

Antibacterial properties of *Persicaria minor* (Huds.) ethanolic and aqueous-ethanolic leaf extracts

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ABSTRACT

Persicaria minor known as small water-pepper is used traditionally for the treatment of dandruffs and stomach indigestion. Therefore, this study was designed to evaluate the antibacterial activity of plant leaf material. 30% aqueous-ethanol and 100% aqueous were used for solvent extraction. Both extracts were evaluated for total protein and polysaccharide contents and results were compared. The extracts were then tested against four strains of bacteria; *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 11229, *Staphylococcus aureus* ATCC 6538 and *Pseudomonas aeruginosa* ATCC 1544, at different concentrations using disc-diffusion and microplate dilution assays with penicillin being used as a positive control standard. Both extracts showed antibacterial activity against *S. aureus*, *E. faecalis*, and *E. coli*, respectively with aqueous-ethanolic extract being more potent. However, none of the extracts were active against *P. aeruginosa*. Results from this study truly illustrated high potential of *P. minor* leaves to be used topically as antibacterial agent for controlling of tested colony.

INTRODUCTION

The utilization of medicinal plants as raw materials in the development of drug is well acknowledged as of late. Since the beginning of time, plants have been the backbone of medicinal remedy of which is a vital part of every society where the Food and Drug Administration and control have approved many plant herbs for therapeutic purposes (Basu, 2004; Kraisintu, 2003). The potential plant herb for the present study is *Persicaria minor* (Huds.) from the family of *Polygonaceae* collectively known as smart-weeds (Thomson *et al.*, 2013). *P. minor* which is previously known as *Polygonum minus*, is an herb whose leaves are used extensively in Southeast Asian cooking. The plant has many common names depending on the country. *P. minor* is recognised as “*daun laksa*” in Singapore, while in Indonesia, Malaysia and Vietnam it is called

“*Daun kesum*” (Vimala *et al.*, 2011). *Persicaria minor* is used as a vegetable for source of protein (Thomson *et al.*, 2013). It has a pungent taste and therefore used as a spice with medicinal importance especially as antioxidant agent (Vimala *et al.*, 2011). The leaves have generally been controlled to treat the following: Indigestion, stomach associated wounds and fungal infections.

In addition to its traditional uses, most of Chinese Buddhists are largely taken the leaves for the fact that it reduces sexual desire, thus the Monks usually grow *P. minor* or *P. odorata* as their garden plant and consume as a supportive stride in their celibate life (Deepti, 2013; Hunter, 1996). Furthermore, *P. minor* has been reported to have potentiality as antioxidant, antibacterial, anti-fungal, anti-diarrheal, anti-inflammatory, anticytotoxic, antiulcer and antigen-toxicity activities (Uyub *et al.*, 2010; Qader *et al.*, 2011; Wasman *et al.*, 2010). *P. minor* has been studied extensively for its chemical compositions associated with the medicinal properties of the plant. Some studies have reported that *P. minor* possesses antimicrobial properties due to the presence of aldehydes and terpenes as the main significant components of essential oil available in the leaves (Mackeen *et al.*,

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1997; Matasyoh *et al.*, 2008; Ridzaun *et al.*, 2013; Sosongko *et al.*, 2011). However, to date, there is paucity of data available on *P. minor* on various pathogenic bacteria. Thus, the current study was aimed to evaluate the antibacterial activities of different standardized leaf-extracts of *P. minor* against some bacterial pathogens and scientifically support its traditional claim for preventive health care.

MATERIALS AND METHODS

Collection and preparation of plant material

Fresh leaf sample of *Persicaria minor* (Fig. 1) was collected from plantation in Johor Bahru, Malaysia on 17th September 2014. The plant was verified by Forest Research Institute of Malaysia (FRIM). The young leaves from the plant were excised, washed and shed-dried at room temperature for 4-5 days. The dried leaves were then taken into the laboratory for crushing and grinding into powder for extraction and further analysis.



Fig. 1: *Persicaria minor* (Huds.)

Extraction of plant material

The prepared grounded leaves of *P. minor* were soaked into two different solvents such as 100% aqueous and 30% aqueous-ethanol solvents for 4 days. 100g of air-dried, coarsely powdered plant material was extracted successively with 1000 mL (ratio; 1:10) each of distilled water and 30% aqueous-ethanol based on solvent polarity using maceration. Briefly, the aqueous-ethanol solvent for extraction was prepared by mixing 700 mL of distilled water and 300 mL of absolute ethanol, to obtain 30 % aqueous-ethanol (Ogbe *et al.*, 2012). The mixture was left in a shaker for 4 days. After 4 days, the extracts were filtered through filter paper No. 1 and allowed to dry in a water bath at 60°C for solvent elimination. The final extracts obtained were kept in a refrigerator at 4°C for total protein and polysaccharide contents and antibacterial screening.

Chemicals, solvents and instruments

Both chemicals and solvents used were of analytical grade including ethanol (Merck), distilled water (Universiti Teknologi Malaysia), dimethylsulfoxide (DMSO), Bovine Serum

Albumin, Fraction V (Sigma Aldrich), glucose (Sigma Aldrich), nutrient broth (NB), nutrient agar (NA), Folin-Ciocalteu reagent (Sigma Aldrich), 96-well plates and 45 UV/VIS Spectrophotometer.

Standardization of the plant extracts

Total protein estimation

Total protein content was analyzed using a standard method described by Lowry *et al* (1951). In this procedure, 50 mg of each extract was weighed and dissolved in 10 mL sterile distilled water in a 15 mL centrifuge tube and agitated with vortex mixer for 2 minutes. Centrifugation at 2700 rpm for 10 minutes was followed and the supernatant solution of each extract was utilized for analysis. 0.1 mL of supernatant was taken and mixed with 0.9 mL distilled water in a test-tube to make a 1 mL solution. 3 mL of reagent C which was prepared by mixing 50 mL of reagent A with 1 mL of reagent B. Reagent A contains 2% sodium trioxocarbonate in 0.1 Normal sodium hydroxide; also reagent B contains 0.5% copper-II sulfate in 1% potassium sodium tartarate. So when reagent C was added, a 0.2 mL Folin-Ciocalteu reagent was also added and taken to incubation at room temperature for 30 minutes. Bovine Serum Albumin (Fraction V) was utilized as a standard ranging from 12.5 to 100 µg/mL. The extracts together with the standards were prepared on triplicates basis and absorbance against BSA concentration of the protein was measured at 600 nm alongside a blank constant full of all the reagents with the exception of the sample extracts. Therefore, total protein contents were estimated from linear regression equation gotten from the graph of a standard curve.

Total polysaccharide estimation

In this procedure, 200 mg of each plant extract was weighed and dissolved in 7 mL hot ethanol 80% in a centrifuge tube to remove sugar. Centrifugation at 2700 rpm for 10 minutes was followed after the sample mixture is agitated with a vortex mixer for 2 minutes. With residual pellets, the procedure was repeated four times until washing did not give color with anthrone reagent. The residual pellets were dried at a particular temperature in a water bath. The dried residue was extracted using 5 mL of distilled water together with 5 mL of 25% hydrochloric acid (HCl) at 0°C for 20 minutes. Centrifugation of the tubes at 2700 rpm for 10 minutes was conducted and clear supernatant filtrate was kept. Another extraction were also repeated to obtain the same filtrate and kept. The filtrate were mixed with distilled water and made up the volume 100 mL in a sterile conical flask. A 0.1 mL of supernatant was transferred into a centrifuge-tube and mixed up with 0.9 mL distilled water. When 4 mL of anthrone reagent were added, the tubes was heated in a water bath at about boiling point of water for 8 minutes. After cooling at room temperature, the color intensity which was observed to be green were measured at absorbance wavelength 630 nm against a blank constant containing full of all the reagents except sample. The same was also adopted with glucose standard solutions prepared in a range of 20 to 100 µg/mL. All test-samples together with the standard

solutions were prepared on triplicates basis, and glucose concentration were estimated from regression linear equation, generated from the graph of a standard curve. The estimated total starch in $\mu\text{g/mL}$ was determined by multiplying the glucose content calculated from the graph with conversion factor 0.9.

Preparation of different concentration of the extracts and standard agent used for the study

A standard method of Cheesbrough (2012) was adopted for the preparation of different concentrations. Stock solutions of 30% aqueous-ethanol and 100% aqueous crude extracts were prepared by dissolving 1g each in 5mL of dimethylsulfoxide (DMSO) in sterile universal bijou bottles to get 200 mg per 1mL DMSO stock. Stock solutions for all the extracts were also reconstituted by two-fold serial dilution into three varied concentrations (100 mg, 50 mg and 25 mg in 1mL of DMSO). Penicillin standard antibiotic (10 mg/mL) as positive control and 10% dimethylsulfoxide (DMSO) as negative control were used for the study during antibacterial sensitivity testing.

Bacterial organisms and culture media

The test organisms used for the antibacterial evaluation of *P. minor* extracts were *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 11229, *Staphylococcus aureus* ATCC 6538 and *Pseudomonas aeruginosa* ATCC 1544. They were all obtained as stock pure culture of American Type Culture Collection (ATCC) strains from Postgraduate Bioassay Laboratory, Universiti Teknologi Malaysia (UTM). The type of media that was utilized for qualitative and quantitative antibacterial screening were Nutrient agar (NA) and Nutrient broth (NB) which were all prepared according to the manufacturers' specifications.

Standardization of bacterial stock culture

The culture stock of each test organism was sub-cultured onto new fresh nutrient agar plates for 24 hours at 37°C to get the active strains accordingly. The organisms contained in suspended broth media were subjected to agitation for some minutes prior to incubation for 24 hours at 37°C in order to get the most active strains needed for quantitative antibacterial testing (minimum inhibitory concentration and minimum bactericidal concentration). Turbidity was also compared with that of McFarland standard turbidity.

Antibacterial screening test

Kirby-Bauer disk diffusion technique was adopted for screening of antibacterial activity of the plant extracts. The grown active colonies from the culture plates obtained after 1-day incubation period were isolated using an inoculating wire-loop and mixed with 5 mL of sterile saline solution 0.9% contained in different test-tubes for each test organism. The mixture was agitated by a vortex mixer for 1 minute and the turbidity were compared and readjusted with that of McFarland standard turbidity. The nutrient agar plates were divided into four regions

and labeled with different concentrations. A standardized suspension of each bacterial culture from saline solution were isolated and streaked evenly in different directions on agar plate with the aid a sterile cotton bud. Later, the sterilized 8 mm in diameter of punched disc-papers containing 0.02 mL of the plant extract were placed with the aid of a pair of forceps onto the agar plates at an equidistant position and left for few minutes at room temperature for pre-diffusion. All test plates were taken for incubation in an upright position at 37°C for 18 to 24 hours. Discs containing the same amount of DMSO (10%) served as negative control while standard antibiotic discs of penicillin (10 μg) were used as positive control. The diameters of growth inhibition zones were measured in millimeter (mm). All tests and analysis were done in duplicates and hence, the data of antibacterial activity of *P. minor* leaf-extracts were expressed as mean \pm standard deviation.

Minimum inhibitory concentration (MIC)

The MIC of both test-extracts of *P. minor* leaves were investigated by broth dilution method in mg/mL using 96-well microplate (Karakoca *et al.*, 2013). 100 μL from each sample stock (200 mg/mL) was drawn and two-fold serial dilutions was carried-out to obtain six different concentrations; 100, 50, 25, 12.5 and 6.25 mg/mL. The least concentration at which no visible microscopic growth or turbidity is observed on the well plate bottom was taken as the MIC level measurement. Penicillin antibiotic standard was used as positive control.

Minimum bactericidal concentration (MBC)

Based on MIC results obtained, 10 μL of solution from the last-clear well of each of the test samples and their controls, respectively was pipetted on to the surface of nutrient agar plates and spread gently with a sterile glass rod to obtain uniformity on the surface. The inoculated plates incubated in an upright position for 24 hours at 37°C. The MBC was determined immediately after incubation as the least concentration at which 99% of the bacteria were killed.

RESULTS AND DISCUSSIONS

Extraction of *Persicaria minor* leaves

Standardization of *Persicaria minor* leaves extracts

Standardization of *P. minor* leaves was carried-out quantitatively for total protein and total polysaccharide contents in both extracts of *P. minor*. The total protein contents were estimated from the graph of a standard calibration curve of BSA using linear regression equation ($Y = 0.001x + 0.004$, $R^2 = 0.994$). The total polysaccharide contents were determined from the graph of a standard curve of glucose from linear regression equation ($Y = 0.009x - 0.0027$, $R^2 = 0.997$) in which absorbance was plotted against glucose concentration. Both types of extracts exhibited different contents of proteins and polysaccharides. The contents of proteins were found to be higher in aqueous-ethanol extract as compared to 100% aqueous extract. In the case of polysaccharide

contents, aqueous extract were found to be higher in comparison with aqueous-ethanol extracts. This indicates that aqueous ethanol is a better solvent for total protein extraction and water is better for total polysaccharide extraction. The less solubility nature of polysaccharide in aqueous-ethanol solvent is attributed to its structural characteristics (Hussain *et al.*, 2008). Thus, the combined nature of the solvent (water + ethanol) may be the reason for low solubility as total polysaccharide content is less in 30% aqueous-ethanol extract.

Table 1: Extraction yields of *Persicaria minor* leaves.

Name of Extract	Weight of extract (g/ 100g of sample)	Percentage yield (%)
30% aqueous-ethanol	10.6	10.6
100% aqueous	9.5	9.5

Table 2: Total protein and polysaccharide contents of *P. minor* leaf extracts.

Name of Extract	Total Protein (µg/mL)	SD	Total Polysaccharide (µg/mL)	SD
30% aqueous-ethanol	1713.67	± 0.01	13.9	± 0.04
100% aqueous	810.56	± 0.01	17.6	± 0.08

All tests, measurements, and values are presented in the mean value of three replicates (mean±SD).

Antibacterial test evaluation

Based on the results of antibacterial screening (Table 3), a strong activity of both extracts of *P. minor* was shown against some pathogenic bacterial strains. Crude aqueous-ethanol and aqueous extracts were examined for susceptibility at four different concentrations: 25 mg, 50 mg and 100 mg and 200 mg/mL of DMSO using disc diffusion method.

Table 3: Antibacterial evaluation of *Persicaria minor* leaf-extracts based on Disc Diffusion Technique (DDT) against the Pathogenic Bacteria.

Extraction Solvent/Standard Agent Used	Conc. (mg/mL)	Mean diameter Zone of Inhibition (mm) ± SD			
		<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>	<i>Enterococcus faecalis</i>
		ATCC	ATCC	ATCC	ATCC
30% aqueous-ethanol	200	19.50±0.50	18.00±1.00	N	19.33±1.25
	100	16.00±1.00	12.00±1.00	N	12.70±0.47
	50	11.50±0.50	7.75±1.13	N	8.33±0.47
	25	9.50±1.50	7.50±1.50	N	N
100% aqueous	200	16.60±0.50	16.45±0.50	N	15.70±1.25
	100	12.00±0.50	11.75±1.13	N	10.30±0.94
	50	9.40±0.03	7.75±1.25	N	7.30±0.94
	25	N	N	N	N
Penicillin	10	25.50±0.50	23.50±0.5	20.70±1.33	25.33±0.47
DMSO	-	N	N	N	N

Note: Diameter of 6mm (size of the disc)= indicates no activity(N), diameter <8.0mm indicates low sensitivity, diameter >8.0mm indicates high sensitivity. Penicillin serves as standard positive control, and DMSO (dimethylsulfoxide) standard negative control.

The bacterial isolates that were susceptible to both extracts were *Staphylococcus aureus*, *Escherichia coli* and *Enterococcus faecalis*. However, the aqueous-ethanol extract did not show any activity at concentration of 25 mg for *Enterococcus faecalis*. The most susceptible organisms for aqueous-ethanol extraction were *Staphylococcus aureus* and *Escherichia coli*, followed by *Enterococcus faecalis*. In the case of aqueous extract,

it was observed that *Staphylococcus aureus*, *Escherichia coli* and *Enterococcus faecalis* were susceptible at all concentrations except at 25 mg where no zones of inhibition were clearly shown for all the three bacteria (Table 3). However, none of the extracts at all concentrations were found to be effective against *Pseudomonas aeruginosa* (Table 3). The highest inhibition zones for *Staphylococcus aureus*, *Escherichia coli* and *Enterococcus faecalis* were observed with the highest concentration of both extracts with the diameter of 19.50 mm, 18.00 mm and 19.33mm for aqueous-ethanol extract and for aqueous extract 16.60 mm, 16.45 mm and 15.70 mm, respectively (Table 3). This illustrated that antibacterial sensitivity testing at highest concentration of 200 mg/mL for both extracts exhibited the strongest antibacterial activity against both *S. aureus*, *E. coli* and *E. faecalis* (Fig. 2 and 3).

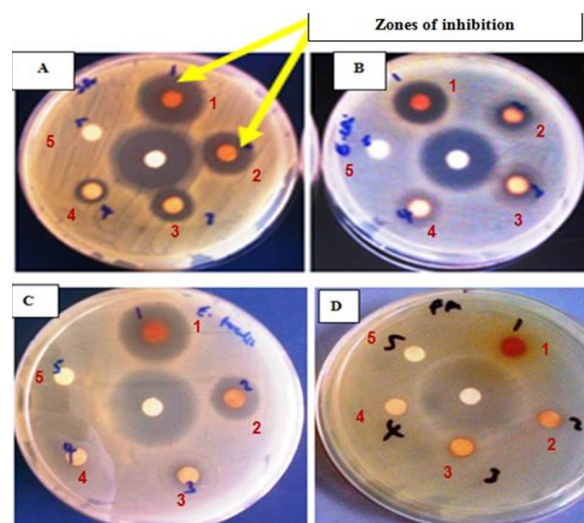


Fig. 2: Zones of inhibition produced by *S. aureus* (A), *E. coli* (B) and *E. faecalis* (C) with non susceptible *P. aeruginosa* (D) against 30% aqueous-ethanol extract of *P. minor* where 1 denotes 200 mg/mL, 2 denotes 100 mg/mL, 3 denotes 50 mg/mL, 4 denotes 25 mg/mL, 5 denotes DMSO.

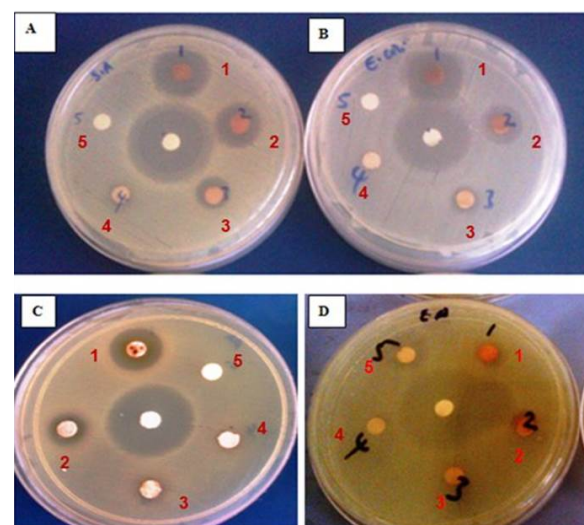


Fig. 3: Zones of inhibition produced by *S. aureus* (A), *E. coli* (B) and *E. faecalis* (C) with non susceptible *P. aeruginosa* (D) against water extract of *P. minor* where 1 denotes 200 mg/mL, 2 denotes 100 mg/mL, 3 denotes 50 mg/mL, 4 denotes 25 mg/mL, 5 denotes DMSO.

The standard antibiotic agent used for the study, penicillin, inhibited the growth of all four bacterial strains signifying that the bacteria are not resistant to penicillin. It has shown the highest mean of inhibition zone in *Staphylococcus aureus* and *Enterococcus faecalis* with diameter of 25.50 mm and 25.33 mm, followed by *Escherichia coli* with mean diameter of 23.50 mm, respectively. The lowest is observed in *Pseudomonas aeruginosa* with mean diameter of 20.70 mm. There was no zone of inhibition observed on the disk impregnated with DMSO (negative control).

Minimum inhibitory concentration (MIC)

The results of MIC were evaluated as the lower concentration of the extracts at which no visible macroscopic growth or turbidity was observed on the well plates bottom. Therefore, the extraction of *Persicaria odorata* using 30% aqueous-ethanol exhibited the most remarkable antimicrobial activities as compared to aqueous extract with MIC values of 50 mg and 100 mg/mL for *Staphylococcus aureus*, *Escherichia coli* and *Enterococcus faecalis*, respectively. Whereas, water extract revealed 100 mg/mL for *S. aureus*, *E. coli* and *E. faecalis* (Table 4).

Table 4: Results of Minimum Inhibitory Concentration (mg/mL) of *P. minor* leaf-extracts obtained from Micro-dilution procedure.

Type of Extract Used	Bacterial Isolates Utilized		
	<i>Staphylococcus aureus</i> ATCC	<i>Escherichia coli</i> ATCC	<i>Enterococcus faecalis</i> ATCC
30% aqueous-ethanol	50	50	100
100% aqueous	100	100	100
Penicillin Agent	6.25	12.5	6.25

Note: the higher the MIC value (100 mg/mL) the lower the activity of the extract thus, aqueous-ethanol extract has a better activity against *S. aureus* and *E. coli* (50mg/mL) than aqueous extract in which the MIC value against all the bacterial strains is the same (100 mg/mL).

Minimum bactericidal concentration (MBC)

The MBC was determined by sub-culturing the test dilution (used in MIC) on to a fresh agar medium and incubated again for 18-24 hours at 37°C. The concentration of *P. minor* extract in mg/mL that completely killed the bacterial strains has been taken as MBC. Based on MBC results, no visible growth of all three bacteria was observed on the plates after incubation period of 1 day except for *E. coli* plates where little growth was observed in the case of aqueous-ethanol extract at 50 mg/mL. This shows that MBC value is the same as MIC and thus, confirms the antibacterial activity of the plant extracts quantitatively. For *E. coli* strain, the MBC value is one level higher than the MIC, which is 100 mg/mL for aqueous-ethanol extract.

Consequences of using different extract concentrations

Based on the results of antibacterial activity of *P. minor* extracts observed from the current study, the mean zone of inhibition is directly proportional with increasing concentration of plant extracts. Thus, the activity of the plant extracts was dose-dependent. This means that, as the concentration of the extract increases the inhibition zones will also increase. This is probably due to the increase in active metabolic compounds present at higher extract concentration tested (Saad *et al.*, 2014). The aqueous-ethanol extract of *P. minor* leaves was found to possess antibacterial properties more effectively against *S. aureus*, *E. coli* and *E. faecalis* at 200 mg and 100 mg/mL disc potency than aqueous extract. This, however, agrees with the findings of Mackeen *et al.* (1997) where they reported that, bacteria (*Bacillus cereus*, *Escherichia coli* and *Pseudomonas aeruginosa*) and fungi (*Aspergillus ochraceus* and *Chryptococcus neoformans*) were sensitive to ethanolic extracts of *P. minor* leaves. However, based on the present study, it is observed that *P. aeruginosa* was found to be non susceptible to both extracts of *P. minor* tested, and this may be due to effect of low dosage or solvents used for the extraction. The antimicrobial properties of *P. minor* as reported by Mackeen *et al.* (1997) and Sosongko *et al.* (2011) are attributed to the presence of bioactive metabolites which are aldehydes, terpenoids, and alcohols among others contained in the plant leaves.

Effect of different extraction solvents

The results of the antimicrobial test obtained have confirmed that the extract obtained from maceration of mixing two different solvents namely water and ethanol as aqueous-ethanol were observed to be more effective than the extract obtained from absolute water. This is likely in light of the fact that, the type of solvent used in the extraction procedure influenced the solubility of the active component of the leaves (Saad *et al.*, 2014; Shanmugan *et al.*, 2014). Thus, 30% aqueous-ethanol had a high power to extract the active antibacterial compounds in the plant which revealed higher activity with higher zones of inhibition in comparison with absolute water solvent.

Moreover, the use of either methanol, ethanol, n-hexane, chloroform or ether may result in high solubility increase of plant material components in contrast to absolute water alone (Amita *et al.*, 2014). Thus, aqueous-ethanol may possibly aid in extraction of novel bioactive compounds in plant materials more than aqueous solvent which includes alkaloids, phenolic, flavonoids, terpenes, tannins, anthrocynins, starches, polypeptides and sterols because of polarity difference exhibiting between the solvents (Sultana *et al.*, 2009). Therefore, the yields of extract and consequential antibacterial activities of plant materials are largely dependent on the nature of extraction solvent, due to the presence of different bioactive compounds of varied chemical characteristics and polarities that may or may not be soluble in a particular solvent.

Susceptibility of bacteria towards the extracts

The results of current study have shown that antibacterial activity between all four bacterial isolates assessed, display

different measurement of diameter in the inhibition zone. The variety of zones of inhibition implies the changeable degree of efficacy and different phytochemical components of the plant herb on the target bacteria (Saad *et al.*, 2014). Hence, amongst the four bacterial isolates tested, only three were sensitive to both extracts of the plant which are *S. aureus*, *E. coli* and *E. faecalis* (being *S. aureus* as the most susceptible in both extracts). However, *P. aeruginosa* was completely non susceptible to both extracts and this may be influenced by the complexity of its membrane surface as gram negative bacteria, or may be due to effect of low dosage or solvents used during extraction. Thus, the ability of a particular plant extract to inhibit or kill some certain species of microorganisms is likely dependent on the strain species of the same or different organisms located at different or the same geographical regions (Akpulu *et al.*, 1994; Teh, 1996). Hence, the types of bacterial strains may therefore contribute to the variation of antibacterial activity.

CONCLUSION

In this study, it has been found that both extracts of *Persicaria minor* leaves (aqueous- ethanolic extract and water extract) possess high potential natural antibacterial activity as they inhibited the growth of *Staphylococcus aureus*, *Escherichia coli* and *Enterococcus faecalis* more effectively as determined by Disc-diffusion, MIC and MBC test. The expression of antimicrobial activity of *P. minor* leaves extracts against the test organisms is a sign that there is likelihood of sourcing alternative antimicrobial or antibiotic agents in the plant for the advancement of more current antibacterial agents to battle various diseases associated with susceptible test bacteria utilized in this study. In this manner, utilizing *P. minor* leaves may have advantageous if consumed routinely especially as vegetable diet in nutrition or as natural antibacterial agent for elimination of various bacterial disease and infections.

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