PHYTOCHEMICAL AND ANTIMICROBIAL INVESTIGATION AND COMPARISON BETWEEN YOUNG AND MATURE *Psidium guajava* LEAVES EXTRACT

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ABSTRACT.Ethnomedicinal properties of *Psidium guajava L.*, or also known as guava leaves has been known since years ago. Nowadays, a lot of guava leaves-based products emerge in industries such as tea and cosmetic. The aims of this study are to examine and compare the variation in the phytochemical constituent as well as the antimicrobial efficacy of young and mature leaves extract. Phytochemical analysis shows the presence of phenol, tannin, terpene, saponin, and flavonoid in the mature leaves methanolic extract. A similar result was obtained in the young leaves extract but no saponin was detected. Total phenols content in young and mature leaves were determined at a total of 31.2 mg and 162 mg GA/g. Both leave extract was carried out to determine the antimicrobial properties by tested against two Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus cereus*) and one gram-negative bacteria (*Salmonella enterica*) through the disk-diffusion method by employing 40 µL of leaf extract solution per disk. Based on the observation, both young and mature extracts exhibited inhibitory activity (<6.0 mm) against the tested bacteria with different sensitivity. At the concentration of 10 mg/mL, mature leaves extract shows higher efficacy on *S. enterica* and *B. cereus* where the inhibitory zone was measured at 9.3 mm and 7.8 mm, respectively, compared to young leaves which is not sensitive to *S. aureus* but the inhibitory zone on *B. cereus* around 7.2 mm while S. aureus at 7.2 mm higher than mature leave extract. This can be concluded that the *P. guajava* mature leave displayed the best to applied as medicinal purposes as its high variety of phytochemical content and high efficacy as antimicrobial activity.

KEYWORDS: *Psidium guajava* L., extraction, phytochemical, antimicrobial, disk-diffusion method

INTRODUCTION

Malaysia is among the most developed country yet still maintaining and conserving a huge area of forest which contributing to the sustainability of the ecosystem in terms of wildlife habitat, biodiversity, clean water source and temperature control. The world acknowledges Malaysia as one of the 12th countries in the world in terms of mega-biodiversity with high endemism (Abdullah *et al*. 2015). Aligned with that, around 2000 plant species were reported to possesswith promising medical benefits and capable of treating various types of diseases (Abas *et al*. 2006). Moreover, a number of local plants are potential to be applied as antimicrobials and antifungals. Salvamani *et al.*, (2016) explained the use of *Amaranthus viridis* leaf to treat hypercholesterolemia. *Centella asiatica*, *Clinacanthus nutans* Lindau and *Gynura procumbens*, a group of local herb can be applied to treat open wound and healed in a short time (Somboonwong *et al*. 2012; Khoo *et al*. 2018; Alam *et a*l. 2016; Zahra *et al*. 2011; Jarikasem *et al*. 2013). Ahmad *et al*., (2011) and Subenthiran *et al.*, (2013) proved the alternative function of *Carica papaya* leaves extract to treat dengue fever and/ or dengue hemorrhagic fever. Malaysian edible plant such as *Piper nigrum*, *Syzygium*

aromaticum, *Murraya koenigii*, *Allium cepa*, and *Persicaria odorata* were reported to inhibit the development of pathogenic bacteria; *Escherichia coli, Edwardsiella tarda, Aeromonas hydrophila, Citrobacter freundii, Vibrio parahaemolyticus, Vibrio vulnificus, Staphylococcus aureus, Streptococcus agalactiae and Streptococcus anginosus* (Najiah *et al.* 2011). Ali *et al.*, (1996) studied from 61 medicinal plants, only three show Co-existing antiviral and cytotoxic activities namely *Polygonum minus, Eugenia michelii* and *Mentha arvensis*.

However, several parameters need to be emphasized to obtain the most optimum result followed by application *in vitro* and *in vivo*. For the example, plant maturity and harvest timing is required to ensure the efficiency of production in the term of yield as well as the optimum amount of phytochemical and physicochemical in the plant. Murukan and Murukan, (2018) determined the young leaf extract exhibited potent antioxidant potentiality when compared with the mature leaf extract of *Tectona grandis*. Chang *et al.*, (2018) study about polyphenolics profile and antioxidant activity in each of *Clausena lansium* leaf development stages such as leaf buds, young, mature and old leaves, and the result shows old leaves was selected as an economical sources of bioactive compounds which can be applied in nutraceutical and pharmaceutical product. The age of coffee leaves affect phytochemical profiles associated with free radical scavenging and anti-inflammatory activities (Chen *et al.*, 2018), and this study in agreement with Nobossé *et al.*, (2018) determined that 45-day-old *Moringa oleifera* leaves show the best phytochemical content and antioxidant properties compared to leaves aged 30 and 60 days

Psidium guajava has commercial value due to the taste, flavor and aroma. The plantation of this fruit is an income to the number of Malaysian farmer. In different perspective, *Psidium guajava* leave was anciently revealed it potential pharmacological uses to treat diarrhea, dysentery, hypertension, diabetes mellitus, and others (Metwally *et al*. 2010; Chiari-Andréo *et a*l. 2017). In this study, *Psidium guajava* leaves was used to compare the presence of phytochemical compound and to test the antimicrobial properties in young and mature leaves, and lastly to determine the potential source for pharmacological application or alternative medication.

MATERIALS AND METHODS

SAMPLE PREPARATION AND EXTRACTION

Young and mature leaves were randomly selected from the same *Psidium guajava* tree at Keningau, Sabah (5°20'48.9"N 116°10'16.5"E). Each leaf were clean by direct wash under tap water flow, then dried for seven days at room temperature. The leaves were ground using benchtop blender to increase the surface area and enhance the rate of extraction. In a conical flask, the ground sample marked as young (YL) and mature leave (ML) were mixed with 95 % methanol at the ratio of 1:5 (w/v), respectively, followed three days incubation with fully sealed using parafilm aid by tin foil coating to avoid evaporation and light contact. Then, the methanol extraction was filtered and dried using a rotary evaporator. The extractant was kept in -20^{o}C until subsequent use (Yoke et al., 2013). Yield for each sample were determined based on the percentage of extracts obtained per raw sample quantity

PHYTOCHEMISTRY ANALYSIS

Phytochemical analysis was carried out by qualitatively test for the presence of saponin, phenol, tannin, terpene, and flavonoid, and quantitative test to determine total phenol compound.

QUALITATIVE TEST FOR SAPONIN

The extractant was dissolved in 5 mL of distilled water, then hand shaking for 15 minutes. A thin layer foam indicating the presence of saponin in the sample (Hossain et al., 2013)

QUALITATIVE TEST FOR PHENOL AND TANNIN

The extractant was mixed with 2 mL of 2% ferum (III) chloride. The presence of greenish blue or black indicating as the presence of both compound.

QUALITATIVE TEST FOR TERPENE

This test also known as Salkowski test. The extractant was mixed with 2 mL of chloroform followed by slowly additional of 2 mL concentrated sulfuric acids. The appearance of reddish brown color at the interphase shows the existence of terpene.

QUALITATIVE TEST FOR FLAVONOID

Several drop of concentrated sodium hydroxide into the extractant to give yellowish color. The changes of the yellowish color to colorless after the additional of diluted hydrochloric acid indicating the presence of flavonoid.

QUANTITATIVE TEST FOR PHENOL

The reactant was prepared by the additional of 0.5 mL extractant, 2.5 mL of 10% reagent Folin-Ciocalteu and 2.0 mL of 7.5% sodium carbonate. The mixture was incubated for 45 minutes at 45°C. Phenol compound was quantified by measuring the absorbance at 765 nm, and referring to the gallic acids standard which expressed as mg GA/g (Jaradat et al., 2015).

ANTIMICROBIAL ACTIVITY DETERMINATION

BACTERIA CULTURE PREPARATION

A gram-negative bacteria; *Salmonella enterica* (ATCC 14028), and two gram positive bacteria; *Staphylococcus aureus* (ATCC 25923) and *Bacillus cereus* (ATCC 11778), were tested for their development capability after exposed with the extract of YL and ML *P. guajava*. Each of the strain were cultured in a nutrient agar plate and incubated for 18 – 24 hours at 37°C. Referring on the application of aseptic technique, a bacterial colony was transferred in a 5 mL of nutrient broth followed by orbital shake incubation for 8 hours at 37°C. One millimeter of each organism were sub-cultured by spreading on a plate of nutrient agar (Auwal et al., 2013).

DISK DIFFUSION ASSAY

Disk diffusion assay was used as a preliminary screening to determine the antimicrobial activity. The dried extractant from rotary evaporator was dissolved in a methanol solution to produce the stock solution at the concentration of 100 mg/mL. This stock solution was used to prepare the diluted extraction at 1.25, 2.5, 5, 10 and 20 mg/mL. 6 mm sterile disk was load with 40 µL of extractant for each leaves sample at five different prepared concentrations then dried. For the negative control disk containing 40 µL of methanol while positive control was load with 40 µL of ampicillin (20 µL/mL). Each of treated disk was onto the surface containing cultured bacteria. The plate was incubated for 24 hours at 37°C. The inhibition

zone was measured in the diameter of clear zone development (mm) around the disk and recorded the observation (Balouiri et al., 2016)

RESULTS AND DISCUSSION

Comparing with aqueous solution, alcohol extraction method was previously proving to enhance the yield and phytochemical content. Meaning, alcohol is effective to disrupting the structure of nonpolar cell wall or seed cause the release of polyphenol. Moreover, using aqueous extraction method may affect the number of phenolic compound due to the activity of polyphenol oxidase enzyme, while alcoholic environment able to inhibit the enzymatic activity (Tiwari et al., 2011). Table 1 shows both samples of *Psidium guajava* leaves were extracted using 95% of methanol and the yield for YL is much lower; 4.32 % compared to ML; 8.64%. Next, phytochemical compound was qualitative analysed and determined that saponin was not detected in YL but presence in ML. Other compounds such phenol, tanin, terpene and flavonoid were positively detected. Studied by Achakzai et al., (2009) shows the distribution of secondary metabolite from different age and development stage, and determined that each concentration referring to phytochemical compound are vary. For the example, the mature leave and stalk of *M. azedarach, B. vulgaris, P. gladulosa* and *T. aphylla* contained high concentration of saponin and phenolic compound compared to young stalk. The data was in line with this study, where the total amount of phenolic compound; extrapolated from the gallic acid standard curve, in ML was much higher at 162 ± 2.08 mg GA/g while compared to YL at 31.2 ± 1.04 GA/g.

Table 1: Determination of percentage yield in *Psidium guajava* leaves; YL and ML, followed by qualitative analysis on phytochemical constituents and quantitative measurement of total phenolic compound (TPC) using gallic acid concentrations as the standard curve.

		***Psidium guajava* leaves**	
		YL	**ML**
Yield	%	4.32	8.64
Qualitative*	Saponin	-	+
	Phenol	+	+
	Tanin	+	+
	Terpene	+	+
	Flavanoid	+	+
Quantitative	TPC (mg GA/g extract)	31.2 ± 1.04	162 ± 2.08

*presence of constituent (+), no presence of constituent (-).

ANTIMICROBIAL ASSAY

Psidium guajava leaves were test to determine the antimicrobial activity; *S. aureus, B. cereus* and *S. enterica* via the method of disk diffusion in different plate; YL and ML, where each disk were load with the different concentration of the extractant. The inhibition zone was measured at millimeter scale (mm), and any inhibition zone lower than 6.0 mm considered as not sensitive.

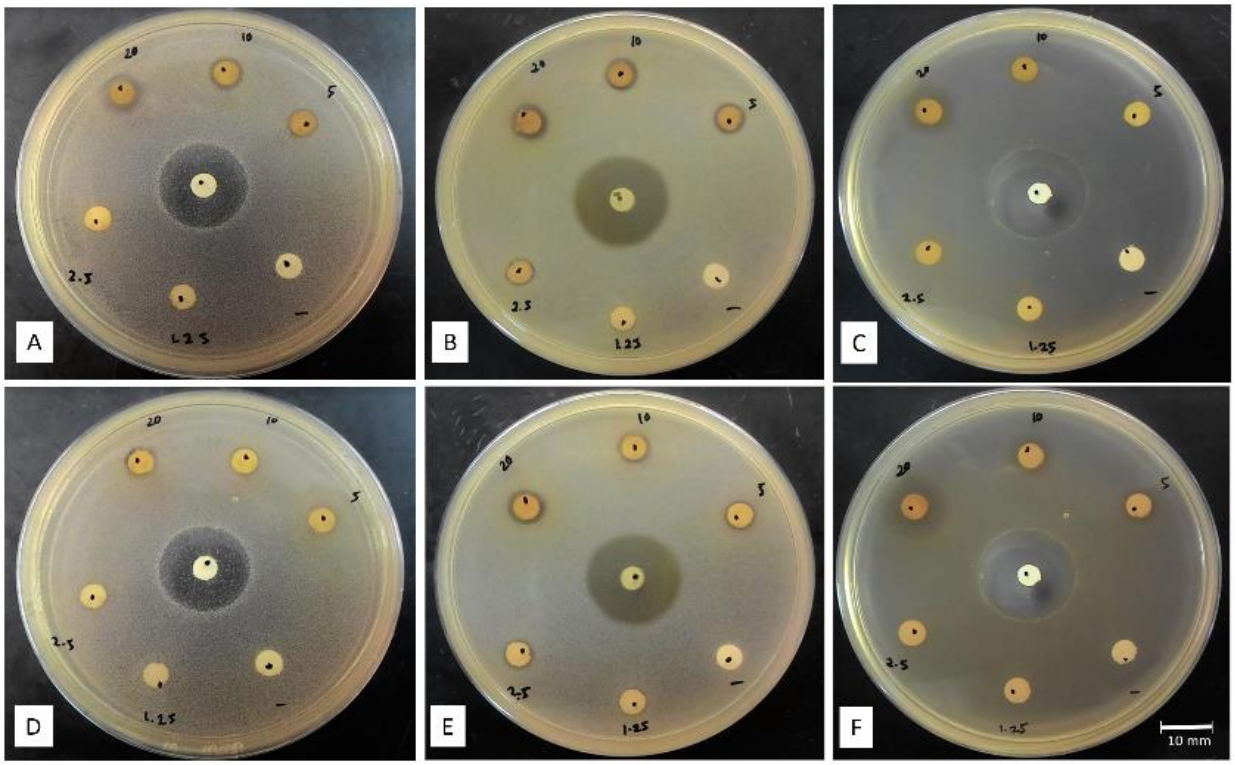

Figure 1: Disk diffusion test to determine the antimicrobial activity by *Psidium guajava* extractant. A, B and C show *S. aureus*, *B. cereus* and *S. enterica* were test with positive and negative control, five different concentration of YL extractant, respectively, while D, E and F show the same test was conducted on *S. aureus*, *B. cereus* and *S. enterica*, respectively, using ML extractant.

Antimicrobial activity was assess based on the formation of inhibition or clear zone, and both samples show antimicrobial capability but different sensitivity. For positive control from all the test plate show the inhibition zone was measured at the average of 24.7 ± 1.2 mm. Negative control considered as unaffected. The minimum concentration of YL extract to suppress the development of *S. aureus*, *B. cereus* and *S. enterica* at the treatment of 5.0, 2.5 and 20 mg/mL with the inhibition diameter of 6.5, 6.5 and 10.3 mm, respectively, while ML extract was determined at 10, 2.5 and 5.0 at the diameter of 6.5, 6.5 and 7.2 mm, respectively (Table 2 and 3). Clearly, ML extractant exhibited higher antimicrobial activity at low concentration treatment. Moreover, at 20 mg/mL treatment using ML, higher inhibition zone was measured at 12.3 mm on *S. enterica* plate but S. aureus only around 7.2 mm but no slightly different with the treatment by YL.

Table 2: Antimicrobial activity determination based on the diameter of inhibition zone after treated with YL extract. NS = Not sensitive.

Bacteria	**Inhibition zone (mm)**						
	20 mg/mL	**10 mg/mL**	**5.0 mg/mL**	**2.5 mg/mL**	**1.25 mg/mL**	**Positive control**	**Negative control**
S. aureus	8.7 ± 0.2	7.2 ± 0.5	6.5 ± 0.1	NS	NS	24.7 ± 1.2	NS

B. cereus	8.3 ± 0.2	7.2 ± 0.1	6.7 ± 0.1	6.5 ± 0.1	NS	24.7 ± 1.2	NS
S. enterica	10.3 ± 0.6	NS	NS	NS	NS		

Table 3: Antimicrobial activity determination based on the diameter of inhibition zone after treated with ML extract. NS = Not sensitive.

Bacteria	**Inhibition zone (mm)**						
	20 mg/mL	**10 mg/mL**	**5.0 mg/mL**	**2.5 mg/mL**	**1.25 mg/mL**	**Positive control**	**Negative control**
S. aureus	7.2 ± 0.6	6.5 ± 0.3	NS	NS	NS	24.7 ± 1.2	NS
B. cereus	8.3 ± 0.1	7.8 ± 0.1	6.7 ± 0.4	6.5 ± 0.2	NS		
S. enterica	12.3 ± 0.4	9.3 ± 0.2	7.2 ± 0.1	NS	NS		

Bacteria are protected from their environment by a layer of menbran, an integrity which is important for survival of bacteria. This membrane consists of basic compounds such as phospholipids and lipopolysaccharides stabilized by divalent Mg and Ca cations (Maris, 1995). The present study shows the presence of phytochemicals known to be able to exhibit medical and physiological properties. For example, tannin is a polyphenolic compound bound proline-rich protein which interferes the synthesis of protein and has been proven to have antibacterial activity (Biswas et al, 2013). On the leaves of plants, tannins are usually found at the upper epidermis layer, however, in evergreen plants, they are equally distributed to all leaf tissues, protecting plants from predators by affecting the taste (Hoffmann, 2003). Tannins possess a great impact on herbivories nutrition because the ability of these compounds form complexes with many types of molecules including carbohydrates, proteins, polysaccharides, bacterial membrane cells and enzymes involved in digestion of proteins and carbohydrates. Tannins are generally regarded as agents that impede the growth of microorganisms, but the mechanisms involved are poorly understood. This polyphenolic compound is reactive to the bacterial cell wall and the secreted enzyme outside the cell. These two interactions may inhibit the transport of nutrients into the cells and infect the growth of the organism (McSweency et al, 2001).

Flavonoids are hydroxyl polyphenolic compounds, known to be produced by plants on the reaction of infections by microbes. This aspect is widely studied and is found to have antimicrobial activity against various microorganisms based on in vitro study. The ability of flavonoid compounds to antimicrobial activity has been linked to form complexes with soluble protein and external cells especially bacterial cell walls (Biswas et al, 2013). Flavonoid compounds are very crucial in plant resistance to pathogenic

microbes. It is implied that the interaction of flavonoid antibacterial activity is based on the capacity of this compound to deactivate microbial bond, adhesion and packing the carrier protein cells. Fat-soluble flavonoids may also disrupt microbial membranes, alter fluid and may obstruct the respiratory chain. Flavonoids also inhibit development of some root pathogens, especially fungi, and in general, iso flavones, flavanes and flavanones are recognized as effective microbial agents (Mierziak et al, 2014). Generally, the aromatic properties of terpene compounds found to be an agents of antimicrobial activity (Biswas et al, 2013). Based on a study conducted by Inoue et al (2004), *S. aureus* is sensitive towards alcoholic terpene which cause severe damage to cell membranes which related to the modes of alcoholism. The initial leakage rate and the number of efflux potassium ions are used as an index for antibacterial activity. This activity depends on the length of the aliphatic chain as well as the configurations of functional group and double bonds.

Saponin is a glycoside compound that able to give significant effect to the developement of gram-positive organisms such as *S. aureus* (Khan et al., 2018). According to a study conducted by Inderjit et al (1999), bacterial growth is inhibited by β-ecsin saponins. Certain members of the Rhizobiaceae family such as *Agrobacterium tumefaciens*, *Rhizobium lily*, and *Bradyrhizobium japonicum* are most sensitive to β-ecsin. Different saponin and aglycone structures have different effects on different genus of bacteria. One of the main physiological effects of saponin on bacteria is the leakage of proteins and certain enzymes from their cells. All saponin compounds with glucose replacement in triose carbon will cause leakage on all tested bacterial strains. The data obtained from this study suggest that bacterial membranes can be affected by saponin which lead to the loss of important activity, especially in gram-positive genera e.g. from Bacillus species. Phenolic compounds considered as a strong antimicrobial compounds where the ability to cause membrane damage resulting to the release of cell contents and subsequently cell lysis, protein precipitation, deactivation of oxidase and dehydrogenase bound membrane, deactivates the enzyme in the cytoplasm by forming an unstable complex and lipophilic molecules are trapped by a phospholipid membrane. For example, when low concentrations, cell constituents such as nucleic acids and glutamic acid are released. If the concentration is high, phenols will block the release, as well as cause the contamination of bacterial protein and membrane cell lysis (Maris, 1995). In hospital, phenol is used as a disinfector during wound treatment.

CONCLUSION

Screening of phytochemicals present in YL extracts and ML was carried out and the presence of phenol, tannin, terpene and flavonoid compounds were identified in both samples while saponin compounds were present only on mature leaf extracts. The amount of phenolic compounds in YL and ML extracts has been determined which is higher in ML extracts than in YL. The antimicrobial properties of YL and ML has been determined which found that both exhibited antimicrobial activity at different sensitivities to different bacterial species. For future studies, it is proposed to isolate phytochemical compounds that have antimicrobial properties and are tested for more patogenic bacterial species for strengthen the result followed by product development using *Psidium guajava* leaves.

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