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Controlling vegetative cells and spores growth of *Bacillus* spp. using ethanolic *Ketapang* (*Terminalia catappa* L.) leaf extract

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ABSTRACT

Terminalia catappa L. is a large, spreading type of tree which usually grows in tropical environment, especially at coastal area with sandy stones. The current study evaluated anti-*Bacillus* potential of the ethanolic *ketapang* (*Terminalia catappa* L.) leaf extract (EKLE) as antibacterial and sporicidal agent against vegetative cells and spores of *Bacillus* spp. The antibacterial activity of EKLE against *Bacillus* spp. (*B. cereus* ATCC33019, *B. pumilus* ATCC14884, *B. subtilis* ATCC6633 and *B. megaterium* ATCC14581) vegetative cells were determined by performing well diffusion assay (WDA), minimum inhibition concentration (MIC), minimum bacterial concentration (MBC) and time-kill curve analyses. The sporicidal activity was tested at different concentrations of EKLE. Then, the extract's stability in terms of antibacterial and sporicidal activities upon exposure to different temperatures and pHs were carried out. Results demonstrated inhibition zones of EKLE against *Bacillus* spp. was in the range of 9.25 ± 0.75 mm - 11.67 ± 0.47 mm. All vegetative cells of *Bacillus* spp. were inhibited with MIC values at 0.63–1.25 mg/mL and can be completely killed with MBC values of 0.63 - >5.00 mg/mL. Time-kill analysis showed all the *Bacillus* spp. tested can be completely killed at concentrations of 2.50–5.00 mg/mL from 1 to 4 h. EKLE concentration of 1% (w/v) completely killed all *Bacillus* spp. spores at different exposure time. The antibacterial and sporicidal activities of EKLE were not affected by exposure to different temperatures (4, 30, 50, 80 and 121 °C) and pHs (3, 7 and 10), revealing the stability of the extract against different conditions. In conclusion, *Terminalia catappa* L. leaf exhibits antibacterial and sporicidal activities against *Bacillus* spp., therefore, the extract can be developed as anti-*Bacillus* agent, paving the way for its utilization in food industry as a natural food preservative.

1. Introduction

T. catappa L. which is also known as Indian almond and tropical almond is mainly found in Asia, where parts of the tree such as

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leaves, kernel, barks and roots have been using traditionally for medicinal purposes to treat liver diseases, scabies, leprosy, bronchitis and bowels across some countries, including Malaysia, Indonesia, Philippines, Taiwan, India and Bangladesh [1–3]. *T. catappa* L. leaf has chemo preventive effect, antibacterial, hepatoprotective, antioxidant, anti-inflammatory, antiparasitic and antifungal activities, showing a huge potential for more studies [4]. A number of studies have found that *T. catappa* L. is a potential source of antibacterial agents as minimal concentration is effective to inhibit bacterial activity, especially for Gram-positive bacteria [5,6], showing more studies using Gram-positive bacteria can be carried to learn its antibacterial properties which to benefit food industry.

Bacillus spp. is one of the crucial pathogens to be studied in food safety because the species is ubiquitous and commonly known to contaminate starchy foods like rice, soy, and vegetables [7]. Spores form by *Bacillus* spp. are known for the ability of being thermoresistant which is a huge problem for food industry as it keeps germinating and growing upon favorable conditions despite various processing steps to kill vegetative cells [8]. The multiple layers of spore and the compositions of different layers are responsible for resistance mechanism of *Bacillus* spores. Food grade antimicrobial agents such as protamine, polylysine, essential oils and organic acids are currently being applied to control outbreaks from spores [9]. Aldehydes like glutaraldehyde and formaldehyde as well as hydrogen peroxide and peroxy acids are well known sporicidal agent, either used individually or combined with disinfectants [10]. Each chemical may have own mechanism to kill spores which includes DNA damage, damaging the layers or spore coats. However, glutaraldehyde kills spores by preventing normal germination before DPA release and by cross-linking their outer layers, including possibly the cortex [11]. Nevertheless, these chemicals are toxic and harmful to human; thus, its usage is limited for sterilization practice of equipment surfaces in food industry.

In an antibacterial study by Allyn et al. it has been reported that *T. catappa* L. leaf extract has antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* [3]. However, the antibacterial activity of ethanolic *T. catappa* L. leaf extracts against *Bacillus* spp. has not been reported, creating a niche for study. Some of antibacterial agents in use to kill vegetative state of *Bacillus* spp. are chlorhexidine and hydrogen peroxide [12]. However, these chemicals are toxic to human in prolong use at high concentration. Therefore, it is necessary to find natural substances which are relatively safe to be used as antibacterial activity against vegetative cells of *Bacillus* spp. Furthermore, the sporicidal activity of this leaf has not been reported yet. In this study, the antibacterial and sporicidal activities of *T. catappa* L. leaf extract against *Bacillus* spp. vegetative cells and spores are determined. The extract's stability at different temperature and pH has been conducted.

2. Materials and methods

2.1. Samples

Leaves of *T. catappa* L. were dried in oven (SMA-112, Smoke Master, Toda, Japan) at 50 °C for two days, followed by grounding to fine powder by using a dry blender (Panasonic MX-GM1011, Osaka, Japan). Then, dried powder was added to 95% (v/v) ethanol at the ratio of 1:4 and kept for 7 days at room temperature with occasional shaking. Whatman filter paper No.1 (Whatman International Ltd., Middlesex, England) was used to filter the plant extracts. Rotary vacuum evaporator (BUCHI Rotavapor R-200, Flawil, Switzerland) was used to concentrate the extracts at 50 °C and at the speed of 150 rpm for about 3–4 h. For further analysis, the crude extract was diluted in dimethyl sulfoxide (DMSO) to obtain 100 mg/mL of concentration and then further diluted in 1:10 (v/v) sterile distilled water to get 10 mg/mL stock solutions.

2.2. Well diffusion assay (WDA)

Well Diffusion Assay method was carried according to Clinical & Laboratory Standards Institute (CLSI) [13]. By using a sterile cotton swab, freshly prepared bacterial inoculum was spread onto an MHA plate. Wells of six mm diameter in agar medium were punched and filled with 50 μL of 10 mg/mL extract. Wells with same volume of CHX of 0.1% (1 mg/mL; w/v) and DMSO (10%; v/v) were served as positive and negative control, respectively. The plates were then incubated at 37 °C for 24 h. The results are based on the measurement of diameter of inhibition zone and recorded in millimeters (mm).

2.3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Minimum inhibitory concentration (MIC) method was carried according to Clinical & Laboratory Standards Institute (CLSI) [13]. Microdilution was carried in sterile 96-well round bottom microtiter plate. The inoculum suspension of bacteria was adjusted from 10^6 to 10^8 CFU/mL. By using micropipette, first column from the left was filled with 200 μL of Mueller-Hinton Broth (MHB), which served as the negative control growth. For positive control, second column from the left was filled with 200 μL of prepared bacterial suspension. The microdilution of two-fold was performed using stock of extract to concentration ranged from 5 mg/mL (12th column) to 0.01 mg/mL (3rd column). CHX and DMSO were used in subsequent rows instead of plant extract with same volume of bacterial suspension. Plate was incubated for 24 h at 37 °C. The least concentration in well which has no turbidity present was observed and determined as the MIC value.

Upon determination of MIC after 24 h, minimum bactericidal concentration (MBC) was carried immediately by sub-culturing suspension from each MIC wells onto MHA plate. Volume of 10 μL from each of the wells from all tested column was pipetted onto the agar plates. Then, the plates were incubated at 37 °C for 24 h or until growth was seen on growth control plates.

2.4. Time-kill analysis

Briefly, the inoculum suspension of bacteria was adjusted to approximately 10^6 CFU/mL. The plant extract stock was diluted with the MHB medium containing inoculum to obtain final concentrations of 0 × MIC, 0.5 × MIC, 1 × MIC, 2 × MIC and 4 × MIC, referring to MIC values obtained for each bacterial species. Cultures with a final volume of 1 mL each were incubated at 37 °C with 200 rpm agitation. At pre-determined time points (0, 0.5, 1, 2 and 4 h), aliquots of 100 μL were removed and transferred to microcentrifuge tubes. The aliquot was serially diluted to 10^{-2} and 10^{-4} in 1% phosphate buffered saline (PBS). Then, spread plate was carried out in MHA plates. The number of colonies formed on the plates after incubation at 37 °C for 24 h was counted and the number of CFU/mL was calculated. The graph of $\log_{10}$ CFU/mL versus time was plotted.

2.5. Stability of T. catappa L. extract on the antibacterial activity at different temperature and pH

The stability of *T. catappa* L. extract on different temperature and pH was evaluated against vegetative cells of *B. cereus* ATCC 33019, *B. subtilis* ATCC 6633, *B. pumilus* ATCC 14884 and *B. megaterium* ATCC 14581, according to the method described by Yusoff et al. [14], with slight modifications. Briefly, the stability of *T. catappa* L. extract was exposed to various temperatures starting from 4 °C, 30 °C, 50 °C, 80 °C and 121 °C for 1 h each. For pH stability, the pH of extract was adjusted to pH 3, pH 7 and pH 10, by using 0.1 M of hydrochloric acid (HCl, Merck, Darmstadt, Germany) or 0.1 M of sodium hydroxide (NaOH, Sigma Aldrich, United States). After treatments, each of the treated extracts were tested for their inhibition zone by carrying out well diffusion assay as described in 2.3.1. Extract at 30 °C with original pH of 6.858 at was used as control.

2.6. Spore preparation

The spores of *B. cereus*, *B. subtilis*, *B. pumilus* and *B. megaterium* were prepared using the method described by Ramli et al. [15], with some modifications. The *Bacillus* strains were grown on nutrient agar (NA) (Oxoid, England) at temperature of 30 °C and stored for over a week. The cells formed were collected by dredging with a sterile cotton swab and were suspended into sterile 1% PBS solution (Oxoid, England), followed by heat shock at 65 °C for 30 min with the purpose of killing vegetative cells present. Then, the solution was centrifuged at the setting of 12,000×*g* for 30 min at 4 °C to collect spores (pellet) and rinsed four times with sterile 1% PBS solution. Viable plate count method was used to obtain the number of viable spores by growing the spores in NA. The spore suspensions were aliquoted at 1 mL into plastic cryopreservation tubes, containing approximately of 10^8 spores/mL and stored at −20 °C until further use.

2.7. Determination of sporicidal activity in T. catappa L. extract against spores of Bacillus spp.

The sporicidal activity of *T. catappa* L. extract was carried out with the four *Bacillus* strains, where all the spore suspensions were thawed and diluted in 1% PBS solution, to prepare spores in the range of 10^6–10^7 spores/mL. Initial spore suspension of *B. cereus*, *B. subtilis*, *B. pumilus* and *B. megaterium* were adjusted to 2.93×10^6, 3.17×10^6, 5.40×10^6 and 2.35×10^6 spores/mL, respectively. *T. catappa* L. extract of 10% from the stock was mixed to the prepared spore suspensions at a final volume of 1 mL in microcentrifuge tubes in order to obtain final concentrations of extract at 0.25, 0.50, 1.00 and 2.50%. For positive control, glutaraldehyde solution (Merck Millipore, Germany) which is available at standard 25% (w/v) was applied. The glutaraldehyde was diluted to the concentration of 1% (w/v) by using distilled water at the ratio of 1:25. The pH of these test solutions was not altered by mixing the extract or glutaraldehyde. Each of the prepared concentrations in microcentrifuge tubes was then incubated for 0, 1, 2, 3 and 4 h in a water bath at 37 °C. Volume of 100 μL aliquot was taken out and pipetted into microcentrifuge tubes. The tubes were then centrifuged at setting of 12, 000×*g* at 4 °C for 5 min (ALC Microcentrifuge 4214, Milan, Italy), followed by rinsing the pellets using 900 μL of 1% PBS solution twice to obtain spores which are free from residue of vegetative cells. Then, the pellets were suspended into 100 μL of 1% PBS solution to perform serial dilution and spread onto NA plates. The plates were then incubated at 37 °C for 24 h until the colonies were formed on the plates. Colonies formed were counted and the average of colony forming unit (CFU/mL) was calculated. Differences between the control (0%) and tested concentrations were obtained by subtracting the $\log_{10}$ CFU/mL. The reduction of spores in CFU was expressed as sporicidal activity.

2.8. Stability of T. catappa L. extract on the sporicidal activity at different temperature and pH

The extract was treated with different temperatures (4, 50, 80 and 121 °C) for 15 min. The pH of extract was adjusted to 3, 7 and 10. The control for this study was temperature of 30 °C with original pH of 6.858. The treated extracts of 2% were then diluted in adjusted spore suspensions, to obtain final concentrations of 1% (w/v) extract at final volume of 1 mL in microcentrifuge tubes. The test solutions were then exposed to incubation at 30 °C for 3 h, to allow sporicidal activity to take place. Volume of 100 μL aliquot was then taken out and pipetted to microcentrifuge tubes, followed by centrifugation at setting of 12,000×*g* at 4 °C for 5 min. Then, the pellets were rinsed using 900 μL of 1% PBS solution twice. Then, the pellets were suspended into 100 μL of 1% PBS solution to perform serial dilution and spread onto NA plates. The plates were then incubated at 37 °C for 24 h until the colonies were formed on the plates. Colonies formed were counted and the average of colony forming unit (CFU/mL) was calculated.

2.9. Statistical analysis

All the tests in this research were performed in triplicates. Data for each group were analyzed and expressed as mean ± standard deviation (SD), generated from Microsoft Excel 2019. One-way analysis of variance (ANOVA) was employed using Minitab 20.3 Statistical Software (Minitab Inc., Pennsylvania, US) and the value of $p < 0.05$ was identified at 95% statistical significance between the treatments by performing Tukey's test.

3. Results

3.1. Well diffusion assay (WDA)

The bacteria in this study exhibited different susceptibility against *T. catappa* L. extract (10 mg/mL) which showed promising effect with inhibition zones of 11.67 ± 0.47, 10.00 ± 0.65, 10.25 ± 0.95 and 9.25 ± 0.75 mm in *B. cereus*, *B. pumilus*, *B. subtilis* and *B. megaterium*, respectively (Table 1).

3.2. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

All the four strains showed sensitivity towards ethanolic *T. catappa* L. extracts and the MIC and MBC values are summarized in Table 2, where the MIC range for *Bacillus* spp. was 0.63–1.25 mg/mL.

3.3. Determination of time-kill curve assay

TKC study was used for all the four *Bacillus* spp. to learn on bactericidal action of ethanolic extract of *T. catappa* L. leaf. Monitoring of bacterial growth and death for of all the four *Bacillus* spp. over 4 h of incubation with five different concentrations, including positive control (no extract) to evaluate effect of antibacterial of the extract are presented in Figs. 1–4.

3.4. Stability of T. catappa L. extract on the antibacterial activity at different temperature and pH

The stability of *T. catappa* L. extract upon exposure to different temperatures and pH was investigated in this study by performing well diffusion assay to determine inhibition zone. Results demonstrated that the antibacterial activity of *T. catappa* L. extract was not affected at temperature 4 °C, 30 °C, 50 °C, 80 °C and 121 °C against the tested *Bacillus* spp. (Table 3), where inhibition zone was in the range of 11.33 ± 0.47–12.00 ± 0.00 mm for *B. cereus*, 10.50 ± 0.41–11.33 ± 0.47 mm for *B. pumilus*, 9.83 ± 0.24–10.50 ± 0.41 mm for *B. subtilis* and 9.17 ± 0.24–9.83 ± 0.24 mm for *B. megaterium*.

The exposure of *T. catappa* L. extract to different pH was also investigated in this study by performing well diffusion assay to determine inhibition zone. Results demonstrated that the antibacterial activity of *T. catappa* L. extract was not affected at pH 3 °C, 7 °C and 10 °C against the tested *Bacillus* spp. (Table 4), where inhibition zone was in the range of 11.33 ± 0.47 to 11.83 ± 0.24 mm for *B. cereus*, 10.67 ± 0.24 to 11.00 ± 0.41 mm for *B. pumilus*, 9.83 ± 0.47 to 10.50 ± 0.41 mm for *B. subtilis* and 9.17 ± 0.24 to 9.83 ± 0.47 mm for *B. megaterium*.

3.5. Determination of sporicidal activity in T. catappa L. Extract against spores of Bacillus spp.

Sporicidal activity of ethanolic *T. catappa* L. crude leave extract against the spores of *B. cereus*, *B. subtilis*, *B. pumilus* and *B. megaterium* were studied at concentrations of 0.25, 0.50, 1.00 and 2.50% w/v at incubation period of 1, 2, 3 and 4 h. This study was carried to learn the efficiency of the extract in being actively sporicidal. It was found that spores of all the *Bacillus* strains were able to

Table 1
Inhibition zone of *T. catappa* L. leaf extract against *Bacillus* spp.

Bacillus spp.	Diameter of inhibition zone (mm) ± SD		
	T. catappa L. leaf extract [1% (w/v)]	CHX [0.1% (w/v)]	DMSO [10% (v/v)]
B. cereus ATCC33019	11.67 ± 0.47	17.75 ± 0.25	n.a
B. subtilis ATCC6633	10.00 ± 0.65	17.00 ± 0.00	n.a
B. pumilus ATCC14884	10.25 ± 0.95	16.50 ± 0.50	n.a
B. megaterium ATCC14581	9.25 ± 0.75	16.50 ± 0.50	n.a

n.a: not active (no inhibition).
Diameter of inhibition zone is in mm (including disc).
Positive control (chlorhexidine: CHX; 0.1%); Negative control (DMSO; 10%).
Results were expressed as means ± standard deviation (SD).

Table 2
Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *T. catappa* L. leaf extracts against *Bacillus* spp.

Bacillus spp.	*T. catappa* L. leaf extract (mg/mL)			CHX (mg/mL)	
	MIC	MBC	Effect	MIC	MBC
B. cereus ATCC33019	0.63	0.63	Bactericidal	0.04	0.08
B. subtilis ATCC6633	1.25	>5.00	Bacteriostatic	0.04	0.04
B. pumilus ATCC14884	1.25	>5.00	Bacteriostatic	0.04	0.04
B. megaterium ATCC14581	1.25	1.25	Bactericidal	0.02	0.02

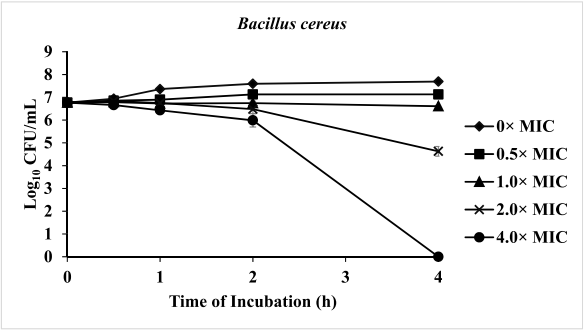

Fig. 1. Time-kill curve of *Bacillus cereus* at 0 × MIC (0 mg/mL), 0.5 × MIC (0.31 mg/mL), 1.0 × MIC (0.63 mg/mL), 2.0 × MIC (1.25 mg/mL) and 4.0x MIC (2.50 mg/mL).

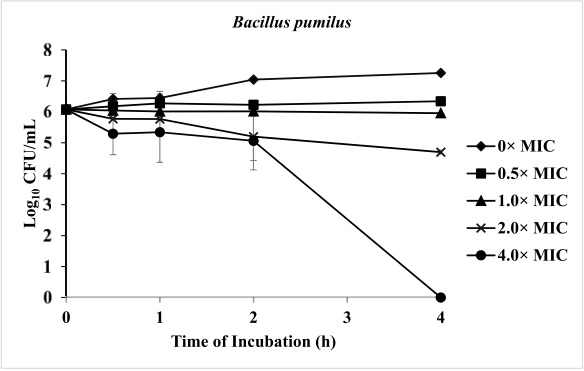

Fig. 2. Time-kill curve of *Bacillus pumilus* at 0 × MIC (0 mg/mL), 0.5 × MIC (0.63 mg/mL), 1.0 × MIC (1.25 mg/mL), 2.0 × MIC (2.50 mg/mL) and 4.0 × MIC (5.00 mg/mL).

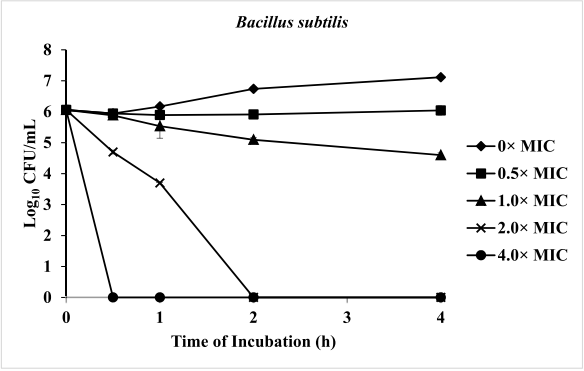

Fig. 3. Time-kill curve of *Bacillus subtilis* at 0 × MIC (0 mg/mL), 0.5 × MIC (0.63 mg/mL), 1.0 × MIC (1.25 mg/mL), 2.0 × MIC (2.50 mg/mL) and 4.0 × MIC (5.00 mg/mL).

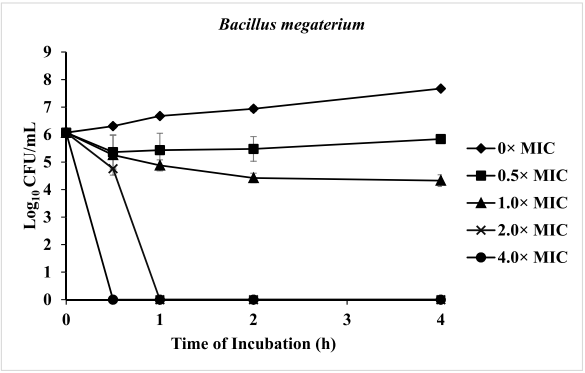

Fig. 4. Time-kill curve of *Bacillus megaterium* at 0 × MIC (0 mg/mL), 0.5 × MIC (0.63 mg/mL), 1.0 × MIC (1.25 mg/mL), 2.0 × MIC (2.50 mg/mL) and 4.0 × MIC (5.00 mg/mL).

Table 3
Inhibition zone of *T. catappa* L. leaf extract against *Bacillus* spp. at different temperatures.

Bacillus spp.	Diameter of inhibition zone (mm)				
	4 °C	30 °C	50 °C	80 °C	121 °C
B. cereus ATCC33019	11.83 ± 0.29[a]	11.33 ± 0.58[a]	11.83 ± 0.76[a]	12.00 ± 0.00[a]	11.50 ± 0.50[a]
B. pumilus ATCC14884	11.17 ± 0.29[a]	10.67 ± 0.58[a]	11.33 ± 0.58[a]	10.83 ± 0.29[a]	10.50 ± 0.50[a]
B. subtilis ATCC6633	10.00 ± 0.50[a]	10.50 ± 0.50[a]	10.17 ± 0.29[a]	9.83 ± 0.29[a]	10.00 ± 0.50[a]
B. megaterium ATCC14581	9.33 ± 0.50[a]	9.17 ± 0.29[a]	9.33 ± 0.29[a]	9.83 ± 0.29[a]	9.50 ± 0.50[a]

Values are the mean ± S.D. of replications. Values with different superscript letters within the same rows are significantly different ($p < 0.05$).

Table 4
Inhibition zone of *T. catappa* L. leaf extract against *Bacillus* spp. at different pH.

Bacillus spp.	Diameter of inhibition zone (mm)			
	pH 3	pH 6.828	pH 7	pH 10
B. cereus ATCC33019	11.83 ± 0.29[a]	11.33 ± 0.58[a]	11.50 ± 0.50[a]	11.67 ± 0.29[a]
B. pumilus ATCC14884	10.67 ± 0.29[a]	10.67 ± 0.58[a]	11.00 ± 0.50[a]	11.00 ± 0.00[a]
B. subtilis ATCC6633	9.83 ± 0.58[a]	10.50 ± 0.50[a]	10.17 ± 0.76[a]	10.17 ± 0.29[a]
B. megaterium ATCC14581	9.50 ± 0.87[a]	9.17 ± 0.29[a]	9.83 ± 0.58[a]	9.33 ± 0.58[a]

Footnotes: Values are the mean ± S.D. of replications. Values with different superscript letters within the same rows are significantly different ($p < 0.05$).

be killed in 1% w/v of crude extract at different incubation hours, resulting in complete killing. The results have been summarized in Tables 5–8 upon conversion to $\log_{10}$ unit.

3.6. Stability of T. catappa L. Extract on the sporicidal activity at different temperature and pH

Stability of *T. catappa* L. leaf extract at 4, 50, 80 and 121 °C was evaluated by incubation at specified temperatures. Then, the treated extracts were mixed with prepared spore suspension of the *B. cereus*, *B. subtilis*, *B. pumilus* and *B. megaterium*. Room temperature of 30 °C and spore suspension without extract were used as the controls. The effect of different temperatures on sporicidal activity of

Table 5
The sporicidal activity of *T. catappa* L. extract against the spores of *B. cereus*.

Concentration (% w/v)	Time (h)			
	1	2	3	4
0.00	5.00 ± 0.07[a]	5.00 ± 0.07[a]	5.00 ± 0.07[a]	5.00 ± 0.07[a]
0.25	4.44 ± 0.02[b]	4.28 ± 0.01[b]	4.25 ± 0.01[b]	4.19 ± 0.02[b]
0.50	3.83 ± 0.13[c]	3.50 ± 0.14[c]	0.00 ± 0.00[c]	0.00 ± 0.00[c]
1.00	2.70 ± 0.03[d]	0.00 ± 0.00[d]	0.00 ± 0.00[c]	0.00 ± 0.00[c]
2.50	0.00 ± 0.00[e]	0.00 ± 0.00[d]	0.00 ± 0.00[c]	0.00 ± 0.00[c]

Values are the mean ± S.D. of replications. Values with different superscript letters within the same column are significantly different ($p < 0.05$).

Table 6
The sporicidal activity of *T. catappa* L. extract against the spores of *B. subtilis*.

Concentration (% w/v)	Time (h)			
	1	2	3	4
0.00	5.02 ± 0.04^{a}	5.02 ± 0.04^{a}	5.02 ± 0.04^{a}	5.02 ± 0.04^{a}
0.25	4.61 ± 0.01^{b}	4.45 ± 0.03^{b}	4.25 ± 0.03^{b}	0.00 ± 0.00^{b}
0.50	4.05 ± 0.01^{c}	3.70 ± 0.04^{c}	3.44 ± 0.04^{c}	0.00 ± 0.00^{b}
1.00	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{b}
2.50	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{b}

Values are the mean ± S.D. of replications. Values with different superscript letters within the same column are significantly different ($p < 0.05$).

Table 7
The sporicidal activity of *T. catappa* L. extract against the spores of *B. pumilus*.

Concentration (% w/v)	Time (h)			
	1	2	3	4
0.00	5.01 ± 0.01^{a}	5.01 ± 0.01^{a}	5.01 ± 0.01^{a}	5.01 ± 0.01^{a}
0.25	4.65 ± 0.01^{b}	4.57 ± 0.02^{b}	4.48 ± 0.02^{b}	4.38 ± 0.01^{b}
0.50	4.45 ± 0.02^{c}	4.28 ± 0.01^{c}	4.20 ± 0.01^{c}	4.06 ± 0.08^{c}
1.00	3.48 ± 0.00^{d}	2.70 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}
2.50	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}

Values are the mean ± S.D. of replications. Values with different superscript letters within the same column are significantly different ($p < 0.05$).

Table 8
The sporicidal activity of *T. catappa* L. extract against the spores of *B. megaterium*.

Concentration (% w/v)	Time (h)			
	1	2	3	4
0.00	5.03 ± 0.01^{a}	5.03 ± 0.01^{a}	5.03 ± 0.01^{a}	5.03 ± 0.01^{a}
0.25	4.57 ± 0.00^{b}	4.45 ± 0.03^{b}	4.37 ± 0.03^{b}	0.00 ± 0.00^{b}
0.50	3.78 ± 0.05^{c}	3.09 ± 0.12^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}
1.00	3.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}
2.50	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}

Values are the mean ± S.D. of replications. Values with different superscript letters within the same column are significantly different ($p < 0.05$).

1% (w/v) *T. catappa* L. extract against spores of the four *Bacillus* have been summarized in Table 9.

Stability of *T. catappa* L. leaf extract at pH 3 (acidic), 7 (neutral), and 10 (alkaline) was evaluated by adjusting their pH by adding 0.1 M hydrochloric acid and 0.1 M of sodium hydroxide. Then, the treated extracts were mixed with prepared spore suspension of the *B. cereus*, *B. subtilis*, *B. pumilus* and *B. megaterium*. The original pH of prepared extract in 10% DMSO was 6.858, thus, it was used as the control along with a spore suspension without extract. The effect of different pH on sporicidal activity of 1% (w/v) *T. catappa* L. extract against spores of the four *Bacillus* have been summarized in Table 10.

Table 9
Effect of temperature on the sporicidal activity of *T. catappa* L. extract against spores of *B. cereus*, *B. subtilis*, *B. pumilus* and *B. megaterium*.

Temperature (°C)	Inoculum/Extract	Colony count (spores/mL)			
		Bacillus cereus	*Bacillus subtilis*	*Bacillus pumilus*	*Bacillus megaterium*
4	Inoculum	6.08 ± 0.09^{a}	6.12 ± 0.02^{a}	6.21 ± 0.03^{a}	6.01 ± 0.02^{a}
	1% extract	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
30	Inoculum	6.08 ± 0.09^{a}	6.12 ± 0.02^{a}	6.21 ± 0.03^{a}	6.01 ± 0.02^{a}
	1% extract	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
50	Inoculum	6.08 ± 0.09^{a}	6.12 ± 0.02^{a}	6.21 ± 0.03^{a}	6.01 ± 0.02^{a}
	1% extract	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
80	Inoculum	6.08 ± 0.09^{a}	6.12 ± 0.02^{a}	6.21 ± 0.03^{a}	6.01 ± 0.02^{a}
	1% extract	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
121	Inoculum	6.08 ± 0.09^{a}	6.12 ± 0.02^{a}	6.21 ± 0.03^{a}	6.01 ± 0.02^{a}
	1% extract	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}

Values are the mean ± S.D. of replications. Values with different superscript letters within the same column are significantly different ($p < 0.05$).

Table 10
Effect of pH on the sporicidal activity of *T. catappa* L. extract against spores of *B. cereus*, *B. subtilis*, *B. pumilus* and *B. megaterium*.

pH	Inoculum/Extract	Colony count (spores/mL)			
		Bacillus cereus	*Bacillus subtilis*	*Bacillus pumilus*	*Bacillus megaterium*
3	Inoculum	6.19 ± 0.01^{a}	6.14 ± 0.05^{a}	6.11 ± 0.09^{a}	6.02 ± 0.09^{a}
	1% extract	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
6.858 (original)	Inoculum	6.19 ± 0.01^{a}	6.14 ± 0.05^{a}	6.11 ± 0.09^{a}	6.02 ± 0.09^{a}
	1% extract	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
7	Inoculum	6.19 ± 0.01^{a}	6.14 ± 0.05^{a}	6.11 ± 0.09^{a}	6.02 ± 0.09^{a}
	1% extract	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
10	Inoculum	6.19 ± 0.01^{a}	6.14 ± 0.05^{a}	6.11 ± 0.09^{a}	6.02 ± 0.09^{a}
	1% extract	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}

Values are the mean ± S.D. of replications. Values with different superscript letters within the same column are significantly different ($p < 0.05$).

4. Discussion

4.1. Well diffusion assay (WDA)

It has been shown in many studies where Gram-positive bacteria are more susceptible with antimicrobial component of plant origin [16,17]. This is because Gram-positive bacteria has peptidoglycan that makes up about 50% of bacterial cell wall which is permeable to molecules in the size range of 30 000 to 57 000 Da, fitting the size of antimicrobe molecules [18]. The highest inhibition zone was observed in *B. cereus*, demonstrating highest antibacterial activity in the ethanol extract as inhibition zone and antibacterial activity are directly proportional. This result is supported by a study reported by Manzur et al. where *B. cereus* had the highest antibacterial activity at 11.50 ± 0.28 and 9.50 ± 0.28 mm compared to *B. subtilis* with inhibition zone of 9.00 ± 1.15 and 8.50 ± 0.86 mm at methanol and acetone extracts of *T. catappa* L., respectively in disc diffusion assay [1]. Data suggest *B. cereus* and *B. subtilis* are more susceptible towards ethanolic *T. catappa* L. leaf extract as it showed convincing inhibition zone compared to some plant-based extracts.

Agar well diffusion assay is being carried out as preliminary screening of antimicrobial activity in many plant extracts studies [19,20]. This method is known for being cost effective and its simplicity. However, the data obtained from this assay is considered inadequate as the assay is focused on qualitative data and acts as preliminary screening for antibacterial activity, where the antibacterial activity of the extracts could not be accurately measured. Therefore, MIC and MBC assays are needed to determine the antibacterial activity of the ethanolic *T. catappa* L. extract.

4.2. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC range for *Bacillus* spp. was 0.63 mg/mL - 1.25 mg/mL which was expectable as they are Gram-positive bacteria strains because in a number of studies, it has been proven that Gram-positive bacteria has better susceptibility towards plant extracts, needing much lower MIC value to inhibit growth compared to Gram-negative bacteria. In a study, extract of *Wedelia chinensis* was used on pathogenic bacteria, consisting of both Gram-positive and Gram-negative bacteria. *B. cereus* and *B. subtilis* had MIC values of 3.13 and 6.25 mg/mL, respectively, which were lower compared to Gram-negative bacteria such as *E. coli* and *P. aeruginosa* where MIC value for both bacteria were 25 mg/mL [21].

T. catappa L. extract showed bacteriostatic and bactericidal effects at 0.63 mg/mL for *B. cereus*. For *B. subtilis*, *B. pumilus* and *B. megaterium*, MIC value was slightly higher (1.25 mg/mL) than *B. cereus* but lower concentration compared to some studies done on plant extracts [19,20], showing a better growth inhibition effect. In a study, methanolic extract of *Zingiber officinale* rhizome was used against some pathogenic bacteria to learn the antibacterial activity in which, *B. cereus* and *B. subtilis* had MIC values of >4 and 4 mg/mL, respectively [22]. Hence, the MIC values obtained in this study showed lower concentration of ethanolic *T. catappa* L. extract is sufficient to inhibit growth of *Bacillus* spp. For both *B. subtilis* and *B. pumilus*, concentrations needed for bactericidal effect were more than 5 mg/mL. However, the MIC and MBC of the positive control (CHX) used demonstrated lower values in all the four strains compared to those of *T. catappa* L. extracts.

4.3. Time-kill curve assay

At positive control (0 × MIC), the bacteria population kept increasing in all four strains as there were no extract to have any antibacterial activity. At 0.5 × MIC and 1.0 × MIC, the bacterial population was slightly lower than positive control at the end but the reduction was not significant in most strains. This trend shows the concentration had slight bacteriostatic effect but wasn't sufficient to prevent growth for the next 3 h. *B. subtilis* and *B. megaterium* showed bactericidal effect with concentration of 2.0 × MIC at second and first hours, respectively as the killing of entire population was observed. Bactericidal effect of *B. cereus* and *B. pumilus* can be seen at fourth hour in the concentration of 4.0 × MIC. The results showed killing the *Bacillus* spp. is concentration and time dependent because higher extract concentration resulted in faster reduction of the population as complete killing of vegetative cells.

In a study conducted by Sowmya and Raveesha [6], UHPLC-MS/MS was used to identify the components present in *T. catappa* L. leaf, in which, polyphenol rich components were isolated and evaluated for the antimicrobial potential. Their findings concluded the bioactive compounds present in *T. catappa* L. leaf is even effective towards multidrug-resistant bacteria which can lead to the potential

of developing antimicrobial molecule for drug resistance issues. This supports the findings in this study on complete killing of *Bacillus* spp. by the application of *T. catappa* L. leaf extract.

4.4. Stability of T. catappa L. extract on the antibacterial activity at different temperature and pH

Though the extract was exposed to different temperatures and pH, antibacterial activity could be still observed at all the tested *Bacillus* spp., statistically no significant trend could be observed for each strain. All the strains have demonstrated a good antibacterial activity at extract concentration of 10 mg/mL at all the tested temperatures and pH. Though the study on temperature and pH stability of plant extract is limited, a study on *Syzygium polyanthum* leaf extract against different temperature and pH was reported by Ramli et al. [23]. A number of strains were tested after exposing the extract to different temperatures and pH but the extract still possessed antimicrobial activity, showing its stability.

Natural polyphenolic compounds found in plants can be damaged structurally to extreme pH conditions, causing them to lose their functional properties as antimicrobial agent. In a study conducted by Friedman and Jürgens [24], pH exposure of different polyphenolic compounds, demonstrated different outcome on their stability. For instance, caffeic, chlorogenic, and gallic acids were not stable at alkaline condition but chlorogenic acid was stable at acidic condition. Meanwhile, catechin and ferulic acid were not affected by any pH conditions. Therefore, it is believed that the bioactive compounds found in *T. catappa* L. leaf extract that contributes to the antibacterial activities are not degraded by extreme change in temperature and pH, showing the extract can be applied for antibacterial activity in food industry at different processing conditions.

4.5. Determination of sporicidal activity in T. catappa L. extract against spores of Bacillus spp.

In a study by Kusuma et al. [25], it was found that plant sources such as cinnamon bark and fingerroot managed to kill spores of *B. cereus* completely at 5% w/v extract concentration which was the minimum concentration used in their study. It was suggested, the extracts from plant, containing secondary metabolites managed to penetrate through inner membrane of the spores of *B. cereus*, lysing them.

Two resistance factors of *Bacillus* species are the coats of the spore which act as barrier towards several factors and the permeability of inner membrane of small molecules into core. Spores' resistance to chemical is due to low permeability in inner membrane which has been a big challenge in preventing food spoilage and food-borne diseases in food industry for a long time [26]. Coat of spore is made up of protein where it consists of 50–80% of total spore protein in which some proteins are heavily cross-linked, leading to the chemical barrier property. The outer membrane of a spore is not a great protective layer as it is permeable to many chemicals and not heat resistance. However, the functionality of outer layer is still unclear due to distinct structure in different bacteria spores.

On the other hand, inner membrane is impermeable to most of the chemicals to protect DNA of the spore in core. The permeability characteristics of inner membrane have been suggested to be different from plasma membrane of vegetative cells, making it harder to kill spores by using antibacterial agents [26,27]. This explains the higher concentration or the longer time used to completely kill the spores of *Bacillus* strains compared to concentration needed to completely kill vegetative cells in this study. For instance, *B. cereus*, needed 2.50 mg/mL to achieve complete killing in vegetative state, showing the extract worked more efficiently because in sporicidal assay, it needed extract concentration of 5.00 mg/mL to demonstrate such result. Similarly, *B. pumilus* only required 5 mg/mL for complete killing of vegetative cells but complete killing of spores needed double of the concentration (10 mg/mL).

On the other hand, the concentration of extract for complete killing in both *B. subtilis* and *B. megaterium* vegetative cells and spores were the same, which was, 2.50 mg/mL. However, the incubation period for *B. megaterium* had increased from 1 h (vegetative state) to 4 h (spores) for complete killing. This can be explained by the structure of spore's inner membrane, consisting immobile lipid molecules which switches to mobile state upon germination into vegetative cells. Due to the immobile state of the lipids, water molecules or even lipophilic molecules such as methylamine moves slow in the inner membrane of spore [28].

In a study conducted by Rukayadi et al. [29], a bioactive compound, macelignan was isolated from nutmeg and tested for sporicidal activity where the compound managed to reduce *B. cereus* spores by > 3 log units (99.9%). This has indicated plant source has the potential to be effective sporicidal agent which can be useful in food industry as natural preservative. Recently, a study of methanolic *S. polyanthum* L. leaf (Indonesian bay leaf) extract on spores of *B. pumilus* and *B. megaterium* was performed with crude extract to determine antibacterial and sporicidal activities of *Bacillus* spp. in the basis of bioactive components listed in previous studies. The extract of 1% (10 mg/mL) managed to completely kill the spores after exposure of 1 h [30]. This finding is similar to current study where crude plant extract has been used to determine sporicidal activity.

The bioactive components responsible for antibacterial activities such as saponins, tannins and alkaloids have been already identified and reported in several studies for this extract [31]. Therefore, they could be also responsible for the sporicidal activities, resulting in complete killing of the *Bacillus* spores in the current study. Glutaraldehyde was used as positive control in this study as it is one of the well-known antimicrobial agents used in sporicidal studies even though it is not food grade [9]. The results obtained revealed *T. catappa* L. leaf has significant sporicidal activities against spores of *B. cereus*, *B. subtilis*, *B. pumilus* and *B. megaterium* at concentration in the range of 0.25–1.00% (2.5–10 mg/mL) at different incubation period for different strains of *Bacillus* spores.

4.6. Stability of T. catappa L. extract on the sporicidal activity at different temperature and pH

All the four *Bacillus* spores were completely killed which was similar to the control (30 °C for temperature study and pH 6.858 for pH study), showing the extract was stable upon exposure to different temperatures and pH. The study on temperature or pH stability of

T. catappa L. was never reported before, creating a need to understand if the extract will still exhibit its sporicidal activity upon exposure to various thermal and pH treatments in food processing line. Though limited studies on plant extract stability have been reported, a study by Ramli et al. [15] on effect of temperature and pH on the sporicidal activity of *Syzygium polyanthum* L. extract against spores of *B. cereus* ATCC 33019 can support current finding because the sporicidal activity remained great as the controlled temperature in their study. Similarly, a study performed by Lau et al. [32] on sporicidal activity of *Eugenia polyantha* Wight against *B. cereus* and *B. subtilis*, concluded the extract's sporicidal activity was not affected by different treatment temperatures and pH to the extract.

A microbial study carried by Shubhi et al. [33] on plants extracts of neem, amla, aloe, assam tea and clove against some bacteria showed all the extracts were stable in a wide range of temperature tested, showing some plant extracts are stable in different temperature exposure. On contrary, a study carried out by Ibrahim and Kebede [34], concluded medicinal plants are affected by high temperature at various exposure period, which was demonstrated by reduced microbial activity in their study. Plant extracts are full with bioactive components which are beneficial to humankind, especially when it comes to herbal plants. However, the bioactive components can lose its functional properties upon exposure to heat treatments in food industry at various processing steps such as blanching, roasting, boiling, heating and pasteurising, which comprises stages from farm to fork [35]. However, these findings concludes that *T. catappa* L. extract will not lose its significance as sporicidal agent in food industry despite exposure to heat in food processing treatments.

Therefore, this assay has shown the bioactive compounds found in *T. catappa* L. leaf extract that contributes to the sporicidal activities are not degraded by extreme pH conditions, showing the extract can be applied for both antibacterial and sporicidal activity in food industry at different temperature and pH processing conditions. Therefore, further research on development of this extract as natural preservative to control food spoilage caused by the germination and growth of *Bacillus* spp. can be performed to study on extending the shelf life of the food. Its application to food and food products, associated with *Bacillus* contamination such as rice, pasta and potatoes will be greatly recommended. In conjunction, sensory evaluation can be carried out to understand if the application of the extract has significant effect on organoleptic properties of the food studied.

5. Conclusions

T. catappa L. leaf, a traditionally used herbal plant by ancient folks, is a potential candidate as natural food preservative due to its functionality in antibacterial and sporicidal activity against vegetative cells and spores of *Bacillus* spp. The extract demonstrated sporicidal activity which can be utilized by using it as a lead compound in development of natural preservative substances to be applied in food industry. The extract was stable in terms of its sporicidal functionality when it was exposed at different temperatures and pHs at 1% (10 mg/mL) concentration, which showed it can withstand different processing conditions in food industry. The extract can be utilized by using it as a lead compound in development of natural preservative substances to be applied in food industry as anti-*Bacillus* agent.

Author contribution statement

Kierrthanah Madhavan: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Yaya Rukayadi: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Noor Azira Abdul Mutalib: Contributed reagents, materials, analysis tools or data.

Data availability statement

Data included in article/supplementary material/referenced in article.

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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