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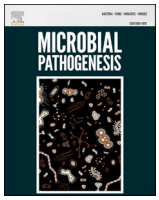

Antibacterial potency of mid-polar extracts obtained from Malaysian plant *Parkia speciosa* against human pathogenic bacteria

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A R T I C L E I N F O

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A B S T R A C T

Background and objectives: Plants contain a wide variety of bioactive compounds, which have attracted the interest of researchers in finding novel sources of natural medicine. In the following paper, we aim to evaluate the antibacterial potential of extract fractions associated with *Parkia speciosa* pods and beans against human pathogenic bacteria.

Methods: Antimicrobial activity was determined with disc diffusion and broth microdilution assays against eight skin colonising microorganisms including *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Salmonella enterica*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia* followed by further fractionation of the pods ethyl acetate fraction by column chromatography along with preparative thin-layer chromatography. Quantification of bacterial death mechanism was elucidated by the measurement of hole size in cell wall that has been induced by extract constituents via field-emission scanning electron microscopy (FESEM).

Results: Four fractions showed significant antimicrobial activity against the six microorganisms tested ($p < 0.01$), with inhibition zones ranging from 35.67 to 17.00 mm, and minimum inhibitory concentration ranging from 6.25 to 50.00 mg/ml in which the pods ethyl acetate fraction was the most effective. The methanol fraction isolated from the pods ethyl acetate fraction was much more effective with a four-fold increase from 6.25 to 1.25 mg/ml against S. epidermidis. The disintegration of *S. aureus* was due to chronic cell wall alterations with pore creation, invaginations and morphological disorganisation. Autolysis in bacterial cells via the expression of peptidoglycan-disrupting lysozyme or bacterial murein hydrolase was postulated. A significantly large pore with a mean diameter of 293.7 nm was detected in the cell wall of *S. aureus*.

Conclusion: P. speciosa fraction could be a potential novel source for the development of a natural antibacterial agent.

Abbreviations: BEA, beans ethyl acetate fraction; BW, beans water fraction; CLSI, The Clinical and Laboratory Standards Institute; EA, ethyl acetate; *E. coli*, *Escherichia coli*; FESEM, field-emission scanning electron microscope; g, grams; H, hexane; keV, kilo electron volts; µl, microlitres; mg, milligrams; mg/ml, milligrams per millilitre; ml, millilitres; ml/min, millilitres per minute; mm, millimetres; MIC, minimum inhibitory concentration; nm, nanometers; *K. pneumonia*, *Klebsiella pneumonia*; *P. speciosa*, *Parkia speciosa*; PEA, pods ethyl acetate fraction; PW, pods water fraction; *P. aeruginosa*, *Pseudomonas aeruginosa*; TEM, transmission electron microscope; *V. parahaemolyticus*, *Vibrio parahaemolyticus*; *A. hydrophila*, *Aeromonas hydrophila*; *S. anginosus*, *Streptococcus anginosus*; *S. agalactiae*, *Streptococcus agalactiae*; *C. diphtheriae*, *Corynebacterium diphtheriae*; *P. mirabilis*, *Proteus mirabilis*; *S. pyogenes*, *Streptococcus pyogenes*; *L. monocytogenes*, *Listeria monocytogenes*; *S. Typhimurium*, *Salmonella Typhimurium*; *V. cholerae*, *Vibrio cholerae*; *M. luteus*, *Micrococcus luteus*; TLC, thin-layer chromatography; *S. enterica*, *Salmonella enterica*; *S. epidermidis*, *Staphylococcus epidermidis*; *S. aureus*, *Staphylococcus aureus*; WHO, The World Health Organization; TTC, 2, 3, 5-Triphenyl Tetrazolium Chloride.

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1. Background

Plants produce a variety of bioactive compounds, which enables them to be rich sources of natural medicine, and at present approximately 20 % of plants worldwide are being tested for pharmacological or biological properties that can be applied in drug development [1]. From 1983 to 1994, the systematic antibacterial screening of plant extracts became a sustainable pathway to the discovery of new drugs with the potential to fight against multidrug-resistant bacteria with the World Health Organization (WHO) reporting that these medicinal plants would be the main resource in developing different drugs of medicinal value [2]. Therefore, it is crucial to explore new antimicrobial compounds with a variety of chemical structures and modes of action, which can effectively combat both existing and re-emerging infectious diseases.

Plant-based antimicrobial agents have lower potencies than synthetic antimicrobials reported by various studies [3]. However, antimicrobial resistance against synthetic antimicrobials is on the rise and becoming a critical issue for human health. Investigations into the development of newer drugs with reduced resistance have led to a reassessment of the therapeutic uses of plants. Plant-derived antimicrobials have enormous potential to fight bacteria and fungi without any adverse effects, and possibly overcome antimicrobial resistance [1]. Secondary metabolites that exhibit *in-vitro* antimicrobial activities are abundant in plants including tannins, terpenoids, alkaloids, flavonoids and glycosides [4]. The WHO has reported that plant extracts and their active constituents have been used in traditional medicine by 80 % of the population worldwide [5]. Annually, various types of bacterial infections affect the health of approximately 300 million people [6]. In particular, Malaysia is located in the tropical region of the globe, which provides a suitable environment for human microbial pathogens to grow and replicate.

Parkia speciosa, commonly called Petai Padi is a traditional medicinal plant according to the ethnobotanical record of Malaysia. It belongs to the genus *Parkia* and species *speciosa* of the family Fabaceae, including Leguminosae and Mimosaceae. It can be found on sandy, loamy and podzolic soils based in rainforests. It normally reaches a height of around 40 m and is known for producing long and flat green beans in stalks called pods [7]. The native communities in Malaysia apply the pods externally to wounds and ulcers as folk medicine. *P. speciosa* is reported to exhibit antimicrobial activity that supports folkloric use as a broad-spectrum antimicrobial agent [8]. Several studies have evaluated the phytochemical profile and pharmacological activities, such as antimicrobial and antioxidant activities of *P. speciosa*. A recent study has signified that ethyl acetate extracts of *P. speciosa* have potent antibacterial action against antibiotic-resistant strains of *S. aureus* and *E. coli* [9]. The following study aims to exploit the *in vitro* antibacterial potential of mid-polar extracts of *P. speciosa* found in Malaysia against human disease-causing pathogenic bacteria and for the first time its TLC (Thin-layer Chromatography) profile, along with FESEM analysis of extract effects on bacteria. Also, this study motivates the exploitation of research on the discovery of novel compounds and *in vivo*/ex vivo probing of *P. speciosa* that are relevant to antimicrobial activities.

2. Materials and methods

2.1. Collection and preparation of plant crude extracts

P. speciosa was collected at Hulu Langat from a village of Orang Asli indigenous residents of Malaysia at the following coordinate 3.215651, 101.868217. Pods and beans of *P. speciosa* were dried in the sun. Two hundred (200) g of dried pods and beans each were ground using a blender and were macerated separately with 1.5 L of methanol (Synergy Scientific, Malaysia) for 24 h. All extracts were concentrated using a rotary evaporator (Buchi, model R-200, Flawil, Switzerland) under low pressure at room temperature. Two hundred (200) ml of distilled water was poured into the methanolic crude extract and filtered through Whatman Grade 1 filter paper (ThermoFisher Scientific, Waltham, United States).

2.2. Preparation of fractions

The aqueous filtrate of methanolic crude extract was fractionated with 300 ml each of hexane with ethyl acetate (2:3) (RCI Labscan Limited, Thailand) to obtain ethyl acetate and water fractions. All fractions were rotary-evaporated and subjected to antimicrobial assays.

2.3. Fractionation of compounds

Column chromatography was conducted using pump manager (Buchi, model C-615, Flawil, Switzerland), pump module (Buchi, model C-60, Flawil, Switzerland) and 40 μm silica flash cartridges (Buchi, Flawil, Switzerland). The sample was prepared by mixing 2 g of fraction in 10 g of silica gel (0.063–0.200 mm) (Merck, Darmstadt, Germany) and was packed inside the sample column. The solvent system was utilised as the mobile phase at a 13 ml/min flow rate and 22 bar pressure. Isocratic elution was conducted, followed by gradient elution with solvents of increasing polarity. All column fractions were collected separately and rotary evaporated. The column fractions were tested on purity with TLC and then studied for bioactivity.

2.4. Thin-layer chromatography (TLC) analysis of fractions

One (1) mg of fraction was dissolved in 0.1 ml of methanol. Two (2) μl of the sample was spotted on TLC aluminum plates precoated with silica gel (Merck, Darmstadt, Germany) with a 2–20 μl Gilson pipette. Two hundred (200) ml of the mobile phase was prepared. The spotted plate was run in a developing tank and visualised under visible and UV light at 254 nm.

2.5. Antimicrobial assays

2.5.1. Bacteria

Six bacterial strains that include *Staphylococcus aureus* (ATCC 11632), *Staphylococcus epidermidis* (ATCC 12228) of Gram-positive type and *Salmonella enterica* (ATCC 6962), *Escherichia coli* (ATCC8739), *Pseudomonas aeruginosa* (ATCC 10145), *Klebsiella pneumoniae* (ATCC 13883) of Gram-negative type were obtained from the Microbiology lab associated with the University of Nottingham Campus in Malaysia.

2.5.2. Determination of inhibition zones

The disc diffusion assay was performed to determine inhibition zones according to CLSI. The inoculum size was adjusted between 1.0 and 5.0 $\times$ 10^6 CFU/ml for fungi and 1.5 $\times$ 10^8 CFU/ml for bacteria with 0.5 McFarland turbidity standards [10]. The standardized inoculum was streaked over the entire surface of the 90 mm Mueller-Hinton agar plate (Merck, Darmstadt, Germany) for bacteria. Ten (10) μl of a 100 mg/ml final concentration of each fraction (pods ethyl acetate fraction, pods water fraction, beans ethyl acetate fraction, beans water fraction) was impregnated in sterilised 6 mm blank discs to obtain 1 mg of the fraction. The fraction impregnated disc was applied on to the inoculated agar. Ten (10) μl of methanol-loaded discs were utilised as negative controls, whereas Gentamicin 10 μg disc was used as the positive control (PC). The agar plates were incubated upside down for 24 h at 37 °C. The diameter of the zones of inhibition was measured in millimetres (mm). The assays were repeated in triplicates and their means were evaluated.

2.5.3. Determination of minimum inhibitory concentration

The broth dilution method was conducted to determine the minimum inhibitory concentration (MIC) values according to CLSI standards [10]. A stock solution of each fraction (pods ethyl acetate fraction, pods water fraction, beans ethyl acetate fraction, beans water fraction) was prepared by dissolving 600 mg of fraction in 2 ml of methanol and 1 ml

of dimethyl sulfoxide (DMSO) (RCI Labscan Limited, Thailand). The initial concentration of fraction (200 mg/ml) was diluted with two-fold serial dilutions to achieve concentrations of 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, 3.13 mg/ml, 1.56 mg/ml, 0.78 mg/ml, 0.39 mg/ml and 0.2 mg/ml.

Bacterial inoculum density was adjusted to 1.5×10^5 CFU/ml (0.5 McFarland standard). Five (5) μl of standardised bacterial suspension was transferred to the wells from column 11 to 1. The well containing broth with inoculum acted as the positive control whereas the well containing broth without inoculum was used as the negative control. The assays were tested in triplicate. All plates were incubated for 18 h at 37 °C.

TTC (2, 3, 5-Triphenyl Tetrazolium Chloride) test was conducted where TTC contributed as an indicator of viable bacterial cells. Ten (10) μl of 2 % TTC (Merck, Darmstadt, Germany) was added to a final concentration of 0.02 % in each well of the plate after 18 h of incubation. The plates were then incubated for another 3 h at 37 °C and were observed. The minimum concentration of fraction dilution that prevented TTC colour change indicated complete microbial growth inhibition was recorded as MIC.

2.6. Characterisation of FESEM

The microorganisms that were susceptible to fractions were prepared for FESEM imaging. A small piece was obtained from the inhibition zone and mounted onto the stub. Surface morphological alterations of the bacterial cells were analysed using FESEM (Quanta 400F, Netherland) at an accelerating voltage of 15–20 keV. FESEM was operated at a low vacuum mode and images were captured at magnifications between 12,000 and 50,000. The size distribution of the nano-scale pores created in the bacterial cell walls was measured using the size scaling tool.

2.7. Statistical data analysis

Experimental data are presented as mean ± standard error of the mean (SEM) and a paired sample *t*-test was used to compare zones of inhibition. Data were analysed using the Statistical Package for Social Sciences (SPSS) (version 24.0) software (IBM, USA)

3. Results

3.1. Antibacterial activity of P. speciosa fractions

Results suggest that there was antibacterial activity of *P. speciosa* bean and pods fractions against the six human colonising bacteria tested. Table 1 with Figs. 1 and 5 represent antimicrobial activity from all fractions, where pods ethyl acetate fraction was the highest against all bacterial specimens tested, and mild activity was detected from fractions of pods water beans, ethyl acetate and beans water fraction. MIC of fractions are represented in Table 2.

Mean inhibition zones of four fractions after 24 h of incubation against eight tested microorganisms ranged from 4.67 to 35.67 mm. The mean inhibition zones against two Gram-positive strains ranged from 4.67 to 35.67 mm. Results showed the strongest activity against *S. epidermidis* (35.67 mm) and *S. aureus* (29.33 mm) by pods ethyl acetate fraction. Mean inhibition zones against four Gram-negative strains ranged from 8 to 29 mm. Pods ethyl acetate fraction showed the strongest activity against *S. enterica* (22.33 mm), *E. coli* (29 mm), *P. aeruginosa* (25.33 mm) and *K. pneumoniae* (17 mm). Data indicated that fractions exhibited varying levels of antibacterial activities. MIC values of four fractions against six tested bacteria ranged from 6.25 to 50 mg/ml. Pods ethyl acetate fraction was the most active with the lowest MIC of 6.25 mg/ml against all tested bacteria. Pods water fraction and beans ethyl acetate fraction showed the same MIC of 25 mg/ml against *S. epidermidis* and *P. aeruginosa*. Beans water fraction showed 25 mg/ml against *S. epidermidis* and 50 mg/ml against *P. aeruginosa*. Pods ethyl acetate fraction with the best antibacterial activity was fractionated using column chromatography. Nine column fractions were obtained with varying degrees of antimicrobial activity against *S. epidermidis*. The column fractions performed even better antibacterial activities. MIC of column fractions ranged from 1.56 to 25 mg/ml. The seventh fraction (methanol) which potentially contains mostly polar compounds displayed the highest antibacterial effect with the lowest MIC of 1.56 mg/ml whereas the sixth fraction (ethanol) performed the lowest activity with the highest MIC of 25 mg/ml. A moderate antibacterial activity with the MIC of 12.5 mg/ml was shown by first, second, third and eighth fractions whereas 6.25 mg/ml by the ninth fraction. However, the fourth and fifth fractions were not subjected to the antibacterial tests as no compounds were identified with TLC. The first, second and third fractions which were fractionated using the same solvent system (hexane: ethyl acetate at a ratio of 2:3 displayed the same potency against *S. epidermidis* even though they were found to contain different compounds with TLC.

3.2. Thin-layer chromatography (TLC) profiling of P. speciosa fractions

Under UV light at 254 nm, pods ethyl acetate fraction (A) produced five spots in which four spots with the Rf values 0, 0.37, 0.45, 0.73 and 1 respectively and the spot that did not travel. The methanol fraction (A) produced three spots in which, two spots with the Rf values (0.1, 0.46), including stationary spots (Rf 0.46). Data for TLC of fractions are illustrated in Fig. 2 and Table 3. However, none of the fractions are visible under normal visible light.

3.3. FESEM analysis

The artifacts of cells treated with pods ethyl acetate fraction appeared to be shrinking and forming a pore in the cell wall. A morphology with a large lesion on the bacteria cell wall could potentially indicate pressure generated internally causing a pressure gradient. Bacterial cells appeared to lose turgidity, and the cytoplasmic content leaked out of the cells, possibly due to bacterial membrane disruption

Table 1
Inhibition zones of *P. speciosa* fractions after 24 h of incubation.

Fraction	Inhibition zones (mm)					
	Gram-positive bacteria		Gram-negative bacteria			
	S. aureus	S. epidermidis	S. enterica	E.coli	P. aeruginosa	K. pneumoniae
PEA	29.33 ± 1.53	35.67 ± 1.53	22.33 ± 2.89	29.00 ± 1.00	25.33 ± 1.53	17.00 ± 1.00
PW	0.00	12.67 ± 2.08	0.00	0.00	16.33 ± 1.15	0.00
BEA	0.00	20.37 ± 1.53	0.00	0.00	20.33 ± 1.53	0.00
BW	0.00	4.67 ± 2.08	0.00	0.00	8.00 ± 1.00	0.00
Gentamicin 10 μg (PC)	31 ± 0.78	33 ± 0.75	26 ± 0.56	28 ± 0.73	25 ± 0.34	23 ± 0.35

Mean ± SD from duplicate determinations (n = 6). Fractions concentrations were at 100 mg/ml. A paired sample *t*-test comparing each zone of inhibition between control and fractions indicated statistical significance ($p < 0.01$). Fractions abbreviations (PEA=Pods ethyl acetate fraction, PW=Pods water fraction, BEA=Beans ethyl acetate fraction, BW=Beans water fraction), PC=Positive control.

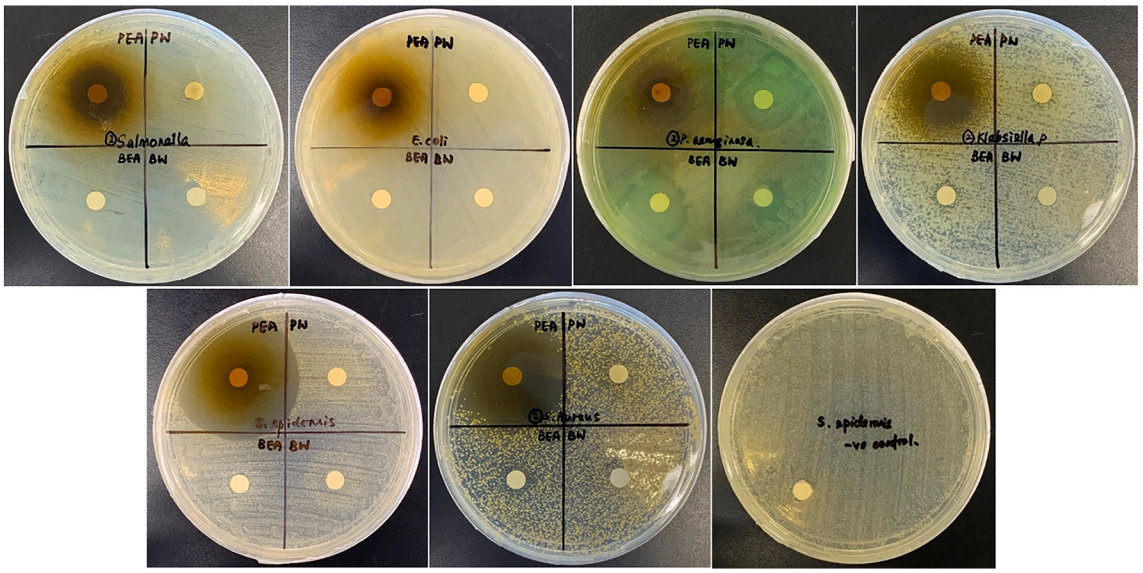

Fig. 1. Zones of inhibition exhibited by *P. speciosa* fractions. Antibacterial activities against Gram-positive bacteria (*Staphylococcus aureus, Staphylococcus epidermidis*), Gram-negative bacteria (*Salmonella enterica, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae*) performed using disc diffusion technique. All discs were soaked in 1 mg of the *P. speciosa* fractions. A negative control test showing microbial growth validated this antimicrobial study.

Table 2
MIC of fractions of *P. Speciosa* methanolic crude extract.

Fraction	Mean MIC (mg/ml)					
	Gram-positive bacteria		Gram-negative bacteria			
	S. aureus	S. epidermidis	S. enterica	E. coli	P. aeruginosa	K. pneumoniae
PEA	6.25 ± 0.00	6.25 ± 0.00	6.25 ± 0.00	6.25 ± 0.00	6.25 ± 0.00	6.25 ± 0.00
PW	–	25.00 ± 0.00	–	–	25.00 ± 0.00	–
BEA	–	25.00 ± 0.00	–	–	25.00 ± 0.00	–
BW	–	25.00 ± 0.00	–	–	50.00 ± 0.00	–

Mean ± SD from triplicate determination (n = 3). Fractions concentration were at 100 mg/ml where two-fold serial dilutions. Fraction abbreviations (PEA=Pods ethyl acetate fraction, PW=Pods water fraction, BEA=Beans ethyl acetate fraction, BW=Beans water fraction).

and complete loss of membrane integrity.

The size distribution of the holes was symmetrical and bell-shaped following the typical pattern for normal distribution. Sizes of the holes had a mean of 293.7 nm with a standard deviation of 40.1 nm. 68.3 % of the size of the cell wall pores formed were between (253.6 nm–333.8 nm) and 95.5 % were between (213.5 nm-373.9 nm). An average of 293.7 nm in diameter of the critical size of the pore created within the bacterial cell wall was predicted to induce bursting or lysis of *S. aureus* cell wall. Data obtained for FESEM analysis are indicated in Figs. 3 and 6.

4. Discussion

Microorganisms like *S. aureus, S. epidermidis, S. enterica, E. coli, P. aeruginosa, K. Pneumonia* are frequently isolated from injuries and burn wounds. These organisms are notoriously known for causing various forms of infections in humans and displaying antibiotic resistance [11]. In the present study, the antimicrobial effect of fractions against these human pathogenic bacteria has been conducted and their inhibitory activities justified. *P. speciosa* was reported to contain flavonoids (quercetin, myricetin, luteolin, kaempferol and apigenin), phenols (gallic acid, catechin and ellagic acid), terpenoids (lupeol), tannins and saponins which are linked to the antimicrobial effect [7].

The highest antimicrobial compounds contained in pods ethyl acetate fraction were suggested, where synergistic effects produced by bioactive compounds to combat bacteria resulted in the highest antimicrobial activity. Beans ethyl acetate fraction, pods water fraction and beans water fraction were only found to be effective against bacteria such as *S. epidermidis* (Gram-positive), *P. aeruginosa* (Gram-negative). These three fractions were assumed to be devoid of certain bioactive compounds that contributed to the antimicrobial effect [12].

However, the absence of antimicrobial activity does not indicate the absence of bioactive constituents in the fraction, or that the fraction does not exert antimicrobial activity against microorganisms. These negative results could be enumerated by the existence of insufficient quantities of active compounds or compounds in different fractions to exhibit antimicrobial activity. These suggestions were established based on the plant's organic molecules in each solvent extracted and the capability of extracts to dissolve in the culture media utilised [12].

Bioactive compounds have varying inhibitory powers against microbes. These bioactive compounds exert a stronger antimicrobial effect against Gram-positive bacteria suggested by the result showing that Gram-positive bacteria were generally more susceptible to the *P. speciosa* fractions than Gram-negative bacteria. This variation might reflect differences between Gram-negative and Gram-positive bacteria in cell surface structures. Structural lipopolysaccharide components in the outer phospholipid membrane of Gram-negative bacteria act as a preventive barrier that causes the cell wall to become impermeable to antimicrobial substances. Gram-positive bacteria only consist of an outer peptidoglycan layer that causes the cell wall to be more permeable to antimicrobial substances than the lipopolysaccharide layer [13]. The cell wall complexity in Gram-negative bacteria is greater than in Gram-positive bacteria rendering them less vulnerable to antimicrobial substances. However, the outer membrane of Gram-negative bacteria

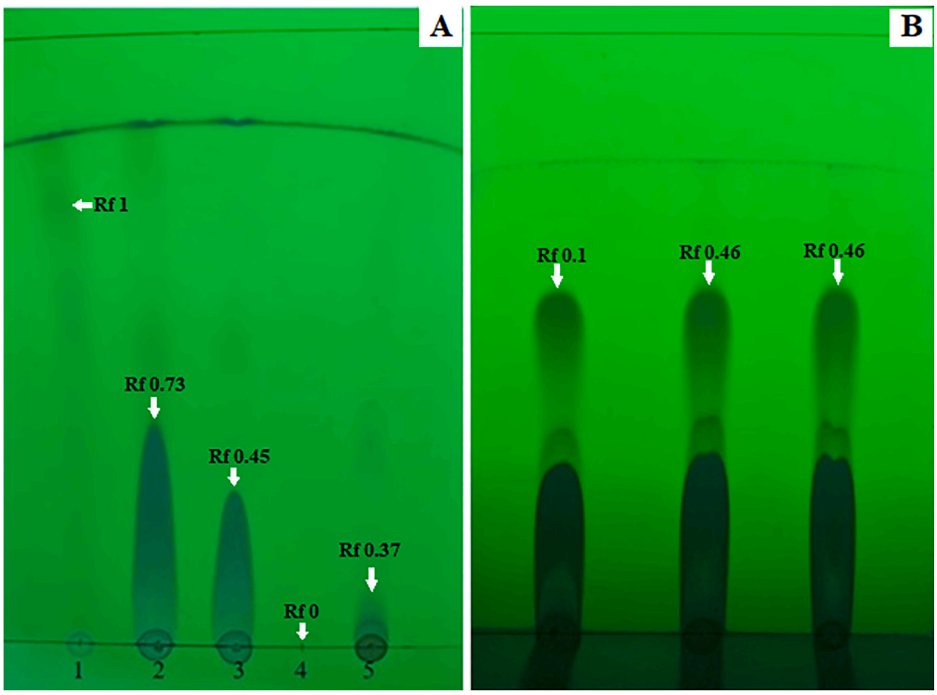

Fig. 2. TLC profiling of *P. speciosa* fractions. TLC separation of pods methanol fraction (A) and collected column fractions of pods ethyl acetate fraction both in hexane: ethyl acetate (2:3) (B) were shown. All plates were visualised under UV light at 254 nm.

Table 3
Mean MIC of column fractions in mg/ml with standard deviations calculated. The weight of each fraction was measured. The broth microdilution test was carried out with two-fold serial dilutions. Concentration of fractions were at 100 mg/ml.

No.	Column Fractions	Volume collected (ml)	Weight (g)	Mean MIC (mg/ml)
1	1st H:EA	250	2.3641	12.50 ± 0.00
2	2nd H:EA	250	2.2615	12.50 ± 0.00
3	3rd H:EA	300	2.5122	12.50 ± 0.00
4	Hexane	250	2.3143	–
5	Acetone	300	2.5107	–
6	Ethanol	300	1.8717	25.00 ± 0.00
7	Methanol	300	2.0750	1.56 ± 0.00
8	Purified water	300	2.5374	12.50 ± 0.00
9	10 % acetic acid	300	0.9714	6.25 ± 0.00

Column fractions abbreviations: H: EA = Hexane: Ethyl acetate (2:3).

contains channels called porins which still allow different molecules such as drugs to enter [14]. Bacteria like *E. coli* have a number of genes responsible for mediating resistance to antibacterial agents through the expression of efflux pumps, activation of antibacterial molecule degrading enzymes and the modification of antibacterial molecule binding sites leading to the inhibition of DNA gyrase and DNA topoisomerase IV synthesis [15].

Ambarwati et al. documented that the power of antimicrobial activity can be categorised using MIC. A smaller MIC value indicates greater antimicrobial activity as a powerful microbial inhibitor is effective at low concentrations. Pods ethyl acetate fraction possessed a broad-spectrum activity against a group of microorganisms that cause human skin diseases, as suggested by these results. This primary fraction revealed the chance of finding new therapeutically effective antibacterial compounds [16]. As a further investigation, the isolation and purification of pods ethyl acetate fraction was carried out to identify the components that contributed to the antibacterial activity. The methanol fraction contained the most concentrated antibacterial ingredients and the active ingredients that possessed antibacterial properties could be

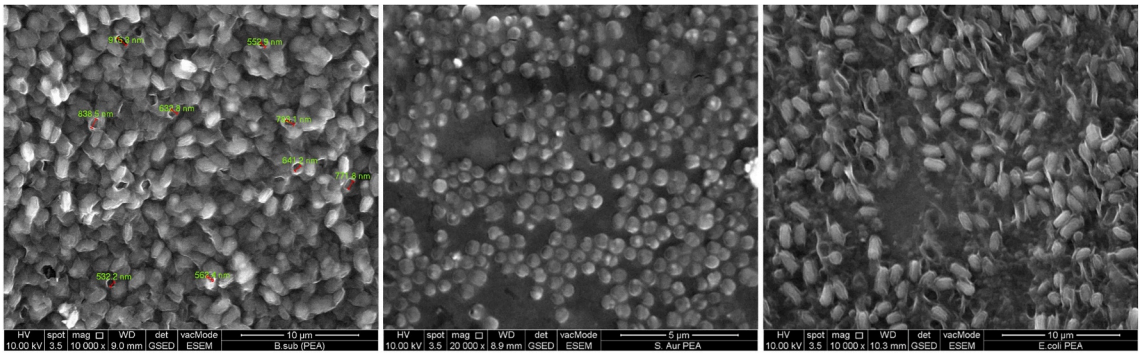

Fig. 3. Morphological changes of *B. subtilis* (a), *S. aureus* (b), *E. coli* (c) cells. FESEM image displayed the region of inhibition zone of S.*aureus* after treated with 100 mg/ml pods ethyl acetate fraction at the magnification of 50,000×.

considered polar. The antibacterial screening of pods ethyl acetate fraction showed significant activity demonstrating the importance of fractionation in searching for plant-based antimicrobials [17].

Categorisation criteria for antibacterial activity are numerous. A fraction is considered to have antibacterial properties when MIC is between 0.1 and 1 mg/ml as reported by Simões et al. [18]. According to Aligiannis et al. a fraction is considered strong when MIC is less than 0.5 mg/ml, moderate when MIC ranges from 0.5 to 1.5 mg/ml, and weak when greater than 1.5 mg/ml. Further purification of the methanol fraction with a MIC of 1.56 mg/ml could be conducted to increase the purity of antimicrobial compounds. In lieu of that, these findings can be deemed as noteworthy [19].

Generally, the inner cell membrane of bacteria is responsible for many important roles including transportation, osmoregulation and respiration, peptidoglycan biosynthesis and cross-linking as well as the synthesis of lipids. Undoubtedly, the integrity of the membrane is essential for all these functions and its disturbance could induce metabolic disruption and death of cells directly or indirectly, besides pore creation. There is limited information about how the compounds in pods ethyl acetate fraction of *P. speciosa* achieved this antibacterial activity. From the observation, the mode of action of pods ethyl acetate fraction against the Gram-positive bacteria, *S. aureus* was postulated to rely on its capability to disintegrate the cell wall and cytoplasmic membrane of bacteria, resulting in lysis and leakage of intracellular components [20].

Penetration of pods ethyl acetate fraction was suggested to induce expression of the peptidoglycan-disrupting lysozyme in the bacterial cells, causing cell autolysis. Lysozyme is the enzyme in charge of breaking down the bacterial cell wall. Lysozyme breaks down the peptidoglycan layer of the cell wall in bacteria via hydrolysis of glucosidic linkages specifically β-1, 4 linkages between N-acetylmuramic acid and 2-acetyl-amino-2-deoxy-D-glucose residues in peptidoglycan. The destabilisation of the bacterial membrane is due to the hydrolysis of these bonds as peptidoglycan causes mechanical resistance to the cell [21]. Progressive breakdown of the bacterial cell wall by lysozyme leads inevitably to protoplasts or spheroplasts, in which a persistent of water inflow from the exterior medium is enhanced leading to an elevated pressure. Due to the internal pressure generated, the cell appeared to be momentarily inflated. For Gram-positive bacteria without an outer membrane, the uptake of excess water produces enough pressure to induce cellular lysis when the gradient is large enough and eventually causes excessive leakage of the cellular content [22].

Alternatively, the bacterial murein hydrolase, as autolysin, could be catalysing cell lysis. Murein hydrolase is an enzyme that precisely cleaves structural components of the cell wall in bacteria. This enzyme is accountable for a hydrolytic activity specifically in different structural components of the peptidoglycan/These involve N-acetylmuramidase, N-acetylglucosaminidase, N-acetylmuramyl-L-alanine amidase, and endo-transglycosidase activities that have significant roles in biosynthesis and production of the bacterial cell wall [23].

Furthermore, the adverse impact on the osmoregulatory ability of the bacterial cell seemed to be connected with a membrane-permeabilising action of the fraction. Pods ethyl acetate fraction was postulated to be capable of interacting with the bacterial membrane leading to a cell membrane destabilisation and permeabilisation, multiple stresses on membrane proteins and eventually leakage of the cell content (Fig. 4) [24]. However, the exact mechanism of action of the fraction is yet to be well established. Several models have been postulated for the interaction of the fraction with the bacterial membrane including 'barrel stave', 'toroidal pore' or 'carpet model'. All these action mechanisms have similarities in which the bacterial membrane is irreparably traumatized in the end, which leads to cell necrosis [25].

Transmission electron microscopy (TEM) could resolve the lack of details in FESEM with the latter providing critical knowledge about the height of the cell and information of its topography [5]. Modifications in bacterial membrane integrity that elucidated the detailed cell death mechanism of *S. aureus* through enzyme-induced lysis could be investigated by TEM. In general, both FESEM and TEM are demanded as complementary approaches to achieve better insight into the antibacterial action of pods ethyl acetate fraction, by displaying not only cell surface action but also intracellular modification [24]. Besides, sample fixation should be carried out for this morphological study for better visualisation of the result in future studies [26]. FESEM might not be adequate to give the needed resolution to make a nano-particle size assessment as the particles were not well resolved and magnified. The accuracy of estimation of the size distribution in the image was affected which can be improved by TEM. TEM has a higher resolution that is commonly used for nano-particle imaging to perform more accurate diameter measurements at a nanoscale [27]. A recent study revealed that pods and seed extracts of *P. speciosa* have antibacterial activity against *B. cereus*, *S. aureus*, *E. coli*, *K. pneumoniae* and *P. aeruginosa* with MICs and MBCs ranging from 0.31 to 2.5 mg/mL [28]. An investigation conducted by Chhikara et al. showed that *P. speciosa* is composed of phytochemicals such as flavonoids, phenolics, alkaloids and terpenoids [29]. Subsequently, a study showed that *P. speciosa* aqueous seeds displayed remarkable inhibitory activity against some pathogenic bacteria that can affect aquatic life such as *S. aureus*, *V. parahaemolyticus. A. hydrophila*, *S. anginosus* and *S. agalactiae* [30]. Fotie et al. showed that ethyl acetate, methanol and aqueous extracts of *P. speciosa* roots exhibit antibacterial activity against human pathogenic bacteria that include *K. pneumoniae*, *C. diphtheriae*, *P. mirabilis*, *S. pyogenes* and *S. typhi* [31]. Interestingly, Wonghirundecha et al. also revealed that ethanolic pod extracts of *P. speciosa* can inhibit food-borne pathogens like *B. cereus*, *L. monocytogenes*, *S. aureus*, *E. coli*, S. Typhimurium and *V. cholerae* [32]. A similar study showed that *P. speciosa* ethanolic seed extract exhibits antibacterial activity against *S. aureus, E. coli*, *B. cereus* and *L. monocytogenes* with inhibition zones ranging from 6.87 to 11.50 mm. The study also revealed the presence of phytochemical secondary metabolites such as alkaloids, terpenoids, phenols and flavonoids in the extracts [33]. HPLC analysis of *P. speciosa* ethanolic pod extracts have indicated the presence of gallic acid, quercetin and caffeic acid, rutin

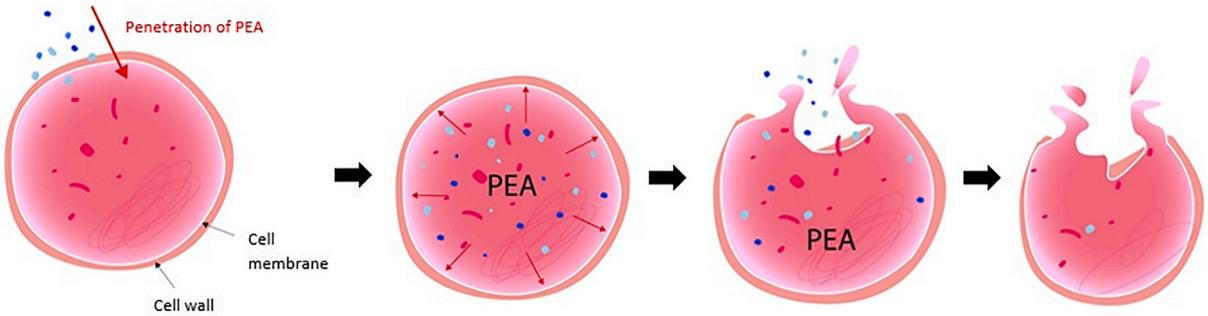

Fig. 4. Morphological variation in *S. aureus* after treated with pods ethyl acetate fraction. Penetration of pods ethyl acetate fraction generated internal pressure within bacteria cells. Bacterial cells lost turgidity and the cytoplasmic content leaked out of the cells leading to lysis of bacteria.

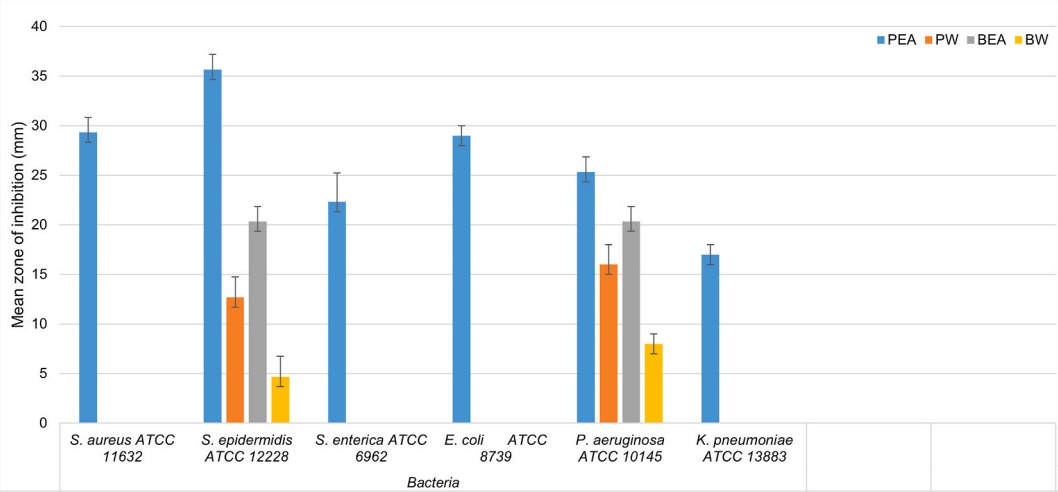

Fig. 5. Antibacterial activity of extract fractions of *P. Speciosa*. Fractions abbreviations (PEA=Pods ethyl acetate fraction, PW=Pods water fraction, BEA=Beans ethyl acetate fraction, BW=Beans water fraction).

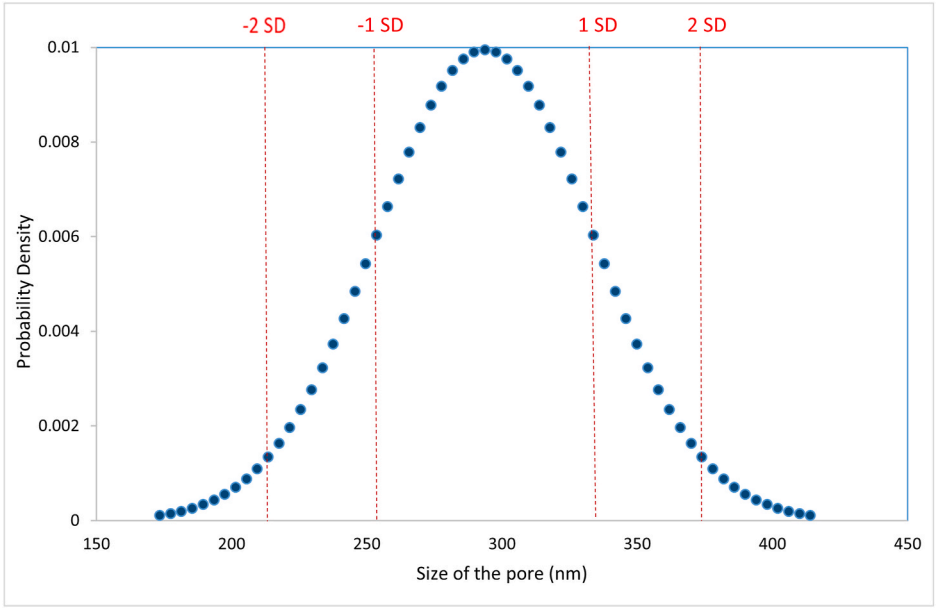

Fig. 6. Size distibution of holes in lysed *S.aureus*. The size distribution of the holes (nm) formed in the bacteria cell wall with sample means (n = 50) approached the shape of normal distribution. The area under the normal curve remained between 1 and 2 standard deviations on each side of the mean indicated 68.3 % and 95.5 % of data respectively.

[34]. Furthermore, Pantong et al. showed that ethanolic seed extract of *P. speciosa* induced inhibition zone for *S. aureus* (2.79 ± 0.11 mm), MRSA (3.19 ± 0.09 mm), B. cereus (3.23 ± 0.16 mm), *S. typhi* (1.23 ± 0.02 mm and *M. luteus* (5.4 ± 0.36 mm) [35].

5. Conclusion

In conclusion, it can be inferred that *P. speciosa* fractions at mid polarity range have broad-spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria, in which methanol and ethyl acetate fractions proved to be promising candidates to be recognized as naturally occurring novel antibacterial agents. As future perspectives and endeavours, further analysis via GC-MS or LC-MS is necessary to determine the identity of the compounds present in mid-polar extracts and their fractions of *P. speciosa* that induced antibacterial activity, particularly in the methanol and ethyl acetate fractions. Moreover, the antibacterial action mechanisms of phytochemical compounds present in these fractions need to be exploited. It is necessary to determine the antifungal and antiviral potentials of these fractions, along with the antibacterial activity of individual compounds that can be isolated from each fraction obtainable via TLC assay. Furthermore, studies can be carried out based on the significance of lead compounds using *in vivo*, ex vivo and nano-medicine drug delivery applications in the future.

CRediT authorship contribution statement

She May Goh: Writing – original draft. **Mackingsley Kushan Dassanayake:** Writing – review & editing. **Chin Chiew Foan:** Methodology. **Christophe Wiart:** Writing – review & editing. **Rachael Symonds:** Writing – review & editing. **Teng-Jin Khoo:** Supervision. **Chien Hwa Chong:** Writing – review & editing. **Omar Ashraf Elfar:** Writing – review & editing.

Ethical approval

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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