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Antimicrobial Activity of *Psidium Guajava* Leaves Extract Against Foodborne Pathogens

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Abstract: *Foodborne disease has been associated with microorganisms like bacteria. This study was designed to evaluate the methanolic extracts of Psidium guajava leaves for antibacterial activity properties. The antibacterial activities of the extract were studied against four different bacteria causing foodborne infection; Staphylococcus aureus, Bacillus cereus, Escherichia coli and Salmonella typhimurium using disc diffusion method. The methanol extracts of P. guajava leaves were active against the Gram-positive bacteria S. aureus and B. cereus with minimal inhibitory concentration (MIC) of 30 mg/ml. However, the extract was inactive against the Gram-negative bacteria E. coli and S. typhimurium although 1000 mg/ml concentration was used. Thus, our results suggest that P. guajava leaves extract contain antimicrobial compound that could be used to treat Gram positive bacterial infections that cause foodborne infection. Furthermore, this study supports its sustainability of medicinal use in the treatment of foodborne infections in worldwide.*

Keywords: *Psidium guajava, foodborne pathogens, Antimicrobial Sensitivity Testing (AST), minimal inhibitory concentration (MIC).*

I. INTRODUCTION

Foodborne outbreak has become a major public health issue in many regions and countries around the world and become one of the main causes of death among infants especially in developing countries (Salgado *et al.,* 2009). Malaysia reported to have high cases of foodborne diseases due to the proper condition and temperature that enhance the growth of most bacteria (Abdul-Mutalib *et al.,* 2015). However, the use of commercial antibiotic as treatment is having become less significant due to its potential to cause toxicity and development of antibiotic resistant (Tacconelli *et al.,* 2018). Recently, various medicinal plants have been recognized by their medicinal value. The plants are use in antimicrobial activity and used throughout past as an alternative approach to preserve food. Shaheena *et al.,* (2019) noted that *P. guajava* bark, fruit, leaves possess potential to treat dysentery, diarrhoea, flatulence, gastric problems and control blood glucose levels.

Psidium guajava (P. guajava) which belong to the family Myrtaceae is plant that cultivated in tropical and subtropical areas. It is commonly called as 'guava' (English), 'guayabo' (Spanish) or 'jambu batu' (Malaysia). The guava tree is medium sized and produce fruits within four years. Guava trees have spread widely throughout the tropics because they can thrive in variety of soils and bear fruits quickly (Kamath *et al.,* 2008). The tree can be easily identified by its spreading branches, small thin, smooth, cooper coloured bark that flakes off, showing a greenish layer beneath.

Since the 1950s, guava, particularly its leaves, has been the subject of diverse research initiatives to identify the chemical identity of its constituents, pharmacological properties, and history in folk medicine (Gutierrez *et al.*, 2008). According to Shaheena *et al.*, (2019) different parts of this plant are used medicinal purposes for the treatment of various human ailments such as wound, ulcers, bowels and cholera. For an example, the extract of guava leaves has a long history of use for medicinal purposes to treat gastroenteritis, diarrhoea, dysentery and a few other conditions (Morton, 1987). Besides, Hirudkar *et al.,* (2019) had tested the antidiarrhea and antimicrobial activities of *P. guajava* leaf extracts. The guava juice showed some positive effects on reducing coughs and the leaves demonstrated some antimicrobial activity on *S. aureus* and *β-streptococcus* groups.

Foodborne disease can be described as any illness resulting from the consumption of food. The bacteria are the most common factors that cause this disease. According to Kamath *et al.*, (2008) the common bacteria causing foodborne diseases are *Staphylococcus*, *Shigella*, *Salmonella*, *Bacillus*, *Escherichia coli* (*E. coli*), *Clostridium* and *Pseudomonas*. *Staphylococcus aureus* is known as one of the major causes of foodborne infections in humans in both community and hospital (Kadariya *et al.*, 2014). Food poisoning is caused by the toxins produced by the bacteria. Meanwhile, *Bacillus cereus* is a spore forming foodborne pathogen commonly associated with food products such as meat, vegetables, soup, rice and other dairy product (Marrollo, 2016). Salmonella are recognized as one of the leading causes of foodborne diseases. These pathogen bacteria have been focused in the food safety industry. *E. coli* are among the common foodborne microorganism that cause infection and intoxication. Food poisoning caused by *E. coli* is usually associated with eating unwashed vegetables and meat contaminated post-slaughter (Yang *et al.*, 2017). Therefore, the aim of this study is to determine antimicrobial activity of *P. guajava* methanol extraction against foodborne bacteria pathogens and to determine the minimal inhibitory concentration (MIC) of *P. guajava.*

II. MATERIALS AND METHODS

Chemical and Reagents: Methanol (Hmbg Chemicals, United Kingdom); dimethyl sulphoxide (DMSO) (Fisher Scientific, United Kingdom); distilled water (dH_2O) (Alam Medic Sdn.Bhd); dehydrate barium chloride (Hamburd Chemical Ltd); concentrated sulphuric acid (HmbG Chemicals, Germany).

Media culture: Mueller Hinton Agar (Merck); Mueller Hinton Broth (Merck); Tryptic Soy Agar (Merck); Tryptic Soy Broth (Merck); Nutrient Agar (Merck).

Bacteria strains: In this study there are four different foodborne bacteria were used. The organisms in these tests include two Gram positive bacteria and two Gram negative bacteria: *S. aureus* (ATCC 25923) and *B. cereus* (ATCC 11778), *E. coli* (ATCC 25922) and *S. typhimurium* (ATCC 14028). All bacteria strains were obtained from the UiTM Puncak Alam Campus, Selangor, Malaysia.

Antibiotics for positive controls: Antibiotic for positive control that have been used in this study were tetracycline (30µg) and streptomycin (25 µg) (Oxoid Ltd, England).

Preparation of *P. guajava* extract

Plant materials: The fresh leaves of *P. guajava* used in this study were collected from Malaysian Agricultural Research and Development Institute (MARDI), Universiti Putra Malaysia, Serdang, Selangor, Malaysia. The taxonomic identification from the plant was Research Centre, MARDI.

Preparation of extract: 200 gram of fresh *P. guajava* leaves were thoroughly washed in water and air dried at room temperature of 220 ^{0}C. Then, the leaves were oven-dried at 60 ^{0}C for 3 days. The plant materials then were finely grounded into powdered form with the help of an electric grinder. The methanol extract was prepared by soaking 100 grams of

powder in 500 ml of 80% methanol (1:5 ratio) for total of 48 hours. After that, solvent was twice filtered through Whatman No. 3. The solvent then was evaporated in oven at 60 ^{0}C to dryness under reduced pressure. After evaporation, the extracts were put into beaker and extracted compound were used for antimicrobial assay. This extract was considered as the 100% concentration of the extract. The extra dry weight was kept in an airtight bottle (Jaiarj *et al.*, 1999).

Preparation of pure concentration of *P. guajava* extract: 500 mg/ml solution of the extract was prepared by dissolving 0.5 g (500mg) of the extracts in 0.1 ml of dimethyl sulphoxide (DMSO) and 0.9 ml dH_2O.

Preparation of different concentration *P. guajava* extract: The modification of concentration of *P. guajava* extract was prepared by dissolving 0.5 g of *P. guajava* extract in 0.1 ml dimethyl sulphoxide (DMSO) and 0.9 ml dH_2O to get the concentration of 500 mg/ml for the screening test against S. *aureus, B. cereus, E. coli* and *S. thypimurium.* Then, the MIC for tested on *S. aureus* and *B. cereus* the 300 mg/ml, 150 mg/ml, 50 mg/ml and 10 mg/ml solutions of the extracts been prepared.

Antibacterial Susceptibility Testing (AST) using Disc Diffusion Technique: Filter paper discs with diameter 6 mm were autoclave before being used. Then, the different concentrations of the *P. guajava* extraction were impregnated into sterilized paper discs. By using micropipette, 20 µl of each concentration were pipette onto that sterile paper Whatman AA paper disks. The discs were dried in oven for two hours before been use in AST.

The positive and negative controls must be done to confirm the efficiency of the AST. The negative controls were made by impregnated 20 µl DMSO onto the sterile filter paper (Nair and Chanda, 2007). A parallel analysis with commercial antimicrobial agents was conducted in order to compare their antimicrobial efficiency with the plant extracts. The positive controlled used included Streptomycin 25 µl g was for *B. cereus,* while Tetracyline 30 µg *for S. aureus, E. coli* and *S. thypimurium.*

Inoculation of Bacteria: Bacteria stock cultures were maintained on Nutrient Agar slant. They were sub cultured monthly and subsequently stored at 4 ^{0}C. The bacteria were cultured on Nutrient Agar for 24 hours at 37 ^{0}C in order to get a single colony that were used to perform AST. For experimental purposes, the organisms were inoculated into 5 ml Tryptic Soy Broth (TSB) and incubated at 37 ^{0}C for 2 to 3 hours. The grow of the four bacteria culture in tryptic soy broth (TSB) were adjusted using distilled water to yield approximately 1.0 x 10^8 CFU/ml by compared with 0.5 McFarland standard. (NCCLS, 2000). With the use of a moist sterile swab the suspensions were spread on plates of Mueller Hinton agar (MHA).

AST Using Disc Diffusion Method: Paper discs that had been impregnated with 500 mg/ml concentration of *P. guajava* leaves extract were placed on the surface of agar with flamed forceps and gently pressed down to ensure contact. After that, positive and negative control were placed. Plates were left to get dry for about 15 minutes and were incubated at 37 ^{0}C overnight. Each test was performed in duplicate and the antibacterial activity were reported as the mean of bacteria inhibition in diameters (mm) that be measured by using ruler. A reading of more than 6 mm indicated there were bacteria growth inhibition.

Determination of Minimum Inhibitory Concentration (MIC) using Disc Diffusion Method: The bacteria inoculums were prepared only for bacteria that were showing the sensitivity against *P. guajava* extract in the AST screening process. There are four different concentrations of *P. guajava* were used in this method. Then the antimicrobial discs were applied onto agar. The zones of inhibition were measured, and all tests were done in duplicate to ensure the reliability.

III. RESULTS AND DISCUSSION

The usage of plant as remedies has been practiced long time ago. The WHO has catalogued more than

20,000 plants species with medicinal properties providing treatments for diseases (Goncalves *et al.,* 2008). *P. guajava* is medicinal plants used in tropical and subtropical countries to treat many disorders such as diarrhoea, cough and gastrointestinal disorder (Hirudkar *et al*., 2019).

Hirudkar *et al*., (2019) have reported that quercetin is the main flavonoid of *P. guajava* leaves that had many pharmacological activities. The author found that the antimicrobial effects of methanol extract from the leaves were probably due to the essential oils present in the plant. Further study should be done in order to identify the specific bioactive components responsible to the antimicrobial activity of guava leaves extract.

In this study, the *P. guajava* leaves used had to be dried first in order to decrease the water content in fresh leaves to prevent the effect of solubility of subsequent separation during methanol extraction. This method is important to stabilize the bioactive component in their exact form.

Ncube *et al.,* (2008) noted that successful of biologically active compounds from plant material is depend on the type of solvent used in the extraction process. The choice of solvent used will give effect on type of the bioactive compounds that we are interested to isolate. Besides, the difference in the antimicrobial activities between organic extracts can also be related to several other factors such as geographic area, extraction methods and laboratory technique. Furthermore, environmental and seasonal conditions also affect the bioactive component from the same type of plant.

According to Parekh *et al* (2005), the most commonly used solvents for investigations of antimicrobial activity in plants are organic compounds (methanol and ethanol) and aqueous (water), but among this two, organic solvents have been found to give more consistent antimicrobial activity compared to water extracts. In this study, methanol had been used for extraction solvent. Many previous studies had shown that methanol extraction of *P. guajava* leaves have potential to give antimicrobial effect (Chah *et al.,* 2006; Nair and Chanda, 2007; Goncalves *et al.,* 2008).

Variation in extraction methods are commonly found in length of the extraction period, solvent used, particle size and solvent to sample ratio. In order to shorten the length of the extraction period, the contact between the methanol solvent and plant material must be longer. So, dried leaves had to be ground into small particles size. By doing this, it will increase the surface area for the extraction thereby increasing the rate of extraction (Ncube *et al.,* 2008). In this study, grinded guava leaves powder was soaked in methanol for total of 48 hours.

Many researchers used homogenization method of plant material in solvent for investigating antimicrobial activity of plant extraction. According to Dilika *et al.,* (2000), homogenized solvents extract had higher activity compared to the shaken extract of the same solvent. The solvent that had been used in this study is methanol and it has been described in Material and Method section.

Furthermore, the solvent-to-sample ratio also can give effects on the quantity and quality of extraction constituents obtained. In this study, the solvent to sample ratio being used is 5:1, where 100 g of powdered guava material were put in 500 ml of methanol. This ratio also has been practiced in previous study done by Lopez *et al.,* (2003) where 20 gram of *Piper betle* Linn. leaves were extracted in 100 ml of 95 % ethanol.

Antimicrobial Sensitivity Test (AST) is used to determine the efficacy of potential antimicrobials from *P. guajava* leaves extracts against several different microbial species that cause foodborne diseases. Agar disc diffusion method was chosen since this technique has been widely used to assay plant extracts for antimicrobial activity in the previous study (Chah *et al.,* 2006).

Before bacteria are inoculated in MHA media, the turbidity of inoculums bacteria must be compared with 0.5 McFarland standards. It has been recommended by NCCLS, (2000) that the turbidity of inoculums used for antimicrobial sensitivity

testing should be adjusted to the concentration of a 0.5 McFarland standard. It is important because when we overlooked during adjustment of the inoculums, it may lead to false susceptible and false resistant results. If the inoculums are too light it may cause false susceptible of bacteria results and if too heavy, it may cause false resistant of bacteria results.

For the screening of *P. guajava* extract at the concentration 500 mg/ml, the result showed that there were antimicrobial activities of the extracts against gram positive bacteria (*S. aureus* and *B. cereus*) compared to gram negative bacteria (**Table 1**). *S. aureus* ATCC 25923 that are common foodborne causative agent had to be the most susceptible of all organism being studied (zone of inhibition is 14 mm). Lutterodt *et al.*, (1999) have also reported the antimicrobial effects of guava leaves extracts and found that there inhibited the growth of the *S. aureus*. This result also supports the finding result of study done by Goncalves *et al.*, (2008) where methanol extraction has obtained better results from hexane and ethyl acetate. For *B. cereus* ATCC 11778, the result gets from this study supported the study done by Nair and Chanda (2007) where methanol extract of the same concentration (500 mg/ml) of *P. guajava* leaves had shown growth inhibition effects on the *B. cereus*. However, in their study, they concluded that the acetone extraction gives a better result on bacteria inhibition compare to methanol extraction.

Most of the extracts were more active against gram-positive bacteria than gram-negative bacteria, which are supported with previous reports that plant extracts are more active against gram-positive bacteria than gram-negative (Cosentino *et al.*, 1999). In this study, there are no growths inhibitions seen in *E. coli* ATCC 25922 and *S. typhimurium* ATCC 14028 at the 500 mg/ml concentration (**Table 1**). The results get still the same, although the concentration of the extracts has been increased into 700 mg/ml and 1000 mg/ml concentration. The results from this study are not parallel compare to the previous study, where at concentration 500 mg/ml of plants extract there were antibacterial activity against the *E. coli* (Nair and Chanda, 2007; Obunbaku and Ilusanya, 2008). *E. coli* O157:H7 and *E. coli* were the most resistant of the organisms tested supporting the findings of Caceres *et al.*, (1990), proving resistant to *P. guajava* extracts.

Furthermore, the results for *S. typhimurium* in this study also is opposite to the findings of Lutterodt *et al.*, (1999) in which the organic extracts of *P. guajava* leaves demonstrated effectiveness against different strains of Salmonellae including *S. typhimurium* but only at concentration of 10 mg/ml. Even though this study has followed a similar concentration used in the previous study, results get are not parallel maybe due to several factors include low aseptic techniques. Besides, the differences in results obtained from different laboratories could be due to methods used in each study.

In this study, Gram negative bacteria have proved generally more resistant than the gram-positive bacteria across guava extracts. According to Parillon and Edward (2006), the resistance of bacteria could be cause by capsule related or due to genetic factors or cell membrane permeability. The surface properties of most bacteria are determined by the exact molecular composition of their plasma membrane and cell wall, including lipopolysaccharide (LPS), and the function of surface structures such as flagella, fimbriae and capsules. It has been found that the gram-negative bacteria cell walls also have other distinct structures such as outer membranes which are semi permeable phospholipid bilayers made up of phospholipids (20-30%), LPS (30%) and protein (40-50%) which collectively contribute to their innate resistance against antimicrobials.

Tetracycline (30 µg) and Streptomycin (25 µg) were used as positive control in this study to make sure that the technique has done well, and the results get are valid. Meanwhile, DMSO was used as negative control because it is used to resuspend the residues. Other than that, investigation of the antimicrobial effect of guava leaves involved a comparison of their effect with commercial antibiotics. From this study, the commercial antibiotics had a larger inhibitory effect than guava leaf extractions. This is also not surprising and proves that commercially antibiotics that had been perfected and tested should

be used for treatment whenever it's available (Goncalves *et al.,* 2008).

By using agar disk diffusion techniques, the MIC of *S. aureus* and *B. cereus* had been determined (**Figure 1**). These techniques also have been practiced by study done by Dilika *et al.,* (2000) to obtain the MIC values. The MIC of *P. guajava* extract against S. *aureus* is 30 mg/ml. The study done by Odunbaku and Ilisanya (2008) had revealed that methanolic extract of *P. guajava* would only show active activity at concentration of at least 500 to 1000 mg/ml while for ethanolic extracts of at least 300 to 500 mg/ml. However, in this study results had shown that at concentration of only 30 mg/ml of methanol extraction the growth inhibition towards *S. aureus* was seen. It means that methanol extracted the active compounds of guava leaves responsible for their antimicrobial activities.

No information on MIC of *B. cereus* towards guava extract is available by previous study. But, for the purpose of comparison, the only closes information on MIC of *B. cereus* towards plant extract is MIC of *B. cereus* on ginger extract done by Natta *et al.,* (2008). The MIC of *B. cereus* against ginger extract is 0.00625 mg/ml but in this study, the MIC of *B. cereus* is 30 mg/ml. It means that ginger extractions are better against *B. cereus* compared to guava extraction.

IV. CONCLUSIONS

The authors conclude that organic extracts from medicinal plants could inhibit certain common foodborne pathogenic microorganisms. Extracts from *P. guajava* leaves inhibited gram positive *(S. aureus and B. cereus)* bacteria been tested but not in gram negative *(E. coli and S. typhimurium)* extract. The results obtained from this study have support the findings of recent studies done by Patel *et al*., 2019 and Shaheena *et al*., 2019. Results revealed that the guava leaves extract can be used as source of natural antimicrobial agents which can be applied to treat foodborne diseases causes by gram positive bacteria and validates its use in traditional medicine. In conclusion, the results showed that all the study objectives were achieved successfully.

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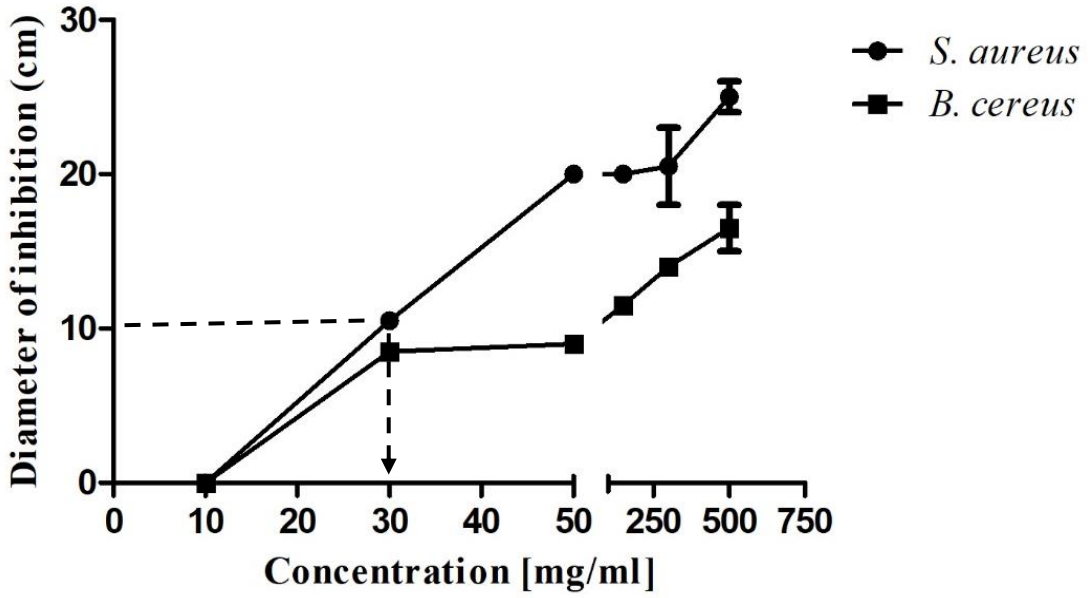
Table 1: Antimicrobial sensitivity testing (AST) of *P. guajava* extract against 4 different foodborne pathogens.

Figure 1: Determination of minimal inhibitory concentration (MIC) against Gram positive foodborne pathogens.



	Mean of inhibition diameter (mm)			
	Staphylococcus aureus	*Bacillus cereus*	*Escherichia coli*	*Salmonella typhimurium*
Tetracycline	30.5	-	24.5	25.0
Streptomycin	-	27.5	-	-
P. guajava extract (500 mg/ml)	25	16.5	0	0
P. guajava extract (700 mg/ml)	-	-	0	0
P. guajava extract (1000 mg/ml)	-	-	0	0

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