extracts on the rice bacterial pathogen *Xoo* that causes BLB. Thus, the objective of this study was to assess the antibacterial activity of *P. betle* leaf extract with the aim of reducing the BLB caused by the plant pathogenic bacteria *Xoo* and to determine the chemical constituents responsible for antibacterial activities by using GC-MS analysis.

MATERIALS AND METHODS

Plant material collection and preparation

A hundred of fresh and matured leaves from the same plant of *P. betle* leaf were collected from UPM Conservatory Park, Institute of Bioscience, Universiti Putra Malaysia (UPM), Serdang, Selangor, Malaysia. The botanist Dr. Mohd Firdaus Ismail from the Institute of Bioscience, Universiti Putra Malaysia (UPM) identified the plant species and the voucher specimen (MFI 0129/19) was deposited in the Herbarium, Biodiversity Unit, Institute of Bioscience, UPM.

The leaves were washed under running tap water and rinsed with sterile distilled water to remove any dirt before being dried in a freeze-dried machine for 24 h to remove moisture from the plant. Using an industrial blender chopper, they were crushed into a fine powder (HR2000, Philiph, Holand). The fine powder was kept in a tightly sealed bottle to avoid contamination.

Extract preparation

Four types of organic solvents were used to extract *P. betle* leaf powder. In the extraction process, methanol, acetone, ethyl acetate and hexane were used for small-scale extraction. The solvents used were analytical grade. Ten grams of *P. betle* leaf powder was weighed and transferred into a 250 mL conical flask. Then, 200 mL of solvent was added to the flask and mixed well. The flasks were tightly covered with the aluminum foil sheet to avoid evaporation of organic solvent and were placed on a rotary shaker for 48 h. After that, the crude extract was filtered using 11 µm Whatman No. 1 filter paper (Whatman, England) and concentrated under a vacuum using a rotary evaporator (Buchi^{R} R210, Switzerland). The residual of the extract was freeze-dried for 24 h and then stored in the freezer at –80 °C to preserve the phytochemicals in the extracts. The extract preparation was done in triplicate and repeated three times.

Preparation of test pathogen

Xanthomonas oryzae pv. *oryzae* (UPMC 691) was obtained from the Microbial Culture Collection Unit (UNiCC), Institute of Bioscience, Universiti Putra Malaysia and was used in this study based on its ability to cause BLB diseases on rice plants (Jonit, 2018). During the experiment, the strain was grown on Luria Bertani (LB) media (Merck, Germany) and incubated for 18-24 h at 37 °C. The stock of the cultures was prepared and kept in a –80 °C freezer for further use.

Determination of antibacterial effect of *Piper betel* L. leaf extract against *Xanthomonas oryzae* pv. *oryzae*

The antibacterial effect of the extract of *P. betle* leaf was determined against a Gram-negative bacterium, *X. oryzae* pv. *oryzae*, by the disc diffusion assay (CLSI, 2018). The test bacteria were streaked onto LB agar media and incubated for 24 h at 37 °C. The colony suspension was prepared by selecting 10-20 isolated colonies in 5 mL of saline solution (0.85% NaCl) and the turbidity of the colony suspension was adjusted to achieve a turbidity equivalent to the 0.5 McFarland standard. This results in a suspension containing approximately 1-2 × 10^8 CFU/mL. The prepared inoculum was vortexed thoroughly and plated on Luria Bertani (LB) agar (Merck, Germany) by streaking the swab over the entire surface of the agar three times, each time at a 60° angle to the previous streaking to ensure an even distribution of inoculum. Twenty µL of extracts were transferred on a sterile blank disc with a concentration of 100, 50, 25 and 12.5 mg/mL dissolved with 100% dimethyl sulfoxide (DMSO). Twenty µL of 100% dimethyl sulfoxide (DMSO) was used as a negative control. The disc containing the extracts was then transferred to the test plates and assayed on a triplicate agar medium plate. After incubation at 37 °C for 24 h, the zone of inhibition was measured by a millimeter ruler. The test was repeated three times.

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) was measured by two-fold serial dilution method (CLSI, 2012). The colony suspension was prepared by selecting 10-20 isolated colonies in 5 mL of saline solution (0.85% NaCl)

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and the turbidity of the colony suspension was adjusted to achieve a turbidity equivalent to the 0.5 McFarland standard. This results in a suspension containing approximately 1-2 × 10^8 CFU/mL. The prepared inoculum was diluted to 1:20 in LB broth to yield approximately 2-8 × 10^6 CFU/mL. In a microtiter plate, hundred µL of inoculum dispense into well in column 1 until 11 and hundred µL of LB broth into well in column 12 as sterility control. Hundred µL the extract was added and mixed into column 1 and serially diluted until column 10 and discarded hundred µL of the solution from column 10. Column 11 was used as growth control. It was incubated for 24 h at 37 °C. The lowest extract concentration that did not cause turbidity in the broth was used as the MIC value.

Determination of minimum bactericidal concentration (MBC)

From the MIC test, the 24 h incubated solution from the microtiter plate was subcultured on LB agar plate to determine the minimum bactericidal concentration (MBC) value by dropping 10 µL of the solution from the well in column 1 until 12. Column 1 until 10 was indicated the concentration of the extract 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL, 0.625 mg/mL, 0.3125 mg/mL, 0.1562 mg/mL, 0.0781 mg/mL, 0.0390 mg/mL, 0.0195 mg/mL and 0.0098 mg/mL, respectively. The solution in column 12 was used as sterility control and 11 as growth control. It was incubated for 24 h at 37 °C. The MBC value was determined by the lowest concentration that showed no observable growth on agar plates.

Time-kill curve analysis

The time-kill curve of methanol leaf extracts of *P. betle* L. was carried out following the procedure described by Tsuji *et al*. (2008). Concentrations of 0× MIC, 0.5× MIC and 1.0× MIC of the extracts were prepared together with an inoculum size of 2-8 × 10^6 CFU/mL in a 10 mL total volume. The suspension was incubated at 37 °C. Aliquots of 100 µL of the medium were taken at time intervals of 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 h. The series of dilution tubes containing 900 µL of saline water (0.85% NaCl) was prepared to obtain a dilution of 10^{-1} to 10^{-7} of the mixture cultures and 100 µL was spread aseptically on LB agar in triplicate and incubated at 37 °C. A control test was performed for the organisms without the extracts and the control for the solvent used. The colony forming unit (CFU) of the organisms was determined. The method was performed in triplicate and the log CFU/mL was plotted against time on a graph.

Gas chromatography-mass spectrometry (GC-MS) analysis

Gas chromatography-mass spectrometry (GC-MS) was used to investigate the chemical components of the plant extracts. A Shimadzu GC-MS QP2010 Plus equipped with a BPX5 fused silica capillary column (Length: 30 m; ID: 0.25 mm; film thickness: 0.25 µm) was used to analyze the extracts. The ionization energy of the mass spectrometer was set at 70 eV. The temperature was programmed to rise from 70 °C (15 min) to 270 °C at a rate of 7 °C/min. Electronic integration was used to determine peak areas and retention times. Area normalization was used to express the relative amounts of each component as a percentage. The chemicals found in the extract were identified and analyzed using computer searches of the National Institute of Standards and Technology's (NIST) 08 mass spectral data library. The spectrums of unknown substances were compared to those of known compounds in the NIST library. The chemicals in the plant extracts were identified by their name, molecular weight and structure.

Table 1: Yield percentage of *P. betle* L. extract with different solvent.

Extracts	Percentage yield (% ± SD)
Methanol	12.14 ± 0.14^{b}
Ethyl acetate	15.04 ± 0.35^{c}
Hexane	10.90 ± 0.36^{a}
Acetone	11.87 ± 0.23^{ab}

Data analysis

The data was analyzed with the SPSS statistical software. Tukey's HSD was used to calculate the mean values and standard deviations (mean ± SD) of a triplicate and repeated three times using one-way analysis of variance (ANOVA). Only variables with a $P<0.05$ confidence level or higher were considered significant.

RESULTS

Yield percentage of extract

Table 1 shows the yield percentage of various crude extracts obtained from 10 g of dried leaf sample using various solvents. The use of small quantities of samples in this analysis was intended to ensure the accuracy of the results. The highest yield percentage was obtained from the ethyl acetate crude extract (15.04%) followed by methanol (12.14%) and acetone (11.87%). The lowest percentage of yield percentage was obtained from hexane crude extract (10.90%). The order of increasing yield in different solvent extraction was hexane < acetone < methanol < ethyl acetate.

Disc diffusion assay

The antibacterial effect of the extracts was determined using a disc diffusion assay. Table 2 represents the antibacterial effect of *P. betle* leaf extract with four extraction solvents. The *P. betle* leaf extracted in methanol, hexane, acetone and ethyl acetate exhibited high inhibitory effect against *Xoo*. This study revealed that *P. betle* leaf extracts showed a significant difference on

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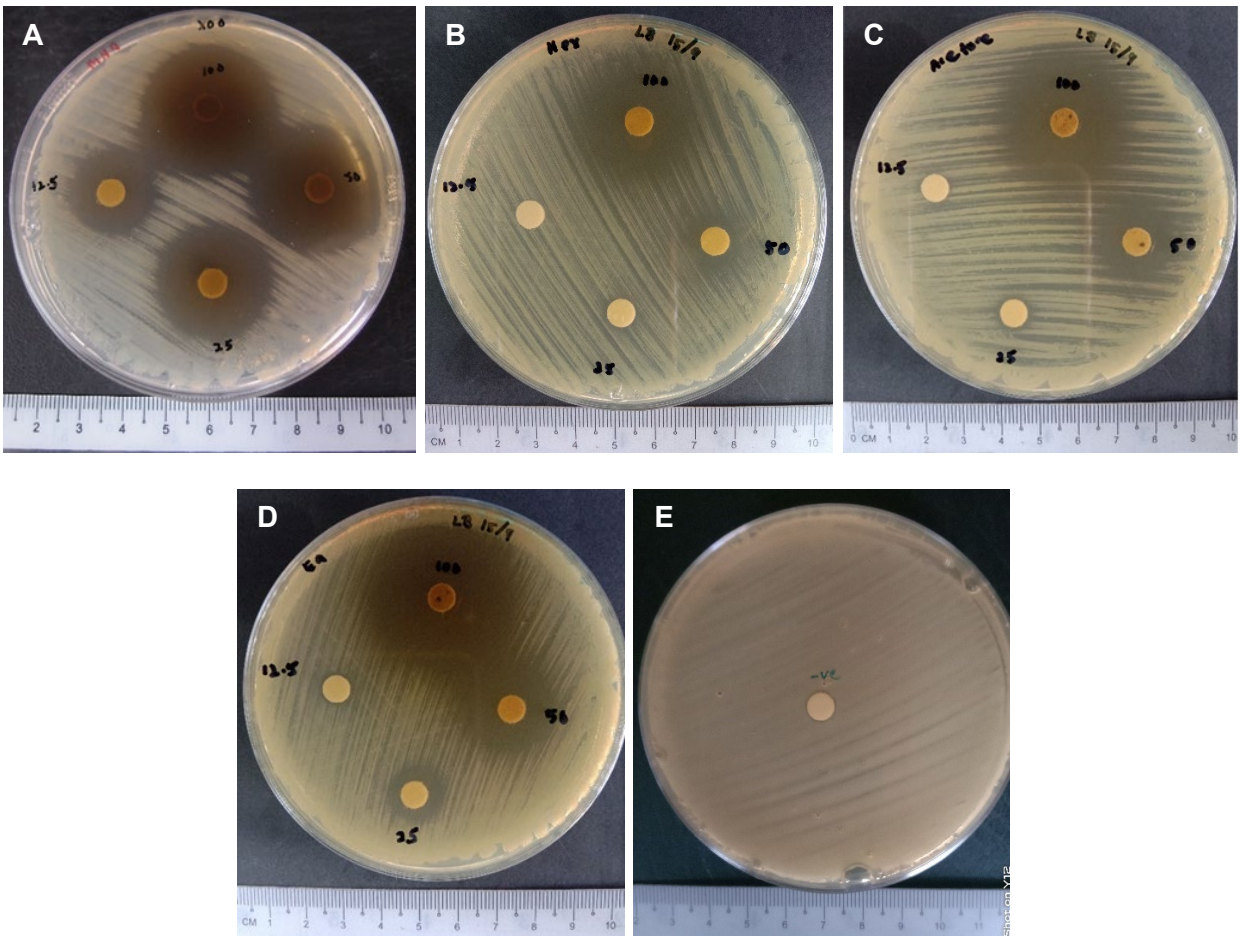

Figure 1: The inhibition zone of *P. betle* L. extract against *Xoo* with different solvents. (A) Methanol extract, (B) Hexane extract, (C) Acetone extract and (D) Ethyl acetate extract with four different concentration (100 mg/mL, 50 mg/mL, 25 mg/mL, 12.5 mg/mL) and (E) Negative control of 100% DMSO.

inhibitory activity against the growth of *Xoo.* In particular, the order of antibacterial efficiency against *Xoo* is: methanol > ethyl acetate > acetone > hexane. Out of four different concentrations, the higher concentration of extract showed greater antimicrobial activity.

There is no overcoming of growth in the zone of inhibition with prolonged incubation. The solvent control of dimethyl sulfoxide (DMSO) had no inhibitory effect on *Xoo* tested. The methanol extract showed the most effectiveness in its antibacterial effect on *Xoo* strains. With a diameter of 32.67 mm, the largest inhibition zone was determined at the concentration of 100 mg/mL, followed by 30.33 mm, 22.00 mm and 20.30 mm for the concentrations of 50 mg/mL, 25 mg/mL and 12.5 mg/mL, respectively (Figure 1).

The antibacterial effect of hexane extract was least effective as compared to methanol, acetone and ethyl acetate extract. The concentration of 12.5 mg/mL of hexane extract did not show any inhibitory activity and similar results were obtained as with the solvent control. The inhibition zone of hexane extract with a diameter of 22.00 mm was observed at the concentration of 100 mg/mL, followed by 10.67 mm and 7.00 mm for the concentration of 50 mg/mL and 25 mg/mL, respectively. *Piper betle* leaf methanolic extracts were found to effectively inhibit the growth of *Xoo* in this study. The higher the concentration used, the higher the resulting inhibition of bacteria (Figure 1).

Minimum inhibitory concentration (MIC)

The lowest extract concentration that did not cause turbidity in the broth during the analysis was used as the MIC value. The minimum inhibitory concentration of the extracts against *Xoo* is depicted in Table 3. The MIC of methanolic extract was the lowest (0.625 mg/mL) as compared with other extracts. The lowest MIC is an indication of the high effectiveness of the extract. The MIC of the hexane extract was the highest (2.50 mg/mL), which indicated the low effect of the extract against *Xoo*. The MIC of acetone and ethyl acetate showed an equal value (1.25 mg/mL).

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Table 2: Antibacterial effect of *P. betle* L. extracts against *Xanthomonas oryzae* pv. *oryzae*.

Extract	Concentration (mg/mL)	Inhibition zone (mm) (Mean ± SD)
Methanol	100	32.67 ± 0.58^{e}
	50	30.33 ± 0.58^{d}
	25	22.00 ± 0.00^{c}
	12.5	20.33 ± 0.58^{b}
Hexane	100	22.00 ± 0.00^{d}
	50	10.67 ± 0.58^{c}
	25	7.00 ± 0.00^{b}
	12.5	0.00 ± 0.00^{a}
Acetone	100	29.67 ± 0.58^{e}
	50	18.65 ± 0.58^{d}
	25	9.33 ± 0.58^{c}
	12.5	6.67 ± 0.29^{b}
Ethyl acetate	100	30.33 ± 0.58^{e}
	50	20.67 ± 0.58^{d}
	25	13.00 ± 0.00^{c}
	12.5	7.00 ± 0.00^{b}
Control (DMSO)	100%	0.00 ± 0.00^{a}

Inhibition zone (mm): Weak activity = 7-10 mm, intermediate activity = 11-15 mm, strong activity = ≥16 mm.

Table 3: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *P. betle* L. extracts against *Xanthomonas oryzae* pv. *oryzae*.

Extracts	MIC (mg/mL) (Mean ± SD)	MBC (mg/mL) (Mean ± SD)
Methanol	0.625 ± 0.0	0.625 ± 0.0
Hexane	2.50 ± 0.0	2.50 ± 0.0
Acetone	1.25 ± 0.6	1.2.5 ± 0.6
Ethyl acetate	1.25 ± 0.6	1.25 ± 0.6

Minimum bactericidal concentration (MBC)

The MBC value was determined by using the lowest concentration that did not demonstrate any observable growth on agar plates. The MBC of the extracts against *Xoo* is depicted in Table 3. The MBC of methanolic extract was the lowest (0.625 mg/mL) as compared with other extracts. The lowest MBC is an indication of the high effectiveness of extract. The MBC value of all the extracts was equal to MIC, which indicated the bactericidal activity of the extract against *Xoo*. The MBC of the hexane extract was the highest (2.50 mg/mL), which indicated the low effect of the extract against *Xoo*. The MBC of acetone and ethyl acetate showed an equal value (1.25 mg/mL). The ratio of MBC and MIC equal to one indicated the bactericidal effects of *P. betle* leaf against *Xoo.* All the extracts displayed a bactericidal effect against *Xoo*.

Time-kill curve of methanolic *P. betle* leaf extract

Based on the MIC results, methanol extract was chosen to proceed for time-kill curve studies. The concentration of

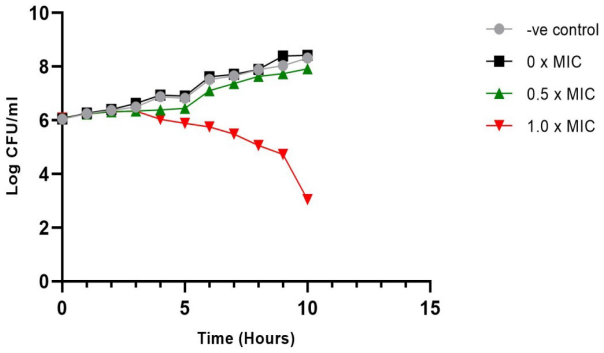

Figure 2: The time-kill curve of *P. betle* L. methanolic leaf extract against *Xoo*.

0× MIC, 0.5× MIC and 1.0× MIC of methanolic *P. betle* leaf extract was used in the analysis. Figure 2 represents the effect of methanolic leaf extract of *P. betle* with different concentrations against *Xoo* with one-hour intervals until 10 h. The 1.0× MIC (0.625 mg/mL) was obtained with a 3 log reduction after 10 h of incubation. The extract was considered to have a bactericidal effect when compared with the initial inoculum.

Chemical constituents of *P. betle* extract using GC-MS

The composition of the chemical constituents of methanolic leaf extract of *P. betle* is presented in Table 4. A gas chromatography-mass spectrometry (GC-MS) analysis of a methanol extract of *P. betle* identified that eugenol acetate was the major component (29.53%), followed by 4-allyl-1,2-diacetoxybenzene (29.51%) and 2,3-dimethyl benzoic acid (22.82%). The minor compounds presented in the methanolic extract are eugenol (3.89%) and chavicol acetate (3.55%) (Figure 3).

DISCUSSION

Piper betle has long been recognized to provide such a variety of health benefits. Most recent studies have focused on the antibacterial effect of *P. betle* leaf extract against Gram-negative and Gram-positive bacteria, including foodborne pathogens and multidrug-resistant bacteria that cause severe infectious diseases in humans. The antibacterial effect of *P. betle* leaf extract against plant pathogenic bacteria that cause a variety of important diseases in crops, fruits and vegetables has received relatively little attention so far, specifically in Malaysian varieties. Thus, the goal of this study was to assess the antibacterial action of a *P. betle* leaf extract to reduce BLB infections caused by *Xoo*, a plant pathogenic bacterium.

Plant-based antibacterial treatments, which contain many antibacterial active components, are known to have therapeutic promise. The type of solvent chosen in the extraction method has a major impact on these compounds' solubility (Nasir *et al*., 2015). Apart from that,

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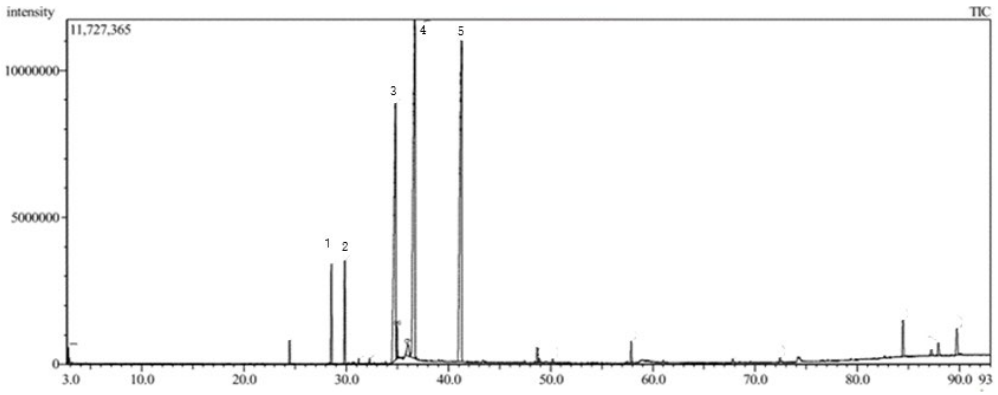

Figure 3: GC-MS chromatogram of methanolic extract of *P. betle* L.

Table 4: Composition of chemical constituents in the *P. betle* L. methanol extract.

No	Component	Formula	Mol. weight	Retention time	% in total extract
1	Chavicol acetate	$C_{11}H_{12}O_2$	176	28.5483	3.55
2	Eugenol	$C_{10}H_{12}O_2$	164	29.8600	3.89
3	2,3-Dimethyl benzoic acid	$C_9H_{10}O_2$	150	34.8233	22.82
4	Eugenol acetate	$C_{12}H_{14}O_3$	206	36.7083	29.53
5	4-Allyl-1,2-diacetoxybenzene	$C_{13}H_{14}O_4$	234	41.2838	29.51

the probability of acquiring the active antibacterial compounds might also be influenced by the amount and variability of the compounds being extracted from the plant materials. The study of percentage yields in the current research revealed that the ethyl acetate extract of *P. betle* had a greater yield (15.04%) while methanolic extract had a moderate yield (12.14%).

The antimicrobial study presented the methanol extract as showing greater inhibitory activity than the other solvent extracts. The results clearly illustrated the effect of different solvents in this study, due to the difference in polarity that resulted in the variations in extracted active compounds. This could be explained by the organic nature of methanol, which is more polar than other solvents and thus has a high capacity to dissolve saturated organic and active antimicrobial compounds (Cowan, 1999). The success of the extraction of compounds from herbal plants largely depends upon the type of solvent used during the extraction process (Nasir *et al*., 2015). Previous studies have also reported that methanol extracts have high antibacterial activities compared to crude extracts obtained from other solvents (Al-Daihan *et al*., 2013; Deshpande and Kadam, 2013; Jayalakshmi *et al*., 2013; Naz and Bano, 2013; Syahidah *et al*., 2017). The largest quantity of gallic acid and hydroxychavicol was obtained from *P. betle* methanol extract, and gallic acid and hydroxychavicol extraction were significantly more efficient using polar solvents (Nguyen *et al*., 2020). According to Felhi *et al*. (2017), the efficiency of methanol is correlated to the intermediate polarity that allows it to solvate low molecular weight organic compounds which possess protonatable functional groups such as COOH and OH. In accordance with the present findings, methanol was a better solvent for extracting antibacterial compounds from *P. betle* leaf in a consistent manner against *Xoo*. Apart from that, other factors such as the quantity of extract used, plant extraction procedure, antibacterial study technique, variation of genes, age of the plant and the environment might have also influenced the efficacy of the herbal extracts in determining the antimicrobial effects.

The antimicrobial effect of the extracts was measured by the disc diffusion assay and revealed that the crude extract of *P. betle* extracted in all solvents (methanol, hexane, acetone and ethyl acetate) appeared to have an inhibitory effect against *Xoo*. The result was supported by a previous report on *P. betle* that showed the significant antibacterial effect on human and animal pathogens due to the existence of a large number of active chemicals like steroids, alkaloids, glycosides, flavonoids, phenols, tannins, saponins and terpenoids (Akiyama *et al*., 2001; Haminiuk *et al*., 2014; Nouri *et al*., 2014; Rekha *et al*., 2014; Xie *et al*., 2015; Sabbineni, 2016; Syahidah *et al*., 2017; Muruganandam *et al*., 2018). The current study's findings provided scientific evidence for *P. betle*'s strong effect on Gram-negative bacteria (Fawad *et al*., 2012; Jayalakshmi *et al*., 2013; Lubis *et al*., 2020; Nguyen *et al*., 2020). According to Nguyen *et al*. (2020) and Marchese *et al.* (2017), the presence of highly bioactive compounds, such as gallic acid, eugenol and hydroxychavicol in the *P. betle* attributed to the antibacterial activities. Eugenol, an amphipathic hydroxyphenyl propene with a free hydroxyl group in its chemical structure, has been shown to aggressively inhibit the growth of most bacteria and fungi (Marchese *et al*., 2017). The most prevalent metabolite with microbial growth inhibitory potential, according to studies on the antibacterial activity of herbal extracts, is a phenolic compound. The activity of the carboxyl group in

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aromatic hydrocarbons could be one explanation for this. These groups form complexes with extracellular and soluble proteins in bacteria, causing them to lose their ability to infect (Cetin-Karaca, 2011). According to Cowan's research, phenol damages three-dimensional proteins, causing Gram-positive bacteria's covalent structure to be disrupted. Thus, the cell wall of the bacterium will be destroyed (Cowan, 1999). Different mechanistic pathways could explain the inhibitory effects of phenolic compounds against pathogenic bacteria. They may interfere with nucleic acid synthesis, causing physiological changes in cell membranes or they may inhibit gene transcription and protein synthesis, which is also required for cell survival. In the case of eugenol, the interaction between the hydroxyl group of eugenol and protein may influence enzyme performance, generating nonspecific membrane permeability and limiting ion and ATP transport. Hydroxychavicol has the potential to damage DNA and impedes cell division, resulting in bacterial cell death (Singh *et al*., 2018).

The antibacterial effect could possibly be attributed to sterol, which is abundant in betel leaf extracts and the surface interaction of the leaf extracts with bacteria's cell walls, destroying bacterial components (Tan and Chan, 2014). Previous research on *P. betle* leaf extracts containing high levels of fatty acids such as stearic acid, palmitic acid and hydroxy fatty acid esters found that they had antimicrobial activity against a wide range of infections (Khan and Kumar, 2011). The investigation by Sugumaran *et al*. (2011a) recorded the antimicrobial effect of plant extract on Gram-positive bacterial strains was found to be more effective due to the simple cell wall along with small pores in the outer layer of the cell, which possess a natural sieve effect against large molecules. The mechanism of flavonoid antibacterial ability is by disrupting the potassium concentration of Gram-positive bacteria, which leads to the dysfunction of their cytoplasm membrane (Xie *et al*., 2015). The finding by Gonelimali *et al*. (2018) suggests that plant extracts significantly affected the cell membrane of Gram-positive and Gram-negative bacteria by the decline in internal pH as well as cell membrane hyperpolarization.

In a different finding by Nguyen *et al*. (2020), the ethyl acetate extract of *P. betle* leaf offered the strongest antibacterial effect on Gram-negative and Gram-positive bacteria. Meanwhile, Sarma *et al*. (2018) found the ethanolic extract of *P. betle* has the highest antibacterial effect and Lakshmi and Naidu (2013) found the chloroform extract showed greater inhibitory activity than the methanolic extract. These different findings must be due to the factors such as habitation, the season of collection, maturity of plants etc. that affect the concentration of their bioactive chemical constituents and antibacterial effect (Jayalakshmi *et al*., 2013). Meanwhile, Stéphane *et al*. (2021) reported that extracting active compounds from plants needs appropriate extraction methods and techniques that provide bioactive ingredients-rich extracts.

The antibacterial activities in the present study were found to be dose-dependent since the inhibitory effects of the *P. betle* leaf extracts were found to proportionately increase with an increase in concentration. Besides, it seemed that the antibacterial assays of the extracts were suitable to be of high concentrations between 25 to 100 mg/mL, with the greatest inhibitory actions. These results reflect the presence of potent antibacterial agents in this extract. The existence of bioactive chemicals could explain the inhibitory effects of *P. betle* leaf extracts at a certain concentration on a certain solvent. According to Nguyen *et al*. (2020), these results must also be related to the high concentrations of gallic acid and hydroxychavicol found in the methanol leaf extract of *P. betle*. However, in this study found that 2,3-dimethyl benzoic acid is the bioactive chemical found in methanol and ethyl acetate extract, which the concentration was higher in methanol extract thus show greatest inhibitory actions compared with other extracts. Thus, finding supported by Synowiec *et al*. (2021) found a high antibacterial effect of benzoic acid, which inhibits the growth of Gram-negative bacteria.

The ratio of MBC and MIC indicated the bactericidal effects of *P. betle* leaves against *Xoo*. All extracts displayed a bactericidal effect against *Xoo*. This must be due to the active effect of the extract on killing the bacteria. Plant extracts with lower MIC and MBC values have a greater antibacterial effect. The MIC value is lower than MBC indicated the bacteriostatic effect, whereas the MIC value equal to MBC was indicated as the bactericidal effect of the extract against the bacteria (Pankey and Sabath, 2004). Overall, the minimum MIC and MBC values of the extracts ranged from 0.625 to 2.50 mg/mL. However, the results were compared with those of Jayalakshmi *et al*. (2013). The MIC value of methanol extract against *Xoo* was 0.104 mg/mL. The methanol extract from betel leaves was discovered to be the most effective extract since it inhibited bacterial strains at the lowest dose.

The use of these lower MIC values from time-kill analysis as a reference to determine effective individualised therapeutic dosages can help to reduce the emergence of resistance while also reducing the negative effects of taking a single agent at a higher dose. The time-kill analysis appeared on the extract with a concentration of 0.625 mg/mL (1× MIC) had a bactericidal effect when a >3 log decrease was reached compared with the initial inoculum. In this finding, the lowest concentration indicated a bactericidal effect and will be the right dose to apply *in-vivo*. The results were comparable to Jesonbabu *et al.* (2011). The MIC of hydroxychavicol (400 g/mL) against *S. aureus* and *E. coli* was determined in time-kill tests, with a 3-log reduction in growth in 10 h compared to the untreated control. The time-kill investigation revealed that methanolic extract killed *Xoo* in a time and concentration-dependent manner.

Gas chromatography-mass spectrometry is an important analytical method in herbal medicine research, particularly for identifying diverse combinations of chemical compounds found in medicinal plants. (Gu *et al*., 2014). The finding from this study showed that 2,3-dimethyl benzoic acid is only present in methanol and ethyl acetate extract, which was higher in methanol

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(22.82%) compared to ethyl acetate (20.29%). Findings from previous research by Yunmei *et al*. (2004) showed that 3,6-dihydroxy-2,4-dimethylbenzoic acid had broad-spectrum antibacterial effect on bacteria. The latest study by Ngurah *et al*. (2020) discovered the 2,4-dihidroxy benzoic acid had antibacterial activity against *Escherichia coli* and *Vibrio alginolyticus*. Cueva *et al*. (2010) and Synowiec *et al.* (2021) found high antibacterial effect of benzoic acid, which inhibits the growth of *E. coli*. This means that the presence of benzoic acid from the extract has an inhibitory effect on Gram-negative bacteria, *X. oryzae* pv. *oryzae*.

This finding also revealed that eugenol acetate was identified in all extracts. However, the percentages for all of them are different. The methanol extract presence had a high percentage of 29.53%, followed by ethyl acetate extract (28.38%), hexane extract (20.14%) and acetone (19.15%). The antibacterial effect of eugenol acetate is because it contains the active phenol (OH) group (Adams, 2007). In addition, it may be due to the chemical composition containing a group of esters, which has a deadly effect on microorganisms (Kon and Rai, 2012). In previous findings by Hateet *et al*. (2016), eugenol acetate identified from *Myrtus communis* showed a broad antibacterial effect against clinical pathogenic bacteria. This different finding may be due to the presence of a different variety of *P. betle*.

Piper betle has several active chemicals such as chavibetol acetate, eugenol acetate, eugenol, allylpyrocatechol monoacetate, allylpyrocatechol (Ramji *et al.*, 2002), chavibetol (Rathee *et al*., 2006), hydroxychavicol, hydroxychavicol acetate (Bhalerao *et al*., 2013), piper betol, piperol A and B (Zeng *et al*., 1997), caryophyllene (Sugumaran *et al*., 2011b), isoeugenol, methyl eugenol (Rawat *et al*., 1989) and phytol (Jantan *et al*., 1994). Besides these, the propenylphenol group's eugenol and hydroxychavicol, as well as the terpene or sesquiterpene group's β-caryophyllene are listed as significant compounds in betel leaves (Singtonggratana *et al*., 2013). The phenolic compounds eugenol and hydroxychavicol have been found as an active antibacterial compound having antibacterial activities that are promising (Syahidah *et al.*, 2017).

CONCLUSION

The antibacterial potential of *P. betle* leaf extracts were discovered in this investigation. Methanolic extracts were the most efficient and the concentration 1× MIC (0.625 mg/mL) was recommended for the treatment of BLB infections in a glasshouse trial. GC-MS analysis identified 2,3-Dimethyl benzoic acid, eugenol acetate and 4-Allyl-1,2-diacetoxybenzene as major compounds from the methanolic extract which are active antibacterial compounds with varying inhibitory activities. According to the findings, the concentration of active chemicals in the extract had a significant impact on its antibacterial effect against *Xoo*. This alternative treatment could effectively prevent bacterial pathogens from developing antimicrobial resistance, protect the rice plant and save rice production from a disastrous disease outbreak. The findings of this study will also help researchers and agriculturists innovate the use of this extract for agricultural purposes, such as an eco-friendly bactericide on crop treatment.

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