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ANTIBACTERIAL ACTIVITY OF *PSIDIUM GUAJAVA* L. METHANOL LEAF EXTRACT AGAINST PLANT PATHOGENIC BACTERIA IN THE GENERA *PECTOBACTERIUM* AND *XANTHOMONAS*

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ABSTRACT : The antibacterial activity of methanol leaf extract of *Psidium guajava* L. was performed on six plant pathogenic bacteria, namely *Xanthomonas citri, Xanthomonas euvesicatoria, Xanthomonas oryzae, Xanthomonas oryzicola, Pectobacterium carotovorum* and *Pectobacterium chrysanthemi* by cup-plate agar diffusion method. Different concentrations gave different range of means diameter inhibition zones where at concentrations o f 25, 50, 100 and 200 mg/ml, the range was 10.00±0.00 mm to 15.00±0.00 mm, 12.00±0.00 mm to 17.00±0.00 mm, 15.00±0.00 mm to 20.00±0.00 mm and 16.00±0.00 mm to 25.00±0.00 mm, respectively. *X. oryzae* gave the highest mean diameter inhibition zone when tested with all concentrations compared to the mean diameter inhibition zones of other plant pathogenic bacteria. The minimal inhibitory concentration (MIC) of the methanol extracts was performed by macrobroth dilution technique and the lowest concentration used that was still able to inhibit the bacterial growth was 0.391 mg/ml for *X. oryzae.*

Keywords: *Psidium guajava* L., cup-plate agar diffusion, inhibition zone, minimal inhibitory concentration (MIC).

INTRODUCTION

For many years, there were many introduction to the various plant that were able to control certain diseases either clinically, pharmacologically, or on other infectious diseases in various field of study. The plant extracts tend to be the better choices when treating clinical isolates that were tested on human, animals and other living organisms, as people are more aware that the chemicals produced in treating diseases from plants extracts tend to be much safer than any other synthetic medicines or antibiotics for controlling infectious diseases. Thorough understanding, the application of plant extracts on plant pathogenic bacteria should also be applied in the same way as they were found to be successful when applied to clinical isolates. It can also reduce the usage of chemical pesticides that are harmful to human, animal and other living organisms when they feed on agricultural products.

There were millions of plants that contain various active ingredients that were able to treat diseases in different ways. But, not all active chemicals in plants can be successfully applied to treat certain diseases that do not react or resistance to certain active compounds exist in the plants, suggesting that wrong plant choices can leads to wrong treatment results or no results obtained at all. In this study, Psidium guajava L.were chosen as the antibacterial agent against plant pathogenic bacteria because these plants have many biological activity potentials of being anti-diarrheal, antimicrobial, antimalarial, hepaprotective effects, wound healing and many more as reported by Gutiérrez, et al., 2008. On the other hands, the reasons for selecting Psidium guajava plant as the antibacterial agent against plant pathogenic bacteria It is also due to its safety aspect as shown by the toxicology test done by Jaiarj, et al., 1999 and Manosroi, et al., 2006 that proved that the plant extracts do not bring any harmful effect on mice and histological results by Martinez, et al., 2001 also indicated that there was lack of genotoxic effects of the extracts on the tested mouse, that strongly suggested that P. guajava L. can be selected as one of the safest and cheapest biological control agent against plant pathogenic bacteria through natural remedies obtained from the plant.

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Xanthomonas sp. and *Pectobacterium* sp. are believed to be the most important plant pathogens that are becoming destructive when affecting specific crops which strengthen the agriculturist trust that these plant pathogenic bacteria cannot be left to spread widely amongst the valuable crops which will lead to serious crop and economic losses. Both of these genera must immediately treated as early as possible before the whole crops are destroyed. The use of antibiotics and chemical pesticides in controlling these detrimental plant pathogens seem to be less effective as these pathogens started to be resistant towards the antibiotics and chemical pesticide used. In order to face this problem, the use of *P. guajava* L. leaf extracts could be another successful method in controlling severe plant diseases. Therefore, the objective of this study was to determine the antibacterial activity of *Psidium guajava* L. methanol leaf extracts against plant pathogenic bacteria.

MATERIALS AND METHODS

Bacteria isolates

All plant pathogenic bacteria used in this study were obtained from the Microbiology Laboratory, Department of Plant Protection, Universiti Putra Malaysia, Selangor, Malaysia. They were *Xanthomonas citri, Xanthomonas euvesicatoria, Xanthomonas oryzae, Xanthomonas oryzicola, Pectobacterium carotovorum* and *Pectobacterium chrysanthemi*. The cultures were maintained on nutrient agar (NA) for continuous viability.

Plant materials

Fresh *Psidium guajava* L. leaves were obtained from the university agriculture park, UPM Serdang. Fresh leaves were rinsed with tap water to wash away any dirt and insects sticking to the leaves. The leaves were then shade dried at room temperature for 2 weeks and were ground into fine powder using a coffee grinder. The fine powder was kept in tight flasks to avoid any contamination.

Preparation of plant extracts

200 g of the *P. guajava* L. fine powder was soaked in 2 L absolute methanol for 2 days. The extract was then filtered through Whatman No. 1 filter paper and was evaporated to dryness using rotary evaporator and stored at 4°C until further use (Egharevba, et al., 2010).

Antibacterial activity

The cup-plate agar diffusion method as recommended by Abdelrahim, et al., 2002 with slight modification was used to test the antibacterial activity. 150 µl of standardized bacterial suspension with O.D. = 0.1 was spread over 20 mm thick Mueller Hinton Agar (MHA) with L-shaped glass rod and left for 5 minutes to dry. Six wells with 0.6 mm diameter was then made with sterile cork borer. Each well was filled with 50 µl of different extracts concentration of 25, 50, 100 and 200 mg/ml. Two other empty wells were filled with 50 µl of Streptomycin sulfate (30 µg/ml) as positive control and 50 µl of absolute methanol as negative control. All plates were then incubated at 30°C for 24 h and were done in three replicates. The clear zones around the wells were measured with a ruler in millimeter (mm) as the zone of inhibition for the extracts.

Statistical analysis

The mean values (mean ± standard deviation) of inhibition zones were statistically analyzed with SAS 9.1 by one-way analysis of variance (ANOVA) using Tukey's studentized range. Significant difference were considered significant at $P<0.05$ (Obeidat, 2011).

Minimal inhibitory concentration (MIC)

For MIC determination, the lowest concentration from the antibacterial activity (25 mg/ml) that was still able to inhibit the bacteria was taken as the starting concentration for making dilution. Eleven test tubes labeled (T1-T11) were each filled with 1 ml Mueller Hinton Broth (MHB) for each bacterial isolate. Two fold serial dilutions (1:1) were made by filling in 1 ml of the extracts to the first test tube and vortexed to mix the solution well. Then 1 ml of the solution from T1 was withdrawn and diluted into second test tube (T2), and the procedure was repeated until test tubes 10 (T10). 100 µl of the standardized bacterial suspension was then inserted into each test tube except for T10 which served as positive control (MHB + plant extracts only). The last test tube (T11) served as the negative control (MHB + bacteria suspension). Finally, 50 µl of 2,3,5-Triphenyltetrazolium chloride (TTC) was added to each test tube. All the test tubes were then incubated for 24 h at 30°C. The test tube that did not change into red color was recorded as the MIC (Motamedi, et al., 2010).

Phytochemical screening of plant extracts

The methanol leaf extracts were then subjected to phytochemical screening based on previous studies for detection of the active chemical constituents such as alkaloid, flavonoid, tannin, saponin, terpenoid (Obadoni and Ochuko, 2001; Edeoga, et al., 2005; Aiyelaagbe and Osamudiamen, 2009; Mariita, et al., 2011), phenol (Chitravadivu, et al., 2009) and glycosides (Chandrappa, et al., 2011).

RESULTS

Antibacterial activity

The antibacterial activity of *Psidium gujava* L. methanol leaf extracts was successfully evaluated *in-vitro* against six plant pathogenic bacteria from the genus *Xanthomonas* (*X. citri, X. euvesicatoria, X. oryzicola* and *X. oryzae*) and *Pectobacterium* (*P. carotovorum* and *P. chrysanthemi*). These bacteria are known to cause detrimental effect on valuable food crops. The extracts exhibited antibacterial activity toward all tested bacterial isolates (Table 1), showing inhibition zone diameter ranging from 10.00±0.00 mm to 25.00±0.00 mm. Streptomycin sulfate (30µg/ml) as positive control showed inhibition towards five bacterial isolates ranging from 14.00±0.00 mm to 22.00±0.00 mm, except for *X. euvesicatoria* which recorded no inhibition zone. There was no inhibition recorded for all plant pathogenic bacteria when tested with absolute methanol as the negative control.

Table 1 Means (n=3)of inhibition zone diameter (mm) of *Psidium guajava* L. methanol leaf extract against plant pathogenic bacteria.

Bacteria species	Positive control	Negative control	Extracts concentration (mg/ml)			
			25	50	100	200
Pectobacterium carotorum	20.00±0.00^{ab}	0	11.00±0.00^{b}	14.00±0.00^{ab}	15.00±0.00^{b}	16.00±0.00^{c}
Pectobacterium chrysanthemi	20.00±0.00^{ab}	0	12.00±0.00^{ab}	14.00±0.00^{ab}	16.00±0.00^{b}	18.00±0.00^{bc}
Xanthomonas citri	18.00±0.00^{b}	0	10.00±0.00^{b}	12.00±0.00^{b}	17.00±0.00^{ab}	19.00±0.00^{bc}
Xanthomonas euvesicatoria	0	0	11.00±0.00^{b}	12.00±0.00^{b}	17.00±0.00^{ab}	20.00±0.00^{b}
Xanthomonas oryzicola	14.00±0.00^{c}	0	12.00±0.00^{ab}	15.00±0.00^{ab}	18.00±0.00^{ab}	20.00±0.00^{b}
Xanthomonas oryzae	22.00±0.00^{a}	0	15.00±0.00^{a}	17.00±0.00^{a}	20.00±0.00^{a}	25.00±0.00^{a}

Diameter of inhibition zones are expressed as mean±SD. The mean ±SD within column with the same letter are not significantly different (Tukey's studentized range test, $\alpha = 0.05$).

At the lowest concentration (25 mg/ml) of leaf extracts used, the highest mean of inhibition zone was recorded for *X. oryzae* (15.00±0.00 mm), followed by *P. chrysanthemi* and *X. oryzicola* (each 12.00±0.00 mm), *P. carotovorum* and *X. euvesicatoria* (each 11.00±0.00 mm), and *X.* citri (10.00±0.00 mm). At the concentration of 50 mg/ml, the highest inhibition zone was recorded also for *X. oryzae* with mean 17.00±0.00 mm, followed by *X. oryzicola (*15.00±0.00 mm), *P. carotovorum* and *P. chrysanthemi* (each14.00±0.00 mm), and *X. citri* and *X. euvesicatoria* (each12.00±0.00 mm).

At concentration of 100 mg/ml, the lowest mean diameter inhibition zones was recorded for *P. carotovorum (*15.00±0.00 mm), while *P. chrysanthemi* 16.00±0.00 mm. *X. citri,* and *X. euvesicatoria* showed diameter inhibition zone of 17.00±0.00 mm, *X. oryzicola* 18.00±0.00 mm, and the highest mean diameter was recorded for *X. oryzae* (20.00±0.00 mm). At the highest extract concentration of 200 mg/ml, the highest mean diameter was recorded for *X. oryzae* (25.00±0.00 mm), followed by *X. oryzicola* and *X. oryzae* (20.00±0.00 mm), *P. chrysanthemi* and *X. citri* (18.00±0.00 mm and 19.00±0.00 mm, respectively), and *P. carotovorum* (16.00±0.00 mm). Figure 1 illustrates the comparison in diameter inhibition zones between plant pathogenic bacterial isolates tested with different concentrations of the extracts.

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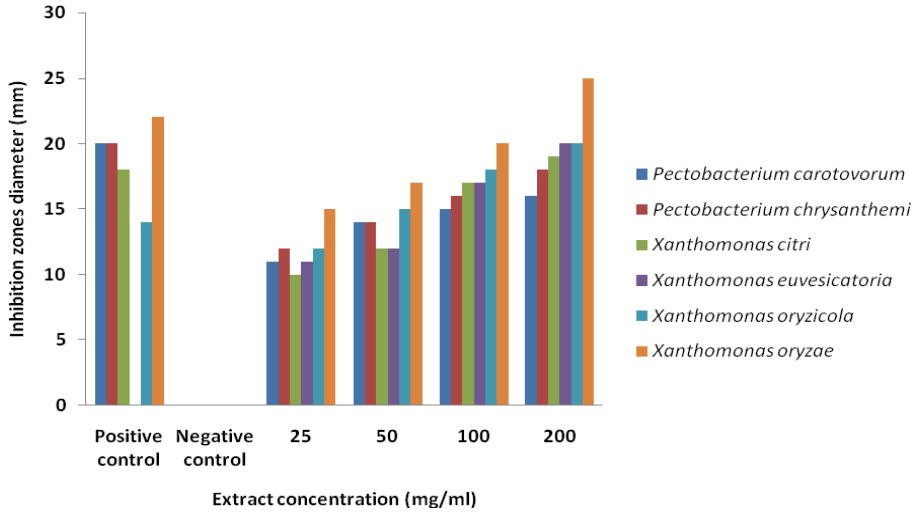

Figure 1 : Inhibition zones diameter of *Psidium guajava* methanol leaf extracts against plant pathogenic bacteria

Minimal inhibitory concentration (MIC)

For the minimal inhibitory concentration (MIC) obtained from all the tested bacterial isolates, the lowest concentration that still inhibited the plant pathogenic bacteria was at 0.391 mg/ml for *X. oryzae* followed by *X. euvesicatoria* with lowest concentration recorded at 0.781 mg/ml, as shown in Table 2. For other tested isolates, the lowest concentration recorded able to inhibit was at the concentration of 1.563 mg/ml. The MIC value taken from each plant pathogenic bacterial isolate was measured using 5% 2,3,5-triphenyltetrazolium chloride aqueous solution that showed no color changes of MHB (yellow), indicated that there was no viable bacterial cell presence, while the change in color of MHB from yellow to red showed the presence of viable bacterial cell, and will not be taken as MIC value.

Table 2 Minimal inhibitory concentration (MIC) of *Psidium guajava* (L.) methanol leaf extracts against plant pathogenic bacteria by macro-broth dilution methods.

Bacteria species	MIC (mg/ml)
Pectobacterium carotovorum	1.563
Pectobacterium chrysanthemi	1.563
Xanthomonas citri	1.563
Xanthomonas euvesicatoria	0.781
Xanthomonas oryzae	0.391
Xanthomonas oryzicola	1.563

For the phytochemical screening of *P. guajava* L. methanol leaf extracts, and in order to determine the presence some major compound known as secondary metabolites, the results obtained were shown in Table 3. It showed the positive results for the presence of alkaloid, saponin, phenol, tannin, flavonoid, terpenoid and steroid, as determined by the color change.

Table 3 Phytochemical screening of *Psidium guajava* (L.) methanol leaf extracts

Chemical Constituents	Positive Results	Methanolic extracts
Saponin	Small bubbles (foam)	+
Alkaloid	Creamish precipitate	+
Phenol	Dark blue	+
Tannin	Blue green color	+
Glycoside	Blue color in the acetic acid layer	-
Flavonoid	Dark yellow	+
Terpenoid	Reddish brown coloration	+
Steroid	Green	+

DISCUSSION

Methanolic leaf extracts of *Psidium guajava* L. were effective against all plant pathogenic bacteria tested. In fact, this study is the first report of using *P. guajava* leaf extracts to control plant pathogenic bacteria. This can probably replace the use of synthetic antibiotics as they can become resistance to the antibiotics when used. Most of the previous research using *P. guajava* L. leaf extracts was conducted on clinical isolates such as *Vibrio cholera* (Rahim, et al., 2010), *Bacillus sp.*, *Staphylococcus aureus, Escherichia coli, Klebseilla sp., Pseudomonas sp., Proteus vulgaris, Salmonella sp.,* and *Shigella sp.* (Buvaneswari, et al., 2011). However, there were no study on the antibacterial effect of this extract on plant pathogenic bacteria and from the results obtained from this study showed that *P. guajava* L. was able to inhibit the plant pathogenic bacteria showing remarkable results when tested at different concentrations. This study proved that, *P. guajava* L. methanol leaf extracts could be developed as next antibacterial agents to control plant pathogenic bacteria as it was able to inhibit the growth of plant pathogenic bacteria. All isolates of *Xanthomonas* sp. gave good inhibition zones when tested with all range of concentration.

The use of 2,3,5-Triphenyltetrazolium chloride (TTC) for the determination of minimal inhibitory concentration (MIC) is possible due to its ability to changed the color of the broth from light yellow to pink, for intermediate growth of the bacterial isolates and producing dark red for heavy growth of bacteria cell inside the tubes. TTC was also able to help observe the existence of any bacterial cell when conducting the experiment involving the observation for visible bacteria cell for MIC determination as suggested by Caviedes, et al., 2002 and Summanen, et al., 1992. For all the bacterial isolates tested for MIC, the majority of the bacteria reduced TTC and gave clearly defined endpoints, that gave results to the last tube that was colorless taken as MIC due to no bacterial cell existence in the tubes. The next tube that changed into pink or red indicated the concentration used in that particular tube was not effective in inhibiting the bacteria, which mean that the extract concentration contained in the tube wastoo low that it became not effective to treat plant pathogenic bacteria itself. As being reported by Eloff, 1998, tetrazolium salts act as electron acceptors and are reduced by biologically active bacteria from a colorless compound to red in case of TTC.

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The antibacterial activity exhibited for all plant pathogens tested might be linked to the existence of plant secondary metabolites such as flavonoid, phenol, triterpenoid, saponin and tannin in the methanol leaf extracts. This might be one of the reasons for the production of inhibition zones as these compounds were well known for their antimicrobial effect as reported by Sofowara, 1986, Akinjogunla, et al., 2010, Ghosh, et al., 2010, Jaiarj 1999, Vieira, et al., 2001, Lozoya, et al., 2002 and Wei, et al., 2000. Phytochemical screening of *P. guajava* L. methanolic leaf extracts obtained in this study gave the same results for the presence of tannin, saponin and flavonoid as reported earlier by Olajide, et al., 1999 and Gutiérrez, et al., 2008.

CONCLUSION

Psidium guajava L. methanol leaf extracts gave significant antibacterial activity against *Xanthomonas* sp. and *Pectobacterium* sp. which indicate that these extracts have the potential being the next natural biological control agent for plant pathogenic bacteria. The major compounds found in this study, can be used to produce new and useful natural chemical product as alternative to the use of synthetic antibiotics and chemicals for the control of plant pathogenic bacteria.

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