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Investigation of Phytochemicals and Antibacterial Activity of Persicaria odorata (L.) Methanolic Leaf Extract Against Selected Bacteria

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ABSTRACT

Multidrug-resistant microorganisms pose serious threats, especially to children from developing countries. Due to this concern, there is a greater need to develop potentially effective antibacterial agents from natural resources. This study aimed to investigate the phytochemical compounds of *Persicaria odorata* (L.) methanolic leaf extract and its antibacterial activity against selected bacteria. Agar well diffusion and broth microdilution were used to test gram-positive (*Staphylococcus aureus* and *Bacillus cereus*) and gram-negative bacteria (*Salmonella enterica serovar typhimurium* and *Shigella flexneri*). The bacteria were treated with *P. odorata* (L.) leaves extracted using methanol and diluted with 10% Dimethyl-sulfoxide (DMSO). *P. odorata* (L.) leaf extract was highly effective against *S. aureus* at a concentration of 1,000 mg/mL. Gram-positive bacteria had the lowest MIC and MBC values. The qualitative phytochemical analysis of the methanolic extract of *P. odorata* (L.) leaves indicated the presence of antibacterial compounds such as phenols, flavonoids, terpenoids, and tannins. In conclusion, *P. odorata* (L.) leaves which have been widely used in cuisines and traditional medicine also possess the potential to serve as an antibacterial agent.

INTRODUCTION

In the modernised era, infectious diseases caused by pathogens pose a significant societal burden worldwide. Another alarming issue is bacterial antimicrobial resistance (AMR) making antibiotic use futile or less effective against many common bacterial infections affecting animals and humans. Data on AMR from Malaysia, as well as other countries, have been analysed. The analysis demonstrated that five bacteria were each involved in more than 500,000 deaths in 2019: *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Three infectious syndromes (lower respiratory infections, bloodstream infections, and peritoneal and intraabdominal infections) caused more than 1 million deaths in 2019 and accounted for 75% of deaths due to bacterial infections (Ikuta et al., 2022). Developed and underdeveloped countries encountered similar challenges in managing the burden of infectious diseases, which can be transmitted from person to person through direct or indirect contact. Weak infection management practices may lead to the development of infectious diseases (Kourtis et al., 2019).

For millennia, traditional medicine that utilises natural resources such as plants and minerals has been widely used to treat illnesses and practised in maintaining patients' health. Approximately 1,200 species of higher plants have been reported to possess medicinal properties but only less than 0.1% of them have been studied (Manaf & Daud, 2016). Each part of the plant is enriched with primary and secondary active metabolites that contribute to a diverse biological activity that is either essential for therapeutic purposes or as precursors for drug synthesis (Manaf & Daud, 2016; A'attiyyah et al., 2018). It has been shown that alkaloid extracts from the leaves, stem bark, and root of *Ochrosia oppositifolia* have antibacterial activity against gram-positive and gram-negative bacteria (Mahmud et al., 2017), while usnic acid and atranorin were the most active chemical constituents to act as antibacterial agents from the lichens of *Ramalina dumeticola* and *Usnea rubrotincta* (Gunasekaran et al., 2016).

Persicaria odorata (Lour.) Soják, also known as *Daun Kesum*, is a perennial fresh culinary herb that belongs to the *Polygonaceae* family. The plant is found in Peninsular Southeast Asia and is widely recognized for its traditional use in medicines, cuisines, pharmacies, and cosmetics (Ridzuan et al., 2017). Leaves of *P. odorata* (L.) successfully treat digestive problems, including flatulence, stomach cramps, and indigestion (Mumtak et al., 2017). Earlier investigations have probed the restorative properties of *P. odorata* (L.), and the outcomes appeared helpful for its broad use as an essential remedial agent, including anti-inflammatory, antioxidant, antimicrobial, anticancer, and antifungal (Okonogi et al., 2016; Chansiw et al., 2019; Ridzuan et al., 2013; Putthawan et al., 2017; Yik et al., 2018). The plant can be of great value as an antibacterial agent for combating the threat posed by bacteria, particularly multidrug-resistant bacteria. This study may act as a starter for further research related to the biological function of *P. odorata* (L.) towards the human population.

The development of new drugs is different from the rapid emergence of various multidrug-resistant bacteria, increasing the burden on healthcare settings. Besides, harmful side effects due to exposure to synthetic drugs in a patient's treatment may lead to higher risk (Rather et al., 2017). Therefore, the need for advanced production of antibacterial derived from bioactive compounds from natural sources is crucial to combat the emergence of these hard-to-treat bacteria, especially in developing countries where the availability of antibiotics and the cost of therapy are critical constraints in public health settings. This study experimented on the antibacterial activity of *P. odorata* (L.) leaves in vitro against selected pathogens (*Staphylococcus aureus*, *Bacillus cereus*, *Salmonella enterica serovar typhimurium*

and *Shigella flexneri*). Moreover, screening for phytochemicals within the leaves was also done qualitatively.

MATERIALS AND METHODS

P. odorata (L.) Leaves Collection

P. odorata (L.) was purchased from the Wholesale Wet Market in Sungai Petani, Kedah (GPS coordinate: 5.642121, 100.490964), and species confirmation was done by submitting a voucher specimen (PID 070319-07) to the herbarium of the Forest and Research Institute, Malaysia (FRIM). The healthy leaves were picked out, washed thoroughly with distilled water, and then dried under the sun. Dried leaves were processed into a powder in an automated blender and then kept at room temperature in an airtight container.

Preparation of Concentrated Extract Stock Solution Using Maceration Technique

For three days at room temperature, 480 g of powdered *P. odorata* (L.) leaves were soaked in 4.8 L of 100% methanol (1:10 weight/volume) while continuously agitated at 70 revolutions per minute (rpm) on an orbital shaker to ensure proper mixing. Whatman filter paper no. 1 and then the white cotton cloth was used to filter the mixtures twice to eliminate any remaining particles. Exhaustive evaporation at 40°C under reduced pressure using a rotary evaporator was used to evaporate the filtrates further, yielding the concentrated extract. The resulting extract was transferred to a universal bottle, weighed using the following procedure, and kept at 4°C.

About 30 g (30,000 mg) of the extract was dissolved into 30 mL of 10% DMSO to obtain a final concentration of 1,000 mg/mL (Samsudin et al., 2018).

Qualitative Phytochemical Screening

Secondary bioactive metabolites are abundant in wild plants and play an important role in plant adaptation. Moreover, it has therapeutically active components in the form of medicines (Shakya, 2016). A phytochemical screening method was performed to detect the secondary metabolites found in the leaves of *P. odorata* (L.).

Phenols (Ferric Chloride Test)

One mL of the concentrated methanolic extract of *P. odorata* (L.) was spiked with a few drops of 1% ferric chloride. Developing colours in the blue, purple, violet, green, or red-brown range indicated the presence of phenol group compounds.

Flavonoids (Alkaline Reagent Test)

Two mL of a concentrated methanolic extract of *P. odorata* (L.) were treated with a few drops of a 20% sodium hydroxide solution (NaOH). After adding NaOH, the solution became a bright yellow.

Terpenoids (Salkowski Test)

First, 5 mL of *P. odorata* (L.) methanolic concentrated extract was mixed with 2 mL of chloroform and 3 mL of concentrated sulfuric acid. The positive result was based on the development of a reddish-brown hue.

Tannins (Ferric Chloride Test)

Two mL of a 10% ferric chloride solution were added to 2 mL of a methanolic extract concentrated from *P. odorata* (L.). Intense green, dark blue or black colouration indicated the presence of tannin in the extract.

Isolation and Confirmation of Bacterial Species

Staphylococcus aureus (ATCC 25923), *Bacillus cereus* (ATCC 11778), *Salmonella enterica serovar Typhimurium* (ATCC 13311), and *Shigella flexneri* (ATCC 12022) from the American Type Culture Collection were all employed as the four bacterial strains. Subculturing of *S. aureus* and *B. cereus* was performed on nutrient agar (NA) and sheep blood agar (SBA), whereas xylose-lysine-deoxycholate (XLD) agar was used for *S. typhimurium* and *S. flexneri*. Gram stain, biochemical test, and iMViC test were used to identify further the four bacterial strains.

To ensure viable bacteria were collected, a bacterial growth curve was performed by measuring the absorbance of each pre-cultured bacteria in Tryptic Soy Broth (TSB) at the wavelength of 600 nm (OD600) against blank (sterile TSB) for every half an hour using an ultraviolet spectrophotometer and the following formula was used for volume determination:

$$C1V1 = C2V2$$

C1 = The absorbance of TSB + bacterial suspension after overnight incubation
V1 = Volume of pre-cultured TSB that will be transferred to obtain a final volume of 50 mL
C2 = Fixed standard value (0.05)
V2 = Final volume (50 mL)

Furthermore, colony count using the spread plate method was also performed using a ten-fold dilution of the pre-cultured bacteria in Tryptic Soy Broth (TSB), and the mean of triplicate plates was calculated and recorded.

Antimicrobial Susceptibility Test (AST)

Bacterial colonies from NA were cultured by suspending three to five similar colonies in 4 mL of sterile Mueller-Hinton Broth (MHB), incubating at 37°C until the turbidity reached 1 to 2 × 10^8 colony forming unit/mL, or the equivalent of 0.5 McFarland standard. After adjusting the turbidity for 15 minutes, 100 µL of each four of the bacterial inoculums were added to Mueller-Hinton agar (MHA), spread equally over the surface of the medium, and rotated 60° to ensure equitable distribution. Sterile blue pipette tips were used to pierce two neighbouring wells in the agar. Pipetting into the well was 60 µL of the concentrated *P. odorata* (L.) extract solution and 60 µL of a 10% DMSO solution. A commercially available antibiotic paper disc that served as a positive control was aseptically put on the bacterial lawn. All plates were incubated for 24 hours of incubation at 37°C. The study was repeated thrice, and the findings were presented as the mean standard deviation.

Minimal Inhibitory Concentration (MIC)

Triplicate broth microdilution MIC was done in 96-well microtiter plates. Microtiter plate wells were numbered 1 – 9. 100 µL MHB was pipetted into wells 2 – 9. Subsequently, 100 µL of *P. odorata* (L.) leaves methanolic concentrated extract (1,000 mg/mL) was pipetted into well 1 and well 2. The stock solution in well 2 was serially diluted with MHB in a two-fold serial dilution by transferring 100 µL of solvents in well 2 into well 3 of the microtiter plate. The steps were repeated until well 9. 100 µL of solvents in well 9 were eliminated to standardize all well volumes. The final concentrations of extract in each well were 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250, 500, and 1,000 mg/mL. At the same time, 100 µL of bacterial solution was pipetted into well 10 to serve as a positive control. Well 11 and well 12 were used for sterility testing by adding 100 µL of a stock solution of methanolic extract from the leaves of *P. odorata* (L.) and 100 µL of MHB, respectively, to make sure no apparent bacterial growth or contamination that might provide a false positive result. The microtiter plate cultures were incubated at 37°C for 16 – 24 hours.

Minimal Bactericidal Concentration (MBC)

The minimum bactericidal concentration (MBC) was determined by subculturing a loop full of bacteria from each dilution of the extract that showed no apparent bacterial growth in the MIC microtiter wells onto an MHA plate (MBC). The plates were kept at 37°C for 16 to 24 hours.

DATA ANALYSIS

IBM SPSS Statistics for Windows, Version 21.0, was used to analyse the data. All ASTs were performed in triplicate, and the results were reported as the mean SEM (SEM). An independent T-test was used to analyse the statistical significance of the zone of inhibition produced by the plant extract in comparison

to the positive control and the variations in antibacterial activity between the bacteria. When the P-value for the differences was less than 0.05, it was regarded as significant.

RESULTS

Phytochemical Analysis

Table 1 summarizes the qualitative phytochemical screening of chemical compounds in the 1,000 mg/mL methanolic leaf extract of *P. odorata* (L.).

Table 1 Results for qualitative screening of phytochemical compounds tested in 1,000 mg/mL of *P. odorata* (L.) methanolic leaf extract which was detected using colour changes

Phytochemicals	*P. odorata* (L.) methanolic leaf extract
Phenols	Present
Flavonoids	Present
Terpenoids	Present
Tannins	Present

Antimicrobial Susceptibility Test Using Agar Well Diffusion Method

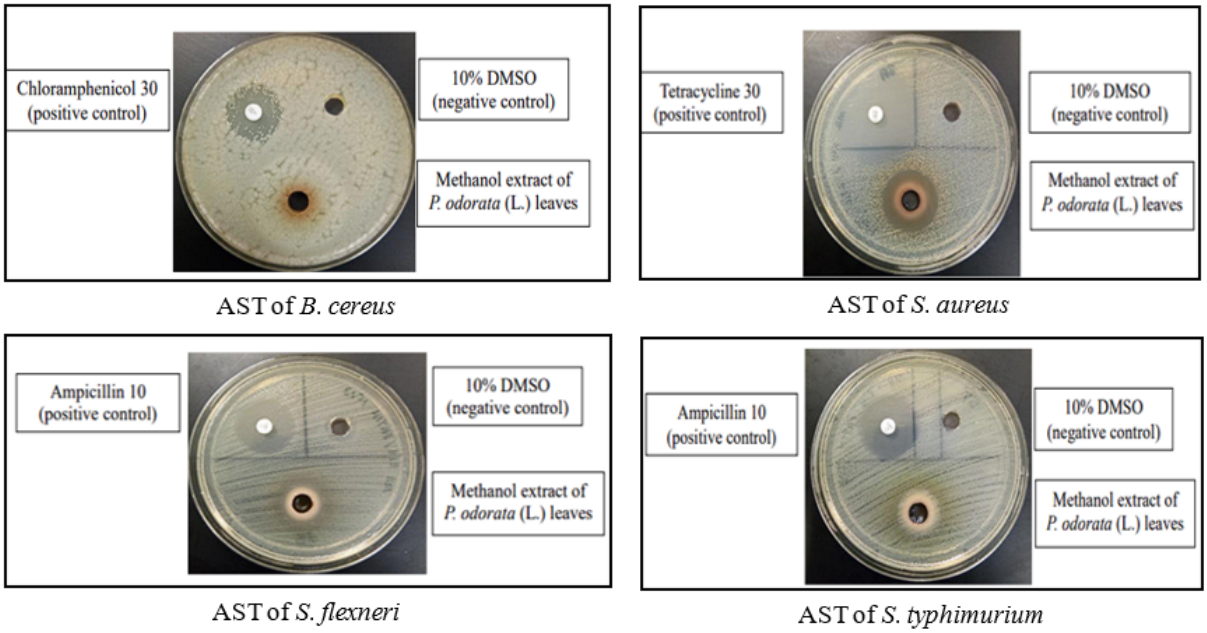
The mean inhibition zone diameter of the 1,000 mg/mL methanolic leaf extract of *P. odorata* (L.) against the selected bacterial strains was presented in Table 2 and shown in Figure 1.

Table 2 Diameter of inhibition zone of 1,000 mg/mL *P. odorata* (L.) methanolic leaf extract against selected bacteria

Bacteria	Zone of inhibition (Mean ± SEM**)					*P*-value*
	***P. odorata* (L.) methanolic leaf extract**	**Positive control**			**Negative control (10% DMSO)**	
		Tetracycline 30	**Chloramphenicol 30**	**Ampicillin 10**		
S. aureus	22.67 ± 0.67	37.33 ± 1.33	–	–	0	0.001
B. cereus	18.00 ± 0	–	20.33 ± 0.33	–	0	0.020
S. typhimurium	18.00 ± 0.58	–	–	27.00 ± 0	0	0.001
S. flexneri	17.00 ± 0	–	–	25.00 ± 0	0	0.000

* Independent T-test of inhibition zone between *P. odorata* (L.) methanolic leaf extract and positive control,
** Standard error of mean

Figure 1 AST using agar well diffusion method showing the absence of an inhibition zone by negative control (DMSO) and presence of inhibition zone by commercial antibiotic discs as positive control across all four bacteria isolates



MIC and MBC Using Broth Microdilution Method

The mean MIC and MBC values in Table 3 reveal that the *P. odorata* (L.) methanolic leaf extract was effective against the *S. aureus* and *B. cereus* at a 15.63 mg/mL concentration. The MIC/MBC ratio indicated the plant extract has bactericidal properties.

Table 3 MIC and MBC of *P. odorata* (L.) methanolic leaf extract against selected bacteria

Bacteria	*P. odorata* (L.) methanolic leaf extract (Mean ± SEM*)		MIC/MBC Ratio
	MIC (mg/mL)	MBC (mg/mL)	
S. aureus	15.63 ± 0	15.63 ± 0	1 (–)
B. cereus	15.63 ± 0	15.63 ± 0	1 (–)
S. typhimurium	250 ± 0	500 ± 0	0.5 (–)
S. flexneri	125 ± 0	500 ± 0	0.25 (–)

* Standard error of mean
(–) Bactericidal
(+) Bacteriostatic

DISCUSSION

This study obtained 13.21% of *P. odorata* (L.) methanolic leaf crude extract using the maceration technique. This technique used solvent-mediated extraction; the conventional method commonly utilised in small research settings, due to its convenience, expanded applicability, productivity, and low production cost (Azwanida, 2015; Safdar et al., 2017). Methanol was selected as an extraction solvent based on its excellent polar, protic solvent, and intermediate polarity characteristics. It produced a higher extraction yield than acetonitrile and extracted a wide range of phytochemicals (Nguyen et al., 2015). A smaller sample size with a longer contact time between the sample and solvent can increase the surface contact area of the sample with extraction solvent (Azwanida, 2015; Dzulkarnain & Abdul Rahim, 2014). Therefore,

the plant extraction technique was slightly modified to achieve a higher yield of the crude extract by soaking finely powdered *P. odorata* (L.) leaves in methanol for a longer period (i.e., three days) and continuous agitation at 70 rpm on an orbital shaker. DMSO is an organosulfur compound utilised widely as a polar, aprotic solubiliser miscible with water to dissolve an extensive number of polar and nonpolar small molecules. 10% DMSO (1:10 v/v) was prepared as it has relatively low toxicity against living cells (Da Violante et al., 2002; De Abreu Costa et al., 2017).

The present study subjected the methanolic extract of *P. odorata* (L.) leaves to qualitative phytochemical analysis. The results demonstrated the presence of phenols, flavonoids, terpenoids, and tannins (Table 1). The presence of secondary metabolites has contributed to diverse biological activity essential for therapeutic purposes or as precursors for drug synthesis (A'attiyyah et al., 2018). The antibacterial properties of polyphenols were reported to stem from their extreme ability to combine with various macromolecules. In contrast, terpenoids exerted antimicrobial activity due to their lipophilic characteristics, allowing direct penetration into the bacterial cells and disrupting protein biosynthesis, nucleic acid replication, and repair mechanisms (Zacchino et al., 2017). The mechanisms of plant phenolic antibacterial occur via the bacterial cell membrane or non-membrane disruption along with the synergism of the compounds with current antibiotics (Rempe et al., 2017). Meanwhile, flavonoids possess several antibacterial mechanisms with enhancement depending on their different structural configuration. Several antibacterial mechanisms proposed include inhibition of nucleic acid synthesis and cell membrane porin, disruption of the functional cytoplasmic membrane and energy metabolism, obstruction of biofilm production, and attenuation of bacterial pathogenicity in which these mechanisms were enhanced as the flavonoid contained hydroxyl groups on specific aromatic rings and hydrophobic substances (Xie et al., 2015). These findings were supported by a study in which the structural relationship of flavonoids with different structural configurations contributed to the enhancement of antibacterial activity, and alteration to these structures, such as methylation, led to the reduction of the antibacterial properties of flavonoids (Ahmad et al., 2015). Meanwhile, the antimicrobial activity of tannins is correlated with bacteria's extracellular enzyme metabolism obstruction and nutrient or substrate starvation, as well as the direct inhibition of oxidative phosphorylation in microbes' metabolism (Agyare et al., 2015). Total tannins exert their antibacterial effect by destroying the bacterial structure, such as malformed cell walls, detached cytoplasm, and strong affinity to aggregate between themselves (Li et al., 2016).

The AST (Table 2) revealed that the *P. odorata* (L.) methanolic leaf extract possesses the highest antibacterial activity against *S. aureus* (22.67 ± 0.67), followed by *S. typhimurium* (18.00 ± 0.58), *B. cereus* (18.00 ± 0) and *S. flexneri* (17.00 ± 0). The absence of an inhibition zone by negative control (DMSO) across all four bacteria isolates indicated nonparticipation of the solvent in the antimicrobial feature of *P. odorata* (L.) leaves (Figure 1). The *P. odorata* (L.) methanolic leaf extract demonstrated effective antibacterial activity, especially on gram-positive bacteria. Nevertheless, its activity was lowest against *S. flexneri* due to the unique structure of the gram-negative bacteria's outer membrane which acts as a powerful permeability barrier against antibacterial agents (Oikeh et al., 2017). In contrast, previous studies only reported the antibacterial activity of *P. odorata* (L.) leaves against gram-positive bacteria, which may be due to the lower concentration of *P. odorata* (L.) methanolic leaf extract, different AST methods and bacterial species used (Ridzuan et al., 2017; Chansiw et al., 2018). The MIC and MBC of the 1,000 mg/mL methanolic leaf extract of *P.*

odorata (L.) demonstrated potent antibacterial activity against the growth of gram-positive bacteria. The lowest concentration was 15.63 mg/mL for both *S. aureus* and *B. cereus* compared to gram-negative bacteria. (Table 3). Bacteriostatic effects were defined as MIC/MBC ratios more than four, whereas bactericidal effects were defined as ratios less than four. Thus, the current findings demonstrated that the *P. odorata* (L.) extract had a bactericidal effect on all four bacterial strains. A previous study with other bacterial species reported that the methanolic extract of *P. odorata* (L.) leaves also exhibited the most significant antimicrobial activities with MICs of 3.125 mg/mL and 12.5 mg/mL for *S. pyogenes* and *S. pneumonia*, respectively (Ridzuan et al., 2013).

Theoretically, the lower the MBC value, the greater the inhibition zone seen in antimicrobial susceptibility tests (Murray, 2015). However, the MBC values of 15.63 mg/mL obtained for the methanolic extract against the gram-positive bacteria in this investigation did not correspond with the inhibitory zone diameter employing a 1,000 mg/mL concentration of the plant extract as compared to the gram-negative bacteria. This result, however, is consistent with a previous study that utilised an ethanolic extract of *Syzygium aromaticum*. Gram-positive bacteria (*B. cereus* and *S. aureus*) exhibited lower MBC values and inhibition zones compared to gram-negative bacteria (*E. coli*) in a contradictory finding (Gonelimali et al., 2018).

The purpose of an antimicrobial susceptibility test is to determine the degree to which an organism is sensitive to or resistant to a particular antimicrobial agent. In this investigation, the well diffusion technique was utilised, in which the antimicrobial compound diffuses into the agar to inhibit the growth of the test organism. The interaction between the antimicrobial agent and the solid medium, as well as inactivation during diffusion, has been demonstrated to be one of the causes of low inhibition zone readings of the tested organism (Bonev et al., 2008). The Clinical and Laboratory Standards Institute also lists several factors that can affect the inhibition zone's diameter, including the susceptibility test medium, the concentration of the test organism, its rate of growth, the concentration of the antimicrobial agent, the antibiotic's diffusion rate in the agar, and the organism's susceptibility to the antibiotic. The creation of bacterial biofilm, on the other hand, has been shown in a prior study to potentially decrease the efficacy of antimicrobial activity because it serves as a significant barrier, blocking the entry of polar and charged antibacterial agents (Macia et al., 2014). *B. cereus* and *S. aureus* propensity to build bacterial biofilms may be one of the factors contributing to the low inhibition zone of the gram-positive bacteria.

CONCLUSION

The antibacterial activity against selected bacterial strains was discovered in the methanolic leaf extract of *P. odorata* (L.). The inhibitory or bactericidal effect was more potent against gram-positive than gram-negative bacteria. The presence of phenols, flavonoids, terpenoids, and tannins suggests that the phytochemicals of *P. odorata* (L.) leaves are responsible for the therapeutic property against infections, making the natural herbal extract a viable initial therapy source.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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