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Antibacterial and antispore activities of Sengkubak [*Pycnarrhena cauliflora* (Miers.) Diels] leaf extract against *Bacillus* spp.

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Abstract

Sengkubak [*Pycnarrhena cauliflora* (Miers.) Diels], a liana plant used as a spice in Dayak cuisine, has been reported to possess antimicrobial activity. However, the antibacterial and antispore activities of its methanolic and ethanolic leaf extracts against *Bacillus* species are not well-known. In this study, the antibacterial properties of *P. cauliflora* leaf extracts were evaluated against four *Bacillus* strains. The susceptibility test was conducted using the disc diffusion assay, minimum inhibitory concentrations (MIC), and minimum bactericidal concentrations (MBC), according to the Clinical and Laboratory Standard Institute's recommendations. Time-kill curve analysis was performed at different exposure durations and concentrations. The results showed that *P. cauliflora* leaf extract exhibited antibacterial activity against all *Bacillus* species tested, with inhibition zones ranging from 8.67±0.58 to 9.33±0.58 mm, MICs ranging from 0.938±0.44 to 1.25±0.00 mg/mL, and MBC values ranging from 3.75±1.77 to 5.00±0.00 mg/mL. Time-kill curve analysis demonstrated that the *P. cauliflora* leaf extract significantly reduced the growth of *Bacillus* spp. vegetative cells and the bactericidal endpoint was reached at 4×MIC after 4 hrs of incubations. Furthermore, *P. cauliflora* leaf extract inactivated *Bacillus* spp. spores significantly after incubation for 4 hrs at concentrations of 0.5%, 1%, 2.5% and 5%. At a concentration of 5%, the extract inactivated 99% of the spores after 1 hr incubation. These findings suggested that *P. cauliflora* leaf extract has potential as an antibacterial agent against *Bacillus* species.

1. Introduction

Bacillus species are notorious for causing spoilage in the food industry, as mentioned by Turnbull in 1996. These Gram-positive, facultative anaerobic, sporulating, rod-shaped bacteria are widely distributed in nature. *Bacillus* species are capable of adapting to environmental changes due to their diverse traits, including the production of a wide range of toxins (Van der Voort and Abee, 2013). They are also known for their ability to form endospores in response to environmental stress, which are dormant structures that help the bacteria survive in unfavourable conditions (Ulrich *et al*., 2018). Despite the fact that *Bacillus* spp. spores can remain in their dormant forms for years, exposure to agents such as particular nutrients can trigger the spores to regenerate within minutes of being exposed (Setlow, 2014). Moreover, most *Bacillus* spp. spores have been found to be heat, radiation, and chemical resistant (Turnbull, 1996). In fact, refrigeration is not an effective method to control the microbiological risks associated with *Bacillus* species (Fernández-No *et al*., 2013). Consequently, *Bacillus* species are often linked to various instances of food contamination in the food industry.

Bacillus cereus can result in two distinct types of gastrointestinal illnesses, namely diarrheal and emetic types (Tewari and Abdullah, 2014). The emetic type leads to nausea and vomiting within 1 to 5 hrs after ingestion. Emetic type is usually linked to the consumption of rice and starchy foods such as noodles and pasta that are held at room temperature for prolonged periods and then quickly fried before eating.

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On the other hand, the diarrheal type is characterized by diarrhoea and abdominal pain that usually develops between 8 to 16 hrs after consuming contaminated food. It is commonly associated with foods such as vegetables, meat, sauces, desserts, pasta, and dairy products. *Bacillus* species are often responsible for food poisoning, which can occur when the spores survive cooking or pasteurization and then proliferate when the food is not stored at the appropriate temperatures (Turnbull, 1996). The spores are able to withstand high heat or pasteurization, and if the food is not kept cold enough, the spores can germinate and multiply, leading to food poisoning. There are various approaches to managing *Bacillus* spore contamination, including the use of chemical sporicidal agents and adopting proper food handling practices. Commercial chemical sporicidal agents are often used to destroy Bacillus spores, but their effectiveness is limited, and they require special precautions due to the handling of toxic formaldehyde and glutaraldehyde (Kida *et al*., 2004). Proper food handling, such as thermal food processing, is one of the most effective and cost-efficient ways to produce safe food that is free from unwanted microbes and enzymatic reactions. However, this method can negatively impact the organoleptic qualities and nutrient content of the food, as mentioned by Cho *et al*. (2008). Thus, the interest in developing natural antibacterial and antispore agents from plant sources that have no adverse effect on the nutritional value or sensory qualities of foodstuffs is growing (Kida *et al*., 2004).

For many years, nature has provided a vast array of therapeutic substances, and numerous modern pharmaceuticals have been developed based on traditional medicinal practices. However, only a small percentage (1%) of the world's natural resources has been explored phytochemically. This is surprising given the vast number of plant species available, which is estimated to be over 500,000 (Palombo, 2011). One of the indigenous plants in Kalimantan is Sengkubak. In a recent study, crude extracts of Sengkubak were found to have antibacterial activity. Sengkubak is a liana plant that native to Sarawak and West Kalimantan with the Latin name of *Pycnarrhena cauliflora* (Miers.) Diels. The leaves are commonly used as spices and flavourings in Dayak cuisine. *P. cauliflora* is a member of the Menispermaceae family and is known to contain a significant amount of bisbenzylisoquinoline alkaloids (Sholikhah *et al*., 2021). According to Bribi (2018), these alkaloids have been found to possess various biological activities, such as antiprotozoal, antiplasmodial, antifungal, antiviral, and antibacterial properties. Hence, the present study aims to investigate the potential antibacterial and antispore activity of *P. cauliflora* leaf extract against *B. cereus*, *B. subtilis*, *B. pumilus*, and *B. megaterium*.

2. Materials and methods

2.1 Sample collection and extraction of Pycnarrhena cauliflora

Pycnarrhena cauliflora was collected from Bario, Sarawak, was air-dried and put in storage at the Laboratory of Natural Products, Institute of Bioscience (IBS), Universiti Putra Malaysia (UPM). *Pycnarrhena cauliflora* has been authenticated by the Department of Pharmacology and Therapy at the Faculty of Medicine of University Gadjah Mada in Yogyakarta, Indonesia, with voucher number 011-01/S.RPB/UII/2013 (Ramadani *et al*., 2018). The soaking method, as described by Rukayadi *et al*. (2008), was used to extract *P. cauliflora*. Absolute methanol was utilised as the organic solvent in the extraction of *P. cauliflora* (R and M Chemicals, 99.8%). To make the powder, 100 g of dried *P. cauliflora* leaves were pulverised. The extraction was done by immersing 100 g of the sample into 400 mL of solvent for 48 hrs at room temperature with occasional shaking. Whatman filter paper size No. 2 was used to filter the plant extract (Whatman International Ltd., Middlesex, England). The extracts were then concentrated at 40°C for 3-4 hrs using a rotary vacuum evaporator (Heidolph VV2011, Schwabach, Germany) to obtain a methanol extract of the *P. cauliflora* leaves. Finally, the extracts were then freeze-dried for 48 hrs to remove any remaining water. The formula below was used to determine the yield of crude extraction:

$$Yield\ (\%) = \frac{W2 - W1}{W0} \times 100$$

Where W_2 represents the weight of the extract and container, W_1 the weight of the empty container, and W_0 the initial dry sample weight.

2.2 Antibacterial activity

2.2.1 Disc diffusion assay

The disc diffusion assay against *Bacillus* spp. was performed using the method recommended by the Clinical and Laboratory Standard Institute (CLSI) (2012). With a sterile cotton swab, the inoculum was prepared and spread on an MHA plate as a single homogenous colony. The inoculated MH agar was topped with sterile paper discs Whatman No. 2 (Whatman International Ltd., Middlesex, England) with a diameter of 6 mm. Each paper disc was infused with 10 µL of 10 mg/mL *P. cauliflora* leaf extract. An amount of 1 mg/mL of CHX was used as a positive control, whereas a concentration of 10% DMSO was utilized as the negative control. The diameter of the inhibitory zone was measured in millimetres (mm) and recorded after 24 hrs of incubation at 37°C. The assay was performed in

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triplicates (n=3). All bacteria were handled aseptically and media were prepared in a class II biosafety cabinet (CLSI, 2012).

2.2.2 Minimum inhibitory concentration and minimum bactericidal concentration

The MIC and MBC were established utilising the techniques recommended by CLSI (2012). The MIC of the extract was identified using broth two-fold standard microdilution method by utilizing a sterile 96-well round bottom microtiter plate (Greiner, Germany). The inoculum suspension of all bacteria species ranged between 10^6 and 10^8 CFU/mL. The first column of the well was designated as negative control growth and filled with 100 µL MHB. The second column is designated as the positive control growth and was filled with 100 µL bacterial suspension. Microdilution was done to obtain varying concentrations which ranged from 5.00 mg/mL in column twelve to 0.0097 mg/mL in column three. The concentrations of CHX vary from 500 µg/mL in column twelve to 0.98 µg/mL in column three. The MIC was established after incubating the plate at 37°C for 24 hrs based on the smallest concentration of fraction which prevents growth in the wells. The suspension from each MIC well was subcultured on MH agar plates in order to establish the MBC. A pipette was used to transfer 10 µL of suspension from columns one to twelve of the wells to an agar plate, and this was followed by a 24-hour incubation of the plates at 37°C until no growth was observed on the control plates. MBC is the lowest antibacterial agent concentration at which no growth occurs on the MH agar plates. The handling of all bacteria and media preparation was done based on the aseptic technique in the class II biosafety cabinet (CLSI, 2012).

2.2.3 Time-kill assay

The time-kill assay of *B. cereus*, *B. subtilis*, *B. pumilus*, and *B. megaterium* was performed using methanol extracts of *P. cauliflora* according to the method described in CLSI (2012), with some modifications. In the first step, approximately 10^6 CFU/ mL of inoculum suspension was prepared; the MHB medium containing inoculum was then used to dilute the extracts to obtain final concentrations of 0×, 0.5×, 1×, 2×, and 4× MIC. The final volume (1 mL) was agitated at 200 rpm and incubated at 30°C. Aliquot in the amount of 100 µL was transferred to new microcentrifuge tubes at particular time point intervals (0, 1, 2, 3 and 4 hrs), and 1% phosphate-buffered saline (PBS) was used to serially dilute the aliquot at a ratio of 1:100 which was then plated onto the MHA. The CFU/mL was recorded as the number of colonies on the plates after incubating for 24 hrs. The graph of Log_{10} CFU/mL against time was plotted. The time-kill assay was carried out two times in triplicate data (n = 2×3).

2.3 Antispore activity

The method described by Kida *et al*. (2004) and Rukayadi *et al*. (2009a) was slightly modified, and it was then used to demonstrate antispore activity. The prepared spore's suspension was diluted in 0.85% NaCl solution with pH6.6 to obtain initial *B. cereus* ATCC33019, *B. subtilis* ATCC6633, *B. pumilus* ATCC14884, and *B. megaterium* ATCC14581 spore suspension of 6.2×10^7, 3.70×10^7, 3.30×10^7 and 7.1×10^7 spores/mL, respectively. The adjusted spore suspension was mixed with the stock extract (10%) to create the final concentration extracts (0.50, 1.00, 2.50 and 5.00%). Standard commercial glutaraldehyde solution, 25%, is used as the experiment's positive control (Merck Millipore, Darmstadt, Germany). To achieve a 1% concentration, the glutaraldehyde solution was diluted in distilled water at a ratio of 1:25. The pH of these test solutions is unaffected by the addition of either the extract or glutaraldehyde. This was followed by incubating 1 mL of each concentration for 0, 1, 2, 3, and 4 hrs in a 30°C water bath. The aliquot in the quantity of 100 µL was transferred to microcentrifuge tubes, centrifuged at 12, 000×*g* at 4°C for 5 mins, and washed twice with 0.9 mL of 0.85% NaCl solution (ALC microcentrifuge 4214, Milan, Italy). The above procedure will verify that no bacteria were introduced into the spores during preparation and that the spores were not contaminated by leftover vegetative cells. The pellets were then suspended in 100 µL 0.85% NaCl solution. The resulting mixture was then serially diluted and spread onto NA plates, which were then incubated at 30°C for a minimum of 24 hrs or until colonies became visible. The colonies that formed were counted, and the mean colony-forming units (CFU/mL) were calculated. To determine sporicidal activity, the Log_{10} CFU/mL values of the test solution were compared with those of the control (0%) with no antimicrobial. Each experiment was conducted twice, with three replicates each (n = 2×3).

2.4 Statistical analysis

The data was analysed by analysis of variance (ANOVA) using MINITAB application software 2018. Additionally, the significance of differences ($p<0.05$) between mean data was determined using Tukey's test. The results of the analysis were expressed as mean values ± standard deviation (SD).

3. Results and discussion

3.1 Yield of Pycnarrhena cauliflora extracts

Extraction is the process of separating desired

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natural products from raw materials. The extraction process generally involves introducing a solvent into the solid matrix to dissolve the desired compound, followed by the dissolution of the compound in the solvent. This is then followed by the diffusion of the dissolved compound out of the solid matrix and finally, the collection of the extracted compound (Zhang *et al*., 2018). There are many procedures to extract compounds from raw materials, which include solvent extraction, distillation, pressing and sublimation. However, the most commonly utilised approach is solvent extraction by maceration. The extraction efficiency can be affected by the characteristics of the solvent, the particle size of the raw materials, the ratio of solvent to solids, the temperature during extraction, and the amount of time it takes for extraction (Sultana *et al*., 2009).

Generally, the efficiency of extraction is determined by the size of the particle, the finer the particle the more efficient the extraction. The tiny particle size will increase the extraction efficiency due to increased solvent penetration and solute diffusion. However, a particle size that is too fine will cause the solid to absorb too much solute and make it hard to filter the solids afterwards. Other than that, solubility and diffusion are enhanced at higher temperatures. Excessive temperatures during extraction can result in the loss of solvents, leading to the extraction of unwanted impurities and the degradation of thermolabile components. In addition, increasing the extraction duration within a specific time range can enhance the extraction efficiency. After equilibrium is attained between the solute inside and outside the solid material, increasing the extraction time does not affect the yield. In most cases, the higher the ratio of solvent to solid, the bigger the amount of material that can be extracted (Zhang *et al*., 2018). In this study, solvent extraction by maceration was used to extract the natural compounds from *P. cauliflora* leaves because it is uncomplicated, practical, and inexpensive. The dried leaves of *P. cauliflora* were extracted by using two different polarities of solvents, which are absolute methanol and ethanol. The yield of the crude extract is presented in Table 1.

From the data in Table 1, the yield of crude extract was 23.17% for methanol and 18.19% for ethanol. It is very important to stress that the use of methanol as a solvent for food applications is not recommended.

Table 1. Yield of *P. cauliflora* leaves extracts.

Solvent extraction	Weight of sample (g)	Yield of extract (g)	Percentage of yield (%)
Methanol	100.00	23.17	23.17
Ethanol	100.00	18.19	18.19

Although methanol was utilised for extraction in this study, care was taken to guarantee that there was no methanol residual in the final crude extract. As the extraction process neared completion, the rotary evaporator's temperature was raised to 85°C for 2×30 s (Madiha *et al*., 2017). Since the boiling point of methanol is 50°C, it is expected that the methanol from the crude will completely evaporate at this temperature. This confirms that the final crude extract of *P. cauliflora* that was prepared for this study did not contain any methanol.

3.2 Disc diffusion assay

Generally, disc diffusion assay involves spreading a lawn of bacteria onto a solid agar medium and then placing a paper disc impregnated with the substance being tested onto the agar (Balouiri *et al*., 2016). The bacteria are allowed to grow for a specified amount of time, after which the diameter of the zone of inhibition (the area around the disc where no bacterial growth has occurred) is measured. This can be used to determine the potency of the substance being tested. In this study, the disc diffusion assay (DDA) is a preliminary screening method for assessing the antibacterial activity of *P. cauliflora* leaf extract against *Bacillus* species. The DDA principle states that the greater the zone of inhibition, the better the antibacterial activity. The disc integrated with crude extracts of *P. cauliflora* from both methanol and ethanol extraction solvents were tested against *B. cereus* ATCC33019, *B. subtilis* ATCC6633, *B. megaterium* ATCC14581, and *B. pumilus* ATCC14884. The ability of *P. cauliflora* extract at a concentration of 1% (10 mg/mL) to inhibit all tested *Bacillus* spp. is presented in Table 2.

Based on Table 2, the inhibition zones for both methanol and ethanol extract range between 8.67±0.58 and 9.33±0.58 mm. The highest inhibition zone was observed for *B. megaterium* at 9.33±0.58 mm for methanolic extract. In general, both extraction solvents showed almost the same inhibition zone results (8.67±0.58 mm) against *B. subtilis*, *B. megaterium* and *B. pumilus*. However, no activity was observed for the ethanolic extract against *B. cereus* at the concentration of 10 mg/mL. The data obtained from DDA is inadequate to confirm the antibacterial activity for both extraction solvents because there are a number of factors that can cause DDA results to be inaccurate. Two of these concerns are the ability of hydrophobic substances to diffuse into the agar as well as the ability of an extract to diffuse through the pores of a disc (Wilkinson, 2006). According to Gangoué-Piéboji *et al*. (2009), a small amount of active compounds may become trapped in the pores of the disc. As a result, these active compounds are unable to travel through the disc and reach the inoculated medium. Other than that, the rate of diffusion through an agar media and the solubility of active compounds both

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Table 2. Inhibition zone of *P. cauliflora* leaves extracts against *Bacillus* spp.

Bacillus spp.	Inhibition zone (mm)			
	Methanol extract (10 mg/mL)	Ethanol Extract (10 mg/mL)	CHX (1 mg/mL)	DMSO (10%)
B. cereus ATCC33019	8.67±0.58	n.a	18±0.00	n.a
B. subtilis ATCC6633	8.67±0.58	8.67±0.58	18±0.00	n.a
B. megaterium ATCC14581	9.33±0.58	9.00±0.00	18±0.00	n.a
B. pumilus ATCC14884	8.67±0.58	8.67±0.58	20±0.00	n.a

Zones of inhibition were measured as the diameter (in mm) and included the 6-mm-diameter disc. Values are presented as mean±SD with triplicates. Positive control (chlorhexidine: CHX; 1 mg/mL). Negative control (DMSO; 10%). n.a: not active (no inhibition).

affect the size of inhibition area (Jun *et al*., 2013). Thus, MIC and MBC determination will further confirm the antibacterial activity of the *P. cauliflora* leaf extracts.

3.3 Minimum inhibitory concentration and minimum bactericidal concentration of Pycnarrhena cauliflora methanolic and ethanolic extract

MIC is a measure of the lowest concentration of a substance that is able to inhibit the growth of a particular type of bacteria. This is typically determined by preparing a series of dilutions of the substance being tested and then incubating them with a bacterial culture. The lowest concentration of the substance that prevents visible growth of the bacteria is the MIC. MBC is similar to MIC, but instead of measuring the lowest concentration of a substance that prevents bacterial growth, it measures the lowest concentration that is able to kill the bacteria. This is typically determined by plating the bacterial culture on agar after incubation with the substance being tested and then determining the lowest concentration of the substance that results in no bacterial growth on the agar.

MIC is defined as the concentration required to prevent at least 99% of bacterial/fungal growth (bacteriostatic). Meanwhile, the MBC is defined as the lowest plant extract concentration required to kill at least 99% of bacteria (bactericidal) (Rukayadi *et al*., 2013). According to Silva *et al*. (2018), antimicrobial activity is good when MIC values are less than 100 µg/mL, moderate between 500 and 100 µg/mL, weak between 1000 and 500 µg/mL, and inactive above 1000 µg/mL. Based on Table 3, the methanol extract of *P. cauliflora* showed good bactericidal activity against *B. cereus, B. subtilis, B. megaterium,* and *B. pumilus*, with a MIC of 0.938 mg/mL. The ethanol extract has MIC activity against *Bacillus* species at a concentration of 1.25 mg/mL and MBC above 5 mg/mL, while the bactericidal activity of the methanol extract against *Bacillus* species was observed at a concentration of 5 mg/mL. Crude plant extracts contain a variety of phytochemical compounds that work together in a synergistic manner and have been found to possess medicinal properties, such as antimicrobial activity. According to Xu *et al*. (2018), the combination of these compounds in a synergistic manner can be more effective against pathogenic bacteria and may prevent the development of antimicrobial resistance. Generally, there was no significant difference between the MIC of the methanol and ethanol extracts against *B. subtilis*, *B. megaterium*, and *B. pumilus*. However, the ethanol extract showed no antimicrobial activity against *B. cereus* at a concentration of 5 mg/mL.

The methanolic extract of *P. cauliflora* leaf was selected for the time-killing assay. The selection of the methanol extract was supported by a previous study in which the methanol extract demonstrated good *in vitro* antimicrobial profile, particularly against *Streptococcus pyogenes, S. mutants*, *Staphylococcus aureus*, *S. epidermidis*, *Salmonella enterica* serovar Typhi, *Shigella flexneri*, *Pseudomonas aeruginosa*, and *Escherichia coli* (Sholikhah *et al*., 2021). However, the study lacks information on the MIC and MBC profile of *P. cauliflora* leaf extracts against the bacteria tested. This is an important aspect to consider as the MIC and MBC values provide crucial information about the potency and effectiveness of the extract against specific bacterial strains. Further investigation is required to determine the

Table 3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *P. cauliflora* leaves extracts against *Bacillus* spp.

Bacteria	Methanol		Ethanol		CHX	
	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)
B. cereus ATCC33019	1.250±0.000	5.000±0.000	>5.000±0.000	>5.000±0.000	0.013±0.010	0.042±0.020
B. subtilis ATCC6633	0.938±0.440	3.750±1.770	1.250±0.000	>5.000±0.000	0.010±0.000	0.042±0.020
B. megaterium ATCC14581	0.938±0.440	3.750±1.770	1.250±0.000	>5.000±0.000	0.013±0.010	0.042±0.020
B. pumilus ATCC14884	1.250±0.000	5.000±0.000	1.250±0.000	>5.000±0.000	0.010±0.000	0.042±0.020

Values are presented as mean±SD with triplicates. Positive control (chlorhexidine: CHX; 1 mg/mL). Negative control (DMSO; 10%). n.a: not active (no inhibition).

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MIC and MBC values for the tested bacteria and evaluate the potential of the methanol extract as a therapeutic agent against these pathogens.

Based on Table 3, the MIC and MBC of the positive control (CHX) were lower than those of the *P. cauliflora* leaf extract. The minimum inhibitory concentration is a widely used parameter for determining the efficacy of antimicrobial agents against microorganisms. It predicts the minimum concentration of an antimicrobial agent required to inhibit the growth of a microorganism at a standard inoculum of approximately 10^5 CFU/mL after an incubation period of 18-24 hrs. This information is used to guide the selection of appropriate antimicrobial agents for treatment. Unfortunately, MIC only provides limited information on the kinetics of the antimicrobial action. Therefore, a time-killing assay was performed to determine the correlation between the rate of bactericidal activity, incubation time, and concentration of the antimicrobial agent.

3.4 Time-kill assay

A time-kill curve is a graphical representation of the effect of an antimicrobial agent on the viability of microorganisms over time (Balouiri *et al*., 2016). The x-axis represents time and the y-axis represents the number of viable microorganisms present (usually measured as colony-forming units, or CFUs). In general, a time-kill curve is obtained by introducing a sample of a particular microorganism into a culture medium containing a range of concentrations of an antimicrobial agent and then measuring the effect of the agent on the microorganism over time. Aliquots of the culture are taken at regular intervals and plated onto agar to determine the number of viable cells present at each time point. The resulting data points are plotted on the graph to show the decline in the number of viable cells over time.

Time-kill curves are used to evaluate the bactericidal activity of an antimicrobial agent, which refers to its ability to kill bacteria (Pankey and Sabath, 2004). The slope of the curve can indicate the rate at which the antimicrobial agent is killing the bacteria, and the shape of the curve can provide information about the mechanism of action of the antimicrobial agent. For example, a steep decline in the number of viable cells at the beginning of the curve may indicate a rapid bactericidal effect, while a more gradual decline may indicate a slower bactericidal effect. The shape of the curve can also indicate whether the antimicrobial agent is bacteriostatic (inhibits bacterial growth) or bactericidal (kills bacteria). Time-kill curves can be useful for comparing the effectiveness of different antimicrobial agents, as well as for determining the appropriate concentration and duration of treatment for a given infection (Foerster *et al*., 2016). Time-kill curves illustrate the relationship between concentration and time for treatment to act on the tested microorganisms. Studies have shown that longer treatment with higher concentrations of an extract leads to a corresponding decrease in the number of viable cells. Bactericidal activity, as defined by Pankey and Ashcraft (2009), is the reduction of at least 99.9% (≥3 $\log_{10}$) in the CFU/mL count compared to the initial inoculum. Table 4 lists the concentrations of *P. cauliflora* extract at 0.5× MIC, 1× MIC, 2× MIC, and 4× MIC.

The time-kill curves of four different types of bacteria *(B. cereus* ATCC33019, *B. subtilis* ATCC6633, *B. pumilus* ATCC14884, and *B. megaterium* ATCC14581) were plotted to examine the relationship between the minimum inhibitory concentration and the bactericidal effect of different concentrations of *P. cauliflora* extract, ranging from 0 to 4× MIC. It was found that the bactericidal endpoint for *B. cereus* (Figure 1), *B. megaterium* (Figure 2), *B. pumilus* (Figure 3), and *B. subtilis* (Figure 4) was reached 4 hrs after incubation with a concentration of 5 mg/mL, 3.75 mg/mL, 5 mg/mL, and 3.75 mg/mL (4 × MIC) of *P. cauliflora* extract, respectively. The observed effectiveness of the extract may be attributed to its high concentration of major secondary metabolites, such as phenolics, alkaloids, flavonoids, tannins, and other identified compounds, which have been previously reported in *P. cauliflora* extracts (Nor Azman *et al*., 2022). Secondary metabolites are chemical compounds produced by plants that are not directly involved in the growth, development, or reproduction of the plant, but play important roles in the ecology and health of the plant (Anulika *et al*., 2016). These compounds often have important functions in the interaction of the plant with its environment and can exhibit a variety of biological activities. One of the primary functions of secondary metabolites in plants is protection. Many secondary metabolites, such as tannins, alkaloids, and flavonoids, act as defence compounds, protecting the plant from herbivores, pathogens, and other environmental stressors (Bartwal *et al*., 2012).

Table 4. Concentration of *P. cauliflora* extract in 0.5× MIC, 1× MIC, 2× MIC, and 4 × MIC.

Bacteria	0.5× MIC (mg/mL)	1× MIC (mg/mL)	2× MIC (mg/mL)	4× MIC (mg/mL)
B. cereus ATCC33019	0.625	1.250	2.500	5.000
B. subtilis ATCC6633	0.469	0.938	1.876	3.752
B. megaterium ATCC14581	0.469	0.938	1.876	3.752
B. pumilus ATCC14884	0.625	1.250	2.500	5.000

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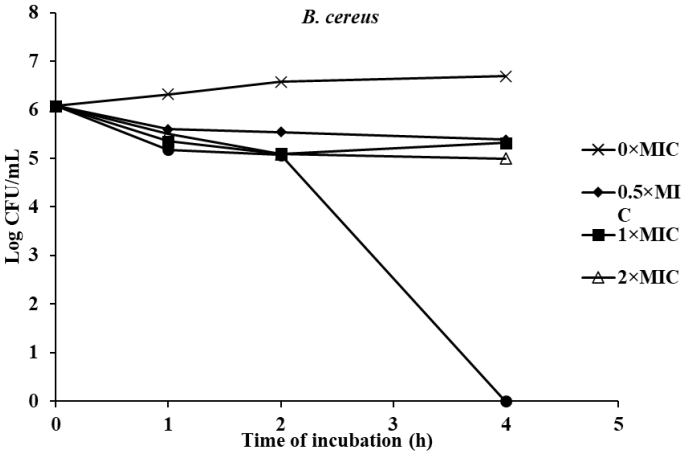

Figure 1. Time-kill curve plots for *B. cereus* ATCC33019 following exposure to *P. cauliflora* at 0× MIC (0 mg/mL), 0.5× MIC (0.625 mg/mL), 1× MIC (1.250 mg/mL), 2× MIC (2.500 mg/mL), and 4× MIC (5.000 mg/mL).

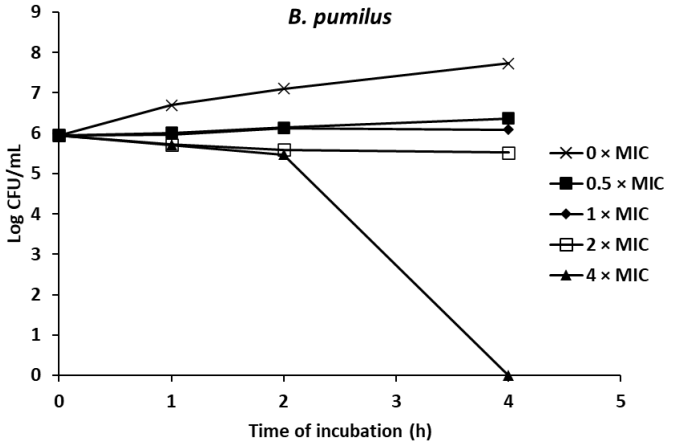

Figure 3. Time-kill curve plots for *B. pumilus* ATCC14884 following exposure to *P. cauliflora* extract at 0× MIC (0 mg/mL), 0.5× MIC (0.625 mg/mL), 1× MIC (1.250 mg/mL), 2× MIC (2.500 mg/mL), and 4× MIC (5.000 mg/mL).

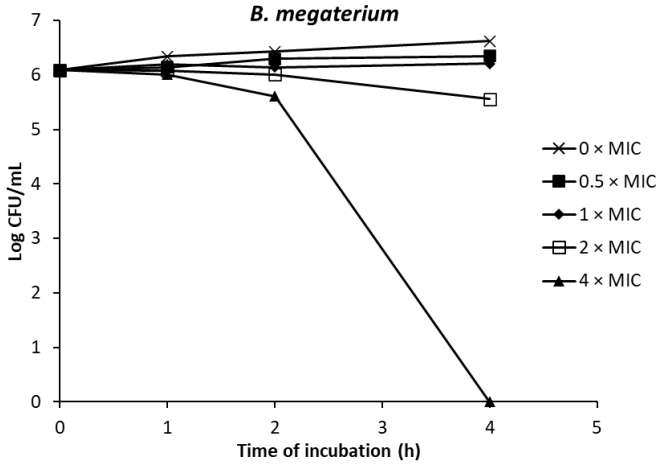

Figure 2. Time-kill curve plots for *B. megaterium* ATCC14581 following exposure to *P. cauliflora* extract at 0× MIC (0 mg/mL), 0.5× MIC (0.469 mg/mL), 1× MIC (0.938 mg/mL), 2× MIC (1.876 mg/mL), and 4× MIC (3.752mg/mL).

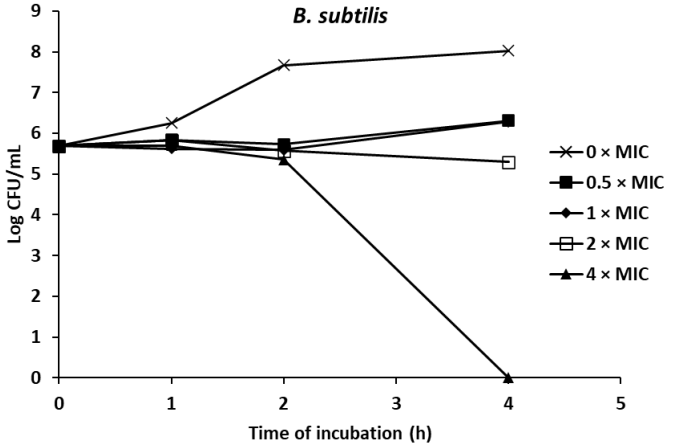

Figure 4. Time-kill curve plots for *B. subtilis* ATCC6633 following exposure to *P. cauliflora* extract at 0× MIC (0 mg/mL), 0.5× MIC (0.469 mg/mL), 1× MIC (0.938 mg/mL), 2× MIC (1.876 mg/mL), and 4× MIC (3.752 mg/mL).

According to previous research, *P. cauliflora* contains tannin, an active compound that has the ability to inhibit the growth of microbes (Chung *et al*., 1998). Crude plant extracts often consist of a mixture of particles with varying chemical structures and compositions, which can affect their biological actions (Saritha *et al*., 2015). The antibacterial action of an extract may be restricted to disrupting the membrane of the bacteria. The antimicrobial action of various proteins and small molecules is believed to lead to the formation of pores in the membrane of bacteria, causing leakage of cellular contents (Yenugu *et al*., 2006). Liang *et al*. (2022) have reported that the antimicrobial action of medicinal compounds can be achieved through a variety of mechanisms, including damage to the cell membrane and wall, inhibiting nucleic acid and protein synthesis, disrupting energy metabolism, inhibiting bacterial efflux pumps, and increasing intracellular osmotic pressure. Additionally, organic acids have been found to exert their antimicrobial effects by altering intracellular pH. Overall, cell membrane damage is considered the most prevalent mechanism of antimicrobial activity. Most medicinal plants contain a variety of complex and diverse alkaloids, which are a group of nitrogen-containing organic compounds known for their strong antimicrobial properties (Kurek, 2019). Alkaloids serve as a prominent source of bioactive compounds in medicinal plants. An example of an alkaloid is berberine, which has been shown to be effective against Gram-positive bacteria, particularly through its impact on the cell division protein filamenting temperature-sensitive mutant Z (FtsZ) (Sun *et al*., 2014).

3.5 Antispore activity

The efficacy of different concentrations (0.00, 0.50, 1.00, 2.50, and 5.00%) of *P. cauliflora* leaves extract in reducing the viability of *B. cereus*, *B. subtilis*, *B. pumilus*, and *B. megaterium* spores after incubation at 30°C for 1, 2, 3, and 4 hrs was evaluated. The impact of varying concentrations of *P. cauliflora* extract on *B. cereus* ATCC33019, *B. subtilis* ATCC6633, *B. pumilus* ATCC14884, and *B. megaterium* ATCC14581 spores at

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different incubation times is presented in Tables 5, 6, 7, and 8, respectively. A substantial decrease in the number of *B. cereus* ATCC33019 spore (Table 5), *B. subtilis* ATCC6633 spore (Table 6), *B. pumilus* ATCC14884 spore (Table 7), and *B. megaterium* ATCC14581 spores (Table 8) was observed after treatment with 0.50% *P. cauliflora* extract. Incubation with 2.50% and 5.00% concentrations of *P. cauliflora* extract mostly resulted in the death of all *Bacillus* spp. spores after 1 hr of incubation. A comparison of 1 hr and 4 hrs incubation periods showed that the strains incubated for 4 hrs had a higher reduction of spores, as the longer contact time allowed the *P. cauliflora* extract to act on the spores. This trend was observed for all concentrations tested. According to some reports, the spore's coat, which is an outer protein layer, contributes to the spore's resilience (Aronson and Fitz-James, 1976; Pandey and Aronson, 1979). This result is particularly interesting, as previous studies found that the outer membrane did not have a large impact on spore resistance, implying that it is not a critical protective layer (Setlow *et al*., 2000; Nicholson *et al*., 2000). Additionally, the inner membrane acts as a strong barrier to control the flow of substances, plays a vital role in preserving the spore's internal structure, particularly its DNA, and has protective mechanisms to defend against anti-sporicidal chemicals (Russell, 1990; Driks, 1999; Setlow *et al*., 2000; Nicholson *et al*., 2000). Dabiri *et al*. (2014) reported in a previous study that the ethanol extract of *Arctium lappa* root, which contains terpenoids, flavonoids, tannins, terpenoids, and several polyphenols, exhibits sporicidal activity against *B. cereus* spores. According to previous research conducted by Nor Azman *et al*. (2022), *P. cauliflora* is known to possess various compounds, including alkaloids, flavonoids, saponin, phenol, tannin, and terpenoids, which are primarily found in its leaves. Thus, the results strongly suggest that the antispore activity observed in the studied extracts was primarily due to the presence of secondary metabolites.

4. Conclusion

Table 5. Sporicidal activity of *P. cauliflora* extract against spores of *B. cereus* ATCC33019.

Concentration (%w/v)	Extract (EX)/ Glutaraldehyde (GLU)	Time (hrs)			
		1	2	3	4
0.0	EX	6.03±0.04^{a}	6.03±0.04^{a}	6.03±0.04^{a}	6.03±0.04^{a}
	GLU	6.24±0.00^{A}	6.24±0.00^{A}	6.24±0.00^{A}	6.24±0.00^{A}
0.5	EX	5.95±0.05^{b}	5.68±0.01^{b}	5.45±0.02^{b}	5.03±0.05^{b}
	GLU	3.74±0.10^{B}	3.62±0.04^{B}	2.46±0.15^{B}	0.00±0.00^{B}
1.0	EX	4.87±0.10^{c}	4.48±0.01^{c}	4.23±0.07^{c}	3.86±0.09^{c}
	GLU	0.00±0.00^{C}	0.00±0.00^{C}	0.00±0.00^{C}	0.00±0.00^{B}
2.5	EX	3.33±0.15^{d}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}
	GLU	0.00±0.00^{C}	0.00±0.00^{C}	0.00±0.00^{C}	0.00±0.00^{B}
5.0	EX	0.00±0.00^{e}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}
	GLU	0.00±0.00^{C}	0.00±0.00^{C}	0.00±0.00^{C}	0.00±0.00^{B}

Values are presented as mean±SD in $\log_{10}$ CFU/mL (n = 2×3). Values with different superscripts within the same column are statistically significantly different at $p<0.05$.

Table 6. Sporicidal activity of *P. cauliflora* extract against spores of *B. subtilis* ATCC6633.

Concentration (%w/v)	Extract (EX)/ Glutaraldehyde (GLU)	Time (hrs)			
		1	2	3	4
0.0	EX	6.15±0.01^{a}	6.15±0.01^{a}	6.15±0.01^{a}	6.15±0.01^{a}
	GLU	6.01±0.02^{A}	6.01±0.02^{A}	6.01±0.02^{A}	6.01±0.02^{A}
0.5	EX	5.63±0.03^{b}	5.45±0.02^{b}	5.28±0.03^{b}	4.91±0.02^{b}
	GLU	3.62±0.05^{B}	3.21±0.18^{B}	2.98±0.00^{B}	0.00±0.00^{B}
1.0	EX	4.78±0.06^{c}	4.39±0.01^{c}	3.69±0.00^{c}	3.58±0.09^{c}
	GLU	0.00±0.00^{C}	0.00±0.00^{C}	0.00±0.00^{C}	0.00±0.00^{C}
2.5	EX	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}
	GLU	0.00±0.00^{C}	0.00±0.00^{C}	0.00±0.00^{C}	0.00±0.00^{C}
5.0	EX	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}
	GLU	0.00±0.00^{C}	0.00±0.00^{C}	0.00±0.00^{C}	0.00±0.00^{C}

Values are presented as mean±SD in $\log_{10}$ CFU/mL (n = 2×3). Values with different superscripts within the same column are statistically significantly different at $p<0.05$.

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Table 7. Sporicidal activity of *P. cauliflora* extract against spores of *B. pumilus* ATCC14884.

Concentration (%w/v)	Extract (EX)/ Glutaraldehyde (GLU)	Time (hrs)			
		1	2	3	4
0.0	EX	5.69±0.07^{a}	5.69±0.07^{a}	5.69±0.07^{a}	5.69±0.07^{a}
	GLU	6.05±0.06^{A}	6.05±0.06^{A}	6.05±0.06^{A}	6.05±0.06^{A}
0.5	EX	5.63±0.03^{a}	5.55±0.02^{a}	5.26±0.07^{b}	5.16±0.04^{b}
	GLU	3.71±0.02^{B}	3.48±0.21^{B}	3.33±0.15^{B}	2.90±0.42^{B}
1.0	EX	4.90±0.11^{b}	4.78±0.06^{b}	4.27±0.02^{c}	4.01±0.07^{c}
	GLU	0.00±0.00^{C}	0.00±0.00^{C}	0.00±0.00^{C}	0.00±0.00^{C}
2.5	EX	3.48±0.21^{c}	0.00±0.00^{c}	0.00±0.00^{d}	0.00±0.00^{d}
	GLU	0.00±0.00^{C}	0.00±0.00^{C}	0.00±0.00^{C}	0.00±0.00^{C}
5.0	EX	0.00±0.00^{d}	0.00±0.00^{c}	0.00±0.00^{d}	0.00±0.00^{d}
	GLU	0.00±0.00^{C}	0.00±0.00^{C}	0.00±0.00^{C}	0.00±0.00^{C}

Values are presented as mean±SD in $\log_{10}$ CFU/mL (n = 2×3). Values with different superscripts within the same column are statistically significantly different at $p<0.05$.

Table 8. Sporicidal activity of *P. cauliflora* extract against spores of *B. megaterium* ATCC14581.

Concentration (%w/v)	Extract (EX)/ Glutaraldehyde (GLU)	Time (hrs)			
		1	2	3	4
0.0	EX	6.14±0.07^{a}	6.14±0.07^{a}	6.14±0.07^{a}	6.14±0.07^{a}
	GLU	6.13±0.05^{A}	6.13±0.05^{A}	6.13±0.05^{A}	6.13±0.05^{A}
0.5	EX	5.42±0.02^{b}	5.14±0.07^{b}	4.90±0.04^{b}	4.81±0.03^{b}
	GLU	3.82±0.03^{B}	3.71±0.02^{B}	3.21±0.18^{B}	2.48±0.27^{B}
1.0	EX	4.78±0.01^{c}	4.60±0.11^{c}	4.35±0.02^{c}	4.00±0.00^{c}
	GLU	0.00±0.00^{C}	0.00±0.00^{C}	0.00±0.00^{C}	0.00±0.00^{C}
2.5	EX	3.68±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}
	GLU	0.00±0.00^{C}	0.00±0.00^{C}	0.00±0.00^{C}	0.00±0.00^{C}
5.0	EX	0.00±0.00^{e}	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}
	GLU	0.00±0.00^{C}	0.00±0.00^{C}	0.00±0.00^{C}	0.00±0.00^{C}

Values are presented as mean±SD in $\log_{10}$ CFU/mL (n = 2×3). Values with different superscripts within the same column are statistically significantly different at $p<0.05$.

Antibacterial activity has been demonstrated in the *P. cauliflora* leaf extract and its fractions against *Bacillus* spp. The DDA results indicate that the methanolic and ethanolic extracts of *P. cauliflora* leaf inhibit the growth of *Bacillus* spp. with inhibition zones ranging from 8.67±0.58 to 9.33±0.58 mm. The highest inhibition zone was observed for *B. megaterium* with the methanolic extract. The methanol extract of *P. cauliflora* has shown better antibacterial activity against *B. cereus*, *B. subtilis*, *B. megaterium*, and *B. pumilus* with an MIC of 0.938 mg/mL compared to the ethanol extract, which exhibited an MIC of 1.25 mg/mL against the *Bacillus* spp. The MBC of the methanolic extract was observed at 5 mg/mL, while the ethanolic extract was above 5 mg/mL. The time-kill assay showed that the bactericidal endpoint for *B. cereus*, *B. megaterium*, *B. pumilus*, and *B. subtilis* was reached at four h after incubation with a concentration of 5 mg/mL, 3.75 mg/mL, 5 mg/mL, and 3.75 mg/mL (4× MIC), respectively. The study evaluated the ability of *P. cauliflora* leaf extract at various concentrations (0.00, 0.50, 1.00, 2.50, and 5.00%) to reduce the viability of *B. cereus*, *B. subtilis*, *B. pumilus*, and *B. megaterium* spores after incubation at 30°C for 1, 2, 3, and 4 hrs. The results indicated that the 1% *P. cauliflora* leaf extracts exhibited antispore activity, and the methanolic extract at a concentration of 2.5% was capable of eradicating all *Bacillus* spp. spores. The *P. cauliflora* leaf extract has antibacterial and antispore activities against *Bacillus* spp. vegetative cells and spores. Thus, it can be developed as a natural anti-*Bacillus* agent.

Conflict of interest

The authors declare no conflict of interest.

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