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Antibacterial activity of ethanolic jambu batu (*Psidium guajava* Linn.) leaves extract against vegetative cells of *Bacillus* spp.

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ABSTRACT

Aims: Jambu batu (*Psidium guajava* Linn.) is a phytotherapic plant used in folk medicine that has active components to treat various diseases. An earlier study has reported on the analysis of its pharmacological properties and was found to possess antibacterial, antifungal, anti-inflammatory and antioxidant activity. The present study aimed to determine the antibacterial activities of *P. guajava* Linn. leaves extracts on the vegetative cells of *Bacillus cereus* ATCC33019, *Bacillus megaterium* ATCC14581, *Bacillus pumilus* ATCC14884 and *Bacillus subtilis* ATCC6633 and to evaluate its effects of different temperatures and pHs on antibacterial activity.
Methodology and results: The susceptibility test used to determine the bacterial growth inhibition were well diffusion assay (WDA), minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and time-kill curve assay. The effects of various parameters on temperatures at 10 °C, 28 °C, 30 °C, 50 °C and 80 °C and pH at 3.0, 5.0, 6.7, 7.0 and 11.0 were investigated. WDA assay of the extracts resulted in 13.75 ± 0.95 and 16.25 ± 0.95 mm of inhibition zone on *B. subtilis* and *B. cereus*, respectively. The extracts can inhibit the growth with MICs value range of 0.195 to 0.781 mg/mL for *B. megaterium* and *B. pumilus*, respectively, and can kill all tested *Bacillus* spp. with MBCs values of 0.781 mg/mL. The killing time analyses showed that *Bacillus* spp. can be killed completely within 4 h at 4× MIC (0.781 to 3.124 mg/mL). The extracts remained stable under a wide range of temperatures and pHs, as there was no significant difference in the MIC and MBC values.
Conclusion, significance and impact of study: *Psidium guajava* Linn. ethanolic leaves extract yielded good antibacterial activities, suggesting that the extract can be utilised or explored as a potential anti-*Bacillus* agent in food applications.

Keywords: Anti-*Bacillus*, antibacterial, jambu batu leaves extract, *Psidium guajava* Linn., stability

INTRODUCTION

Preventing food spoilage and food poisoning typically involves the use of chemical preservations which can lead to side effects on human health and microbial resistance in the food and feed chains (Mostafa *et al.*, 2018). However, the popularity of chemical preservatives has diminished due to increasing consumer health awareness (Gavahian *et al*., 2020). As a result, there is a growing interest in finding natural preservatives that are safe, effective against microbial resistance (Bano *et al*., 2021) and capable of extending shelf life (Pisoschi *et al*., 2018). Plant-derived natural preservatives, rich in bioactive compounds such as polyphenols, phenolics and flavonoids, have shown a potential to provide antimicrobial activity while being less harmful to health compared to synthetic preservatives (Yu *et al*., 2021). These natural compounds have emerged as potential alternatives to combat microbial resistance, addressing the growing concern about environmental and health hazards associated with synthetic products (Scavo *et al.*, 2019). Thus, plant extracts serve as beneficial antimicrobial agents and offer a natural alternative for controlling food-borne diseases, avoiding health hazards associated with chemical preservatives (Mostafa *et al.*, 2018). *Psidium guajava* Linn. (Family : Myrtaceae) is commonly called guave, goyave in French, guave, guavenbaum in German, banjiro in Japanese, goiaba in

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Portugal; arac guaiaba in Brazil, jambu batu in Malay and guava in English (Anand *et al.*, 2016). *Psidium guajava* Linn. leaves are commonly known for its potential antimicrobial and antioxidant activities against both Gram-positive and Gram-negative bacteria, positioning them as promising candidates for herbal medicine development (Fernandes *et al.*, 2014). *Psidium guajava* Linn. has been practiced in traditional treatments such as Ayurveda, Siddha, Unani and Homeopathy in Tegal regency, Central Java, Indonesia, for several health illnesses in herbal tea (Chandra and Wanda, 2017). *Psidium guajava* Linn. leaves also have long been utilized as food crops and medicinal plants in folk medicine worldwide due to their comprehensive chemical compounds and pharmacological properties (Gutiérrez *et al*., 2008). Studies have revealed that *P. guajava* Linn. leaves, fresh fruits and tea extracts offer various pharmacological benefits and therapeutic applications in treating diabetes mellitus, dysentery, diarrhea (Chiari-Andréo *et al.*, 2017), constipation, scurvy, blood pressure, weight loss, better skins tonicity, bowel irregularities, cough, cold (Bulugahapitiya *et al.*, 2021), wound healing, rheumatism, lung problems and ulcers (Gupta *et al.*, 2011). In addition, *P. guajava* Linn. extracts have been explored as an alternative treatment for oral diseases, including cariogenic, dental caries, periodontal disease and biofilm adhesion (Millones-Gómez *et al.*, 2020). *Psidium guajava* Linn. been reported to offer strong natural antibacterial compounds with non-toxic and less harmful effects, making them beneficial for various healthcare applications (Parham *et al.*, 2020). The ethanolic leaves extracts of *P. guajava* Linn. have been reported to have good inhibitory activity and are frequently used as folk medicine against Gram-positive bacteria (Biswas *et al.*, 2013). The aqueous bark, ethanol and methanolic leaves extracts of *P. guajava* Linn. has also been identified to contain antibacterial activity (Abdelgani *et al.*, 2018) against various bacteria such as *B. subtilis*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa* (Soliman *et al.*, 2016). The antimicrobial and antioxidant properties of *P. guajava* Linn. leaves can be attributed to the wide range of phytochemicals present, including terpenoids, flavonoids, alkaloids, tannins, steroids and saponins (Raj *et al.*, 2020). Among the bacterial species of interest are *B. megaterium*, *B. cereus*, *B. pumilus* and *B. subtilis*, known for their enormous genetic diversity (Draganić *et al.*, 2017). *Bacillus* spp. have been implicated in food-borne outbreaks, particularly in starchy and vegetable foods (Glasset *et al.*, 2016) and they facilitate food pathogenicity and deterioration through the production of extracellular enzymes (Özdemir and Arslan, 2019). *Bacillus cereus* is generally oval-shaped, sometimes round and cylindrical shaped endospores which is a Gram-positive, motile and spore forming bacterium (Gharib *et al.*, 2020). *Bacillus cereus* is known to cause various health issues and is frequently associated with food poisoning outbreaks and food industry contamination, such as initial colonization, maturation and dispersal of biofilms in ready-to-eat and dairy products (Galié *et al.*, 2018). Additionally, *B. cereus* has been implicated in nosocomial infections, such as endophthalmitis and bacteremia (Oda *et al.*, 2020). It is also reported that terpinene-4-ol, a natural compound, has demonstrated effective inhibition of *B. cereus* growth, spore germination and biofilm formation (Zhao *et al.*, 2021). The cumulative evidence on the antibacterial and pharmacological attributes to *P. guajava* Linn. leaves make it an intriguing subject of investigation. Hence, the study is to determine the antibacterial activity of *P. guajava* Linn. ethanolic leaves extracts against the vegetative cells of *B. cereus* ATCC33019, *B. megaterium* ATCC14581, *B. pumilus* ATCC14884 and *B. subtilis* ATCC6633 in terms of well diffusion, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and the time-kill assay and to evaluate the effects of different temperature and pH on antibacterial activity of *P. guajava* Linn. leaves extracts.

MATERIALS AND METHODS

Plant sampling and extraction

The fresh leaves of *P. guajava* Linn. contributed to this study were collected from native type trees grown in Taman Pertanian, Universiti Putra Malaysia, Serdang, Selangor, Malaysia. The fresh leaves were chosen between the middle and matured age because this stage of the leaf have undergone significant growth and exposure to environmental factors, leading to the accumulation of secondary metabolites (Zaka *et al.*, 2010). These leaves samples were sent for identification to the Institute of Bioscience (IBS), University Putra Malaysia. The fresh leaves were gently rinsed with tap water, shade air-dried and ground to a fine particle size below 450 µm using a blender before being stored in an air-tight polyethylene plastic container at room temperature on a storage rack. One hundred g of this fine powder were weighed into a 1000 mL Schott bottle. Further 400mL of absolute ethanol grade 99.8% were added to the Schott bottle with the powdered *P. guajava* Linn. leaves inside. These mixtures were then placed inside a sonicator for the phytochemical release process for 30 min with a sonication frequency of 50Hz. This mixture of plant extracts was further filtered using filter paper Whatman No. 2 (Whatman International Ltd., Middlesex, England). The plant extract was transferred to a round bottom flask to further concentrate with a rotary vacuum evaporator (Heidolph VV2011, Schwabach, Germany) at 50 °C, 150 rpm for 3-4 h. The final ethanol extract of *P. guajava* Linn. leaves were transferred to an air-tight glass bottle and stored at 4 °C chilling temperature in a chiller. Stock concentration of 10% (100 mg/mL) *P. guajava* Linn. leaves extract prepared by weighing 100 mg extract dissolved in 1 mL of DMSO. Working concentration of 1% (10 mg/mL) prepared by pipetting 100 µL of stock concentration of 10% (100 mg/mL) *P. guajava* Linn. leaves extract diluted with 1 mL of DMSO.

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Well diffusion assay (WDA)

The method suggested by the Clinical and Laboratory Standard Institute (CLSI) (2012) was employed to carry out the well diffusion assay against *Bacillus* spp. The inoculum was prepared with turbidity adjusted to 10^6 to 10^8 CFU/mL by using the spectrophotometer method and immediately spread on a Mueller Hinton agar (MHA) plate as a single uniform colony with a sterile cotton swab. A sterile self-punched hole with a diameter of 7 mm was made to the inoculated MH agar. Each hole was filled with 10 mg/mL *P. guajava* Linn. leaves extract in the amount of 10 µL. The positive and negative controls in these studies were 0.1% chlorhexidine (CHX) and 10% dimethyl sulfoxide (DMSO), respectively. After a 24 h incubation of the plates at 37 °C, the diameter of the inhibition zone was measured (in mm) and recorded. Analysis was carried out in two times in double data (n = 2 × 2). The handling of all bacteria and the preparation of media were done using an aseptic technique in a class II biosafety cabinet (CLSI, 2012).

Determination of minimum inhibitory concentration (MIC)

The MIC of the *P. guajava* Linn. leaves extract against *Bacillus* spp. were set up as suggested by the Clinical and Laboratory Standard Institute (CLSI) (2012). The determination of MIC of extract against *B. cereus*, *B. megaterium*, *B. pumilus* and *B. subtilis* with vegetative cells was established via the broth microdilution method, which was performed using a 96-well round bottom microtiter plate (Greiner, Germany). The suspension of inoculum of *Bacillus* spp. in this test ranged between 10^6 and 10^8 CFU/mL. The first well column was labelled as the negative control growth and were filled with 100 µL MHB. The second column was labelled as a positive control growth column and the wells were filled with 100 µL of *B. cereus*, *B. megaterium*, *B. pumilus* and *B. subtilis* bacterial suspension. Micro two-fold dilution of varying concentrations that range from 50 mg/mL in column 12 to 0.019 mg/mL in column 3 was established. The plates loaded with *Bacillus* spp. with varying extract concentrations were incubated at 37 °C for 24 h to establish the MIC value. The MIC refers to the minimum concentration of the extract that can inhibit the growth of *Bacillus* spp. in the well.

Determination of minimum bactericidal concentration (MBC)

Minimum bactericidal concentration (MBC) is the minimum concentration of antibacterial agent that prevents the growth of *Bacillus* spp. on the MH agar plates. The established suspension from each MIC well was subcultured on MH agar plates in order to establish the MBC value. A micropipette was used to transfer 10 µL of suspension from MIC well columns 1 to 12 of the wells to the MH agar plates. For 24 h, the plates were incubated at 37 °C to see the *Bacillus* spp. grow on plates. The preparation of the media and handling of *Bacillus* spp. was carried out using an aseptic technique and procedure reported in CLSI (2012).

Determination of time-kill curve

The time-kill assay of *B. cereus*, *B. megaterium*, *B. pumilus* and *B. subtilis* was performed using extracts of *P. guajava* Linn. 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 Mueller Hinton broth (MHB) medium containing inoculum were then used to dilute the extracts to obtain final concentrations of 0×, 0.5×, 1×, 2× and 4× MIC value established. The final volume (1 mL) was agitated at 200 rpm and incubated at 37 °C using a shaking incubator. Well homogenized aliquot in the amount of 100 µL was transferred to a new microcentrifuge tube which contained 1% phosphate-buffered saline (PBS) to be used to serially dilute the homogenised aliquot at a ratio of 1:100 at designated time intervals of (0, 1, 2 and 4 h) and which was then plated onto the MHA. The colony formed (CFU/mL) was recorded after the plates incubation for 24 h at 37 °C. The time-kill curve graph with $\log_{10}$ CFU/mL against time was plotted for *B. cereus*, *B. megaterium, B. pumilus* and *B. subtilis*. The time-kill curve assay was carried out two times in duplicate data (n = 2 × 2). The assay was conducted as suggested by the CLSI (2012).

Stability effect at different temperatures and pH

The effect of varying temperatures (10 ± 2 °C, 28 ± 2 °C, 30 ± 2 °C, 50 ± 2 °C and 80 ± 2 °C) and pH (extract pH 3.0, 5.0, 6.7, 7.0 and 11.0) were tested to determine the stability of *P. guajava* Linn. leaves extract against *Bacillus* spp. The stability effect at different temperatures and pHs assay done according to the method proposed by Durairaj *et al*. (2009) with slight modification. Extract temperature conditionings were done by exposing the extract to the set of temperatures for 15 min in a shaking water bath. The extracts were left to cool down at room temperature before further assay was conducted using the conditioned extract. Hydrochloric acid 0.1 M (HCl, Merck, Darmstadt, Germany) and sodium hydroxide 0.1 M (NaOH, Sigma Aldrich, United States) were used to adjust the designated pH for the assay. The temperatures and pH of the conditioned extracts were then tested to determine the value of MICs and MBCs. The experiment was performed in two independent replications and was repeated two times for each experiment (n = 2 × 2).

Statistical analysis

The antibacterial activity and stability of *P. guajava* Linn. leaves extracts were evaluated, and the findings were provided as the mean ± standard deviation (SD) of duplicate trials (n = 2 × 2).

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Table 1: Inhibition zone of *P. guajava* Linn. leaves extracts against *Bacillus* spp.

Bacillus spp.	Inhibition zone (mm)		
	P. guajava Linn. extract [1% (w/v)]	CHX [0.1%(w/v)]	DMSO [10%(v/v)]
B. cereus ATCC33019	16.25 ± 0.95	17.50 ± 0.70	n.a
B. megaterium ATCC14581	15.50 ± 0.57	17.50 ± 0.70	n.a
B. pumilus ATCC14884	15.50 ± 0.57	17.50 ± 0.70	n.a
B. subtilis ATCC6633	13.75 ± 0.95	16.50 ± 0.70	n.a

n.a; not active. Values are expressed as mean ± standard deviation.

Table 2: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *P. guajava* Linn. leaves extracts against *Bacillus* spp.

Bacillus spp.	MIC		MBC	
	P. guajava Linn. (mg/mL)	CHX (mg/mL)	*P. guajava* Linn. (mg/mL)	CHX (mg/mL)
B. cereus ATCC33019	0.390	1.562	0.781	0.781
B. megaterium ATCC14581	0.195	1.562	0.781	1.562
B. pumilus ATCC14884	0.781	1.562	0.781	1.562
B. subtilis ATCC6633	0.390	3.125	0.781	0.781

RESULTS

Yield of *P. guajava* Linn. leaves extract

Ultrasonic assisted extraction (UAE) method in *P. guajava Linn.* leaves extraction has a significantly high yield of 11.17%, which is an excellent antibacterial agent produced using low extraction temperature in a short time. Optimum total phenolic compounds and extraction yield using ultrasonic-assisted extraction showed maximum zone of inhibition, effective minimal inhibitory concentration and minimal bactericidal concentration against all foodborne pathogens tested (Ida Madiha *et al.*, 2017).

Well diffusion assay (WDA)

Well diffusion assay (WDA) is a preliminary screening for determining the antibacterial activity of selected plants against selected bacteria species. The principle of WDA is greater zone of inhibition is the higher antibacterial activity results. The inhibition zones range between 13.75 ± 0.95 mm and 16.25 ± 0.95 mm. The ability of *P. guajava* Linn. leaves extract to inhibit all tested *Bacillus* spp. within the inhibition zone are as in Table 1. The highest inhibition zone was observed for *B. cereus.* The observed inhibition for 0.1% chlorhexidine CHX is 17.50 mm. Negative controls (10% DMSO) did not show any growth suppression and inhibition zone. The least inhibition zone was observed for *B. subtilis* with 13.75 ± 0.95 mm.

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

A plant extract is regarded to have bacteriostatic potential when the minimum inhibitory concentration or MIC, is required to inhibit at least 99% of bacterial growth. MBC is the lowest concentration of the plant extract necessary to kill at least 99% of the bacteria, which is considered to have bactericidal potential (Rukayadi *et al.*, 2013). Table 2 shows the summary of the MIC and MBC values of *P. guajava* Linn. leaves extracts against tested *Bacillus* spp. The ethanolic extracts of *P. guajava* Linn. exhibited good MIC value of 50 mg/mL for *B. subtilis* (Gupta *et al.*, 2020). The lowest MIC value reported in this study was 0.195 mg/mL for *B. megaterium,* and the highest MIC value reported were 0.781 mg/mL for *B. pumilus*. *Bacillus megaterium* was more susceptible to *P. guajava* Linn. extract compared to another *Bacillus* spp. The bacteria *B. pumilus* reported the most resistance to *P. guajava* Linn. extract compared to other *Bacillus* spp., which less effective in inhibiting the growth. *Psidium guajava* Linn. extract was a good bacteriostatic agent for *B. megaterium* compared to the other *Bacillus* spp. The MBCs value reported in the study for the *Bacillus* spp. were 0.781 mg/mL. *Psidum guajava* Linn. leaves extract effectively killed the *Bacillus* spp. with a concentration of 0.781 mg/mL.

Determination of time-kill assay curve

Antibacterial agent effectiveness was measured by the capacity of the agent to inhibit and kill the bacteria. Time kill curve were used as the most reliable method to determine the killing rate and the length of time the antibacterial agent needs to inhibit and kill the bacteria at a designated concentration of an antibacterial agent (Garmana *et al.*, 2014). Time-kill curves show the relationship between concentration and time. Time-kill curve assay showed an increased trend of cells killed with an increased concentration of the extract as well as contact time period (Abidoye *et al.*, 2019). Table 3 lists the concentrations of leaves extracted from *P. guajava* Linn. in 0× MIC, 0.5× MIC, 1× MIC, 2× MIC and 4× MIC. To determine the correlation between MIC and the

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Malays. J. Microbiol. Vol 20(2) 2024, pp. 167-174
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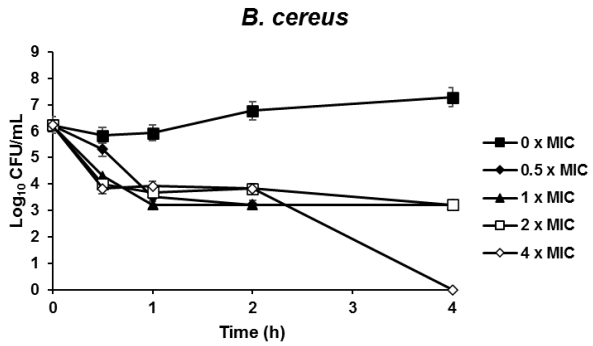

Figure 1: Time-kill curve plots for *B. cereus* following exposure to *P. guajava* Linn. leaves extract at 0× MIC (0 mg/mL), 0.5× MIC (0.195 mg/mL), 1× MIC (0.390 mg/mL), 2× MIC (0.781 mg/mL) and 4× MIC (1.56 mg/mL).

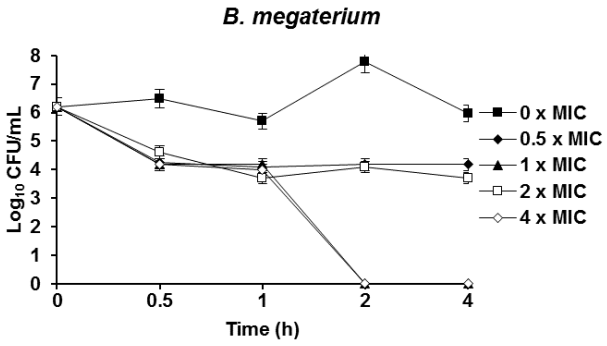

Figure 2: Time-kill curve plots for *B. megaterium* following exposure to *P. guajava* Linn. extract at 0× MIC (0 mg/mL), 0.5× MIC (0.098 mg/mL), 1× MIC (0.195 mg/mL), 2× MIC (0.390 mg/mL) and 4× MIC (0.781 mg/mL).

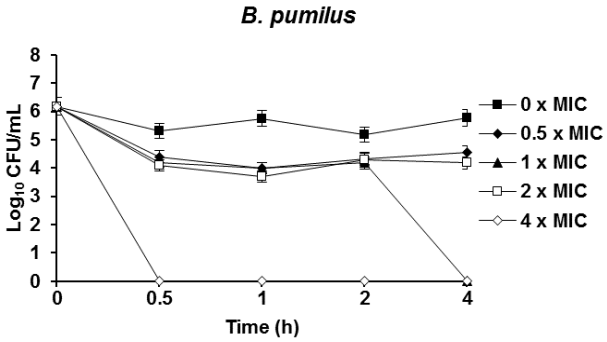

Figure 3: Time-kill curve plots for *B. pumilus* following exposure to *P. guajava* Linn. leaves extract at 0× MIC (0 mg/mL), 0.5× MIC (0.390 mg/mL), 1× MIC (0.781 mg/mL), 2× MIC (1.562 mg/mL) and 4× MIC (3.124 mg/mL).

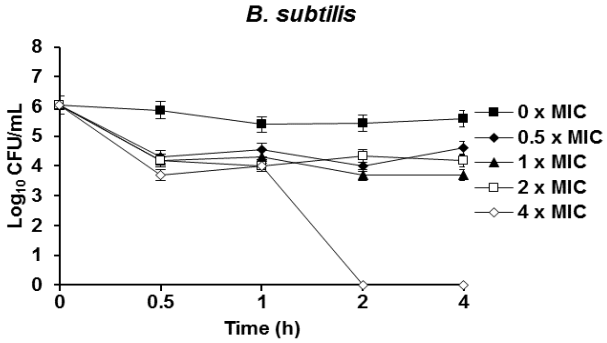

Figure 4: Time-kill curve plots for *B. subtilis* following exposure to *P. guajava* Linn. leaves extract at 0× MIC (0 mg/mL), 0.5× MIC (0.195 mg/mL), 1× MIC (0.390 mg/mL), 2× MIC (0.781 mg/mL) and 4× MIC (1.562 mg/mL).

bactericidal activity of various concentrations of *P. guajava* Linn. leaves extract, plots of the time-kill assay curves for the vegetative cells of *B. cereus*, *B. megaterium*, *B. pumilus* and *B. subtilis* were generated ranging from 0× MIC to 4× MIC. The bactericidal endpoint for *B. cereus* (Figure 1), *B. megaterium* (Figure 2), *B. pumilus* (Figure 3) and *B. subtilis* (Figure 4) were achieved at incubation with a concentration of 0.390 mg/mL at 4 h, 0.195 mg/mL at 2 h, 3.124 mg/mL at 0.5 h and 1.562 mg/mL at 2 h, respectively.

Stability of *P. guajava* Linn. leaves extract at different temperatures

The crude extracts of *P. guajava* Linn. leaves showed more effective under acidic and low temperature (Abubakar, 2009) The stability of *P. guajava* Linn. extracts against heat and pH were analyzed with respect to their antibacterial activity against *Bacillus* spp. The extracts were exposed to temperatures of 10 ± 2 °C, 30 ± 2 °C, 50 ± 2 °C and 80 ± 2 °C for 15 min. The untreated extracts which have temperatures of 28 ± 2 °C were analyzed as control extracts. Table 4 presents the values of MIC and MBC of the heat-treated extracts against the *Bacillus* spp. The result showed that there are no significant changes in the pattern of heat-treated *P. guajava* Linn. extracts in compared to the untreated extracts. Hence, these values present the stability of the extract with heat treatment during the extraction process and heat treatments have no significant difference in the effect of the antibacterial activity of extracts.

Stability of *P. guajava* Linn. leaves extract at different pH

The stability of *P. guajava* Linn. leaves extract against *Bacillus* spp. was analysed under varying pH conditions. The analyses were done at an acidic pH of 3, pH of 5, neutral pH of 7 and alkaline pH of 11. pH 6.7 was the original extract value as control. Table 5 presents the MIC and MBC values for *Bacillus* spp. exposed to different pH of *P. guajava* Linn. leaves extracts. Generally, the

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Table 3: Time kill assay concentration of *P. guajava* Linn. leaves extract at 0× MIC, 0.5× MIC, 1× MIC, 2× MIC and 4× MIC.

Bacillus spp.	0× MIC (mg/mL)	0.5× MIC (mg/mL)	1× MIC (mg/mL)	2× MIC (mg/mL)	4× MIC (mg/mL)
B. cereus ATCC33019	0	0.195	0.390	0.781	1.562
B. megaterium ATCC14581	0	0.098	0.195	0.390	0.781
B. pumilus ATCC14884	0	0.390	0.781	1.562	3.124
B. subtilis ATCC6633	0	0.195	0.390	0.781	1.562

Table 4: MIC and MBC of heat-treated *P. guajava* Linn. leaves extract at a different temperature against *Bacillus* spp.

Bacillus spp.		10 ± 2 °C (mg/mL)	28 ± 2 °C (mg/mL)	30 ± 2 °C (mg/mL)	50 ± 2 °C (mg/mL)	80 ± 2 °C (mg/mL)
B. cereus	MIC	0.390 ± 0.09	0.390 ± 0.09	0.195 ± 0.07	0.195 ± 0.07	0.390 ± 0.09
ATCC33019	MBC	0.781 ± 0.02	0.781 ± 0.02	0.781 ± 0.02	0.781 ± 0.02	0.781 ± 0.02
B. megaterium	MIC	0.390 ± 0.09	0.195 ± 0.07	0.390 ± 0.09	0.390 ± 0.09	0.390 ± 0.09
ATCC14581	MBC	0.097 ± 0.01	0.781 ± 0.02	0.781 ± 0.02	0.390 ± 0.09	0.781 ± 0.02
B. pumilus	MIC	0.781 ± 0.02	0.781 ± 0.02	0.390 ± 0.09	0.390 ± 0.09	0.390 ± 0.09
ATCC14884	MBC	0.781 ± 0.02	0.781 ± 0.02	0.781 ± 0.02	0.781 ± 0.02	0.781 ± 0.02
B. subtilis	MIC	0.390 ± 0.09	0.390 ± 0.09	0.390 ± 0.09	0.390 ± 0.09	0.390 ± 0.09
ATCC6633	MBC	0.781 ± 0.02	0.781 ± 0.02	0.781 ± 0.02	0.781 ± 0.02	0.781 ± 0.02

Table 5: MIC and MBC of heat-treated *P. guajava* Linn. leaves extract at a different pH against *Bacillus* spp.

Bacillus spp.		pH 3.0 (mg/mL)	pH 5.0 (mg/mL)	pH 6.7 (mg/mL)	pH 7.0 (mg/mL)	pH 11.0 (mg/mL)
B. cereus	MIC	0.390 ± 0.09	0.390 ± 0.09	0.390 ± 0.09	0.390 ± 0.09	0.390 ± 0.09
ATCC33019	MBC	0.781 ± 0.02	0.781 ± 0.02	1.562 ± 0.08	1.562 ± 0.09	0.781 ± 0.02
B. megaterium	MIC	0.390 ± 0.09	0.390 ± 0.09	0.195 ± 0.07	0.390 ± 0.09	0.390 ± 0.09
ATCC14581	MBC	0.781 ± 0.02	0.781 ± 0.02	0.781 ± 0.02	0.781 ± 0.02	0.390 ± 0.09
B. pumilus	MIC	0.390 ± 0.09	0.390 ± 0.09	0.390 ± 0.09	0.390 ± 0.09	0.781 ± 0.02
ATCC14884	MBC	0.781 ± 0.02	0.781 ± 0.02	0.781 ± 0.02	0.781 ± 0.02	0.781 ± 0.02
B. subtilis	MIC	0.390 ± 0.09	0.390 ± 0.09	0.390 ± 0.09	0.390 ± 0.09	0.390 ± 0.09
ATCC6633	MBC	0.781 ± 0.02	0.781 ± 0.02	0.781 ± 0.02	0.781 ± 0.02	0.781 ± 0.02

Bacillus spp. exposed to extracts with designated pH values have no significant differences in the value of MIC and MBC. All the *Bacillus* spp. had similar values of MIC and MBC except for strain *B. cereus,* and *B. megaterium* had slightly different values at pH neutral.

DISCUSSION

The ability of *P. guajava* Linn. leaves extract to inhibit all tested *Bacillus* spp. were within the range between 13.75 ± 0.95 mm and 16.25 ± 0.95 mm. *Bacillus cereus* was observed to have the highest zone of inhibition. The observed inhibition for 0.1% chlorhexidine CHX is 17.50 mm. According to Sanches *et al.* (2005), the aqueous extracts of *P. guajava* Linn. leaves were active against the Gram-positive *S. aureus* MICs (500 µg/mL), contributing to the presence of triterpenes (alpha- and beta-amyrin) and sterol (beta-sitosterol). Hoque *et al.* (2007) also reported that food-borne pathogens *B. cereus*, *S. aureus* and *Listeria monocytogenes* were susceptible to *P. guajava* Linn. ethanol extract, with their MIC value showing the highest inhibition for at 0.1 mg/mL. A study done by Braga *et al.* (2014) stated that the high level of phenolic (766.08 mg/g) and flavonoid (118.90 mg/g) content contributed to *P. guajava* Linn. antimicrobial activity against *Streptococcus mutans*, *Streptococcus mitis* and *Streptococcus oralis* at MIC value of 250 µg/mL. Anas *et al.* (2008) reported that the methanolic *P. guajava* Linn. leaves extracts were found to exhibit MIC and MBC at 625 µg/mL and 1.25 mg/mL against multidrug-resistant *S. aureus* and required 4 mg/mL to kill within 10 h of incubation period. Farhana *et al.* (2017) have reported that *P. guajava* Linn. leaves have exhibited antibacterial activity against *S. aureus*, *E. coli*, *B. cereus*, *Shigella sonnei and Salmonella* Typhi after heat treatment at 50 °C, 75 °C and 100 °C suggesting that the temperature does not affect the activity of the extracts. In consensus with a current study, there are no significant changes in the pattern of heat-treated *P. guajava* Linn. extracts compared against the untreated extracts. Hence, these values present the stability of the extract with heat treatment during the extraction process and heat treatments have no significant effect on the antibacterial activity of extracts. In general, the MIC and MBC values of *Bacillus* spp. exposed to extracts with a certain pH value do not differ significantly. Except for strains *B. cereus* and *B. megaterium*, all the *Bacillus* spp. showed identical MIC and MBC values at pH neutral.

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CONCLUSION

In conclusion, it is remarkable to note that the ethanolic *P. guajava* Linn. leaves extract confers significant antibacterial activity against vegetative cells of *B. cereus*, *B. pumilus, B. subtilis* and *B. megaterium*. The ethanolic *P. guajava* Linn. leaves extracts showed stability when tested at different temperatures and pHs against all tested bacteria strains. The resilient nature of endospore-forming bacteria such as *Bacillus* spp. has been an ongoing biosafety concern. Thus, in this study, *P. guajava* Linn. leaves extract showed potential to be developed as a natural anti-bacillus agent and natural preservative.

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CONFLICTS OF INTEREST

The authors have no conflict of interest to declare.

REFERENCES

Abdelgani, A. M., Rukayadi, Y., Shaari, K. and Safinar, I. (2018). Antibacterial and sporicidal activities of methanolic *Sygygium polyanthum* L. leaf extract against vegetative cells and spores of *Bacillus pumilus* and *Bacillus megaterium*. *Journal of Pure and Applied Microbiology* **12(3), 1047-1053.**

Abidoye, R. O., Akinpelu, A. D., Alayande, K. A. and Agunbiade, O. M. (2019). Biocidal potentials of stem bark extracts of *Psidium guajava* (Linn.) on panel of bacterial strains associated with diarrhea and dysentery. *Asian Journal of Pharmaceutical and Clinical Research* **13(2), 84-96.**

Abubakar, E. M. M. (2009). The use of Psidium guajava Linn. in treating wound, skin and soft tissue infections. *Scientific Research and Essays* **4(6), 605-611.**

Anand, V., Manikandan, Kumar, V., Kumar, S., Pushpa and Hedina, A. (2016). Phytopharmacological overview of *Psidium guajava* Linn. *Pharmacognosy Journal* **8(4), 314-320.**

Anas, K., Jayasree, P. R., Vijayakumar, T. and Manish Kumar, P. R. (2008). *In vitro* antibacterial activity of *Psidium guajava* Linn. leaf extract on clinical isolates of multidrug resistant *Staphylococcus aureus. Indian Journal of Experimental Biology* **46(1), 41-46.**

Bano, A., Gupta, A., Rai, S., Sharma, S. and Pathak, N. (2021). Elucidation of bioactive potential of two commonly grown North Indian *Psidium guajava* viz., Lalit and Shweta against pathogenic foodborne and MDR bacteria. *Biointerface Research in Applied Chemistry* **11(6), 14090-14102.**

Biswas, B., Rogers, K., McLaughlin, F., Daniels, D. and Yadav, A. (2013). Antimicrobial activities of leaf extracts of guava (*Psidium guajava* L.) on two Gram-negative and Gram-positive bacteria. *International Journal of Microbiology* **2013, Article ID 746165.**

Braga, T. V., das Dores, R. G. R., Ramos, C. S., Evangelista, F. C. G., da Silva Tinoco, L. M., de Pilla Varotti, F. *et al.* (2014). Antioxidant, antibacterial and antitumor activity of ethanolic extract of the *Psidium guajava* leaves. *American Journal of Plant Sciences* **5(23), 3492-3500.**

Bulugahapitiya, V. P., Kokilananthan, S., Manawadu, H. and Gangabadage, C. S. (2021). Phytochemistry and medicinal properties of *Psidium guajava* L. leaves: A review. *Plant Science Today* **8(4), 963-971.**

Chandra, K. A. and Wanda, D. (2017). Traditional method of initial diarrhea treatment in children. *Comprehensive Child and Adolescent Nursing* **40, 128-136.**

Chiari-Andréo, B. G., Trovatti, E., Marto, J., de Almeida-Cincotto, M. G. J., Melero, A., Corrêa, M. A. *et al.* (2017). Guava: Phytochemical composition of a potential source of antioxidants for cosmetic and/or dermatological applications. *Brazilian Journal of Pharmaceutical Sciences* **53(2), e16141.**

CLSI, Clinical and Laboratory Standards Institute. (2012). Performance standards for antimicrobial disk susceptibility tests; approved standard-eleventh edition. CLSI document M02-A11. CLSI, Wayne, PA, USA.

Draganić, V., Lozo, J., Biočanin, M., Dimkić, I., Garalejić, E., Fira, D. *et al.* (2017). Genotyping of *Bacillus* spp. isolate collection from natural samples. *Genetika* **49(2), 445-456.**

Durairaj, S., Srinivasan, S. and Lakshmanaperumalsamy, P. (2009). *In vitro* antibacterial activity and stability of garlic extract at different pH and temperature. *Electronic Journal of Biology* **5(1), 5-10.**

Farhana, J. A., Hossain, M. F. and Mowlah, A. (2017). Antibacterial effects of guava (*Psidium guajava* L.) extracts against food borne pathogens. *International Journal of Nutrition and Food Sciences* **6(1), 1-5.**

Fernandes, M. R. V., Dias, A. L. T., Carvalho, R. R., Souza, C. R. F. and Oliveira, W. P. (2014). Antioxidant and antimicrobial activities of *Psidium guajava* L. spray dried extracts. *Industrial Crops and Products* **60, 39-44.**

Galié, S., García-Gutiérrez, C., Miguélez, E. M., Villar, C. J. and Lombó, F. (2018). Biofilms in the food industry: Health aspects and control methods. *Frontiers in Microbiology* **9, 898.**

Garmana, A. N., Sukandar, E. Y. and Fidrianny, I. (2014). Activity of several plant extracts against drug-sensitive and drug-resistant microbes. *Procedia Chemistry* **13, 164-169.**

Gavahian, M., Chu, Y. H., Lorenzo, J. M., Khaneghah, A. M. and Barba, F. J. (2020). Essential oils as natural preservatives for bakery products: Understanding the mechanisms of action, recent findings, and applications. *Critical Reviews in Food Science and Nutrition* **60(2), 310-321.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Gharib, A. A., El-Hamid, M. I. A., El-Aziz, N. K. A., Yonan, E. Y. and Allam, M. O. (2020). *Bacillus cereus*: Pathogenicity, viability and adaptation. *Advances in Animal and Veterinary Sciences* **8(1), 38-40.**

Glasset, B., Herbin, S., Guillier, L., Cadel-Six, S., Vignaud, M., Grout, J. *et al.* (2016). *Bacillus cereus*-induced food-borne outbreaks in France, 2007 to 2014: Epidemiology and genetic characterisation. *Eurosurveillance* **21(48), 30413.**

Gupta, G. K., Chahal, J. and Arora, D. (2011). *Psidium guajava* L.: Current research and prospects. *Journal of Research in Pharmacy* **4(1), 42-46.**

Gupta, S., Budhbhatti, N. and Keshava, R. (2020). Antimicrobial activity and phytochemical screening of *Psidium Guajava* (Guava) plant. *International Journal of Pharma and Bio Sciences* **11(4), 162-167.**

Gutiérrez, R. M. P., Mitchell, S. and Solis, R. V. (2008). *Psidium guajava*: A review of its traditional uses, phytochemistry and pharmacology. *Journal of Ethnopharmacology* **117(1), 1-27.**

Hoque, M. D. M., Bari, M. L., Inatsu, Y., Juneja, V. K. and Kawamoto, S. (2007). Antibacterial activity of guava (*Psidium guajava* L.) and neem (*Azadirachta indica* A. Juss.) extracts against foodborne pathogens and spoilage bacteria. *Foodborne Pathogens and Disease* **4(4), 481-488.**

Ida Madiha, Y., Rukayadi, Y. and Norhayati, H. (2017). Effects of extraction conditions on yield, total phenolic contents, and antibacterial activity of methanolic *Cinnamomum zeylanicum* Blume leaves extract. *International Food Research Journal* **24(2), 779-786.**

Millones-Gómez, P. A., Maurtua-Torres, D., Bacilio-Amaranto, R., Calla-Poma, R. D., Requena-Mendizabal, M. F., Valderrama-Negron, A. C. *et al.* (2020). Antimicrobial activity and antiadherent effect of Peruvian *Psidium guajava* (Guava) leaves on a cariogenic biofilm model. *Journal of Contemporary Dental Practice* **21(7), 733-740.**

Mostafa, A. A., Al-Askar, A. A., Almaary, K. S., Dawoud, T. M., Sholkamy, E. N. and Bakri, M. M. (2018). Antimicrobial activity of some plant extracts against bacterial strains causing food poisoning diseases. *Saudi Journal of Biological Sciences* **25(2), 361-366.**

Oda, M., Yokotani, A., Hayashi, N. and Kamoshida, G. (2020). Role of sphingomyelinase in the pathogenesis of *Bacillus cereus* infection. *Biological and Pharmaceutical Bulletin* **43(2), 250-253.**

Özdemir, F. and Arslan, S. (2019). Molecular characterization and toxin profiles of *Bacillus* spp. isolated from retail fish and ground beef. *Journal of Food Science* **84(3), 548 - 556.**

Parham, S., Kharazi, A. Z., Bakhsheshi-Rad, H. R., Nur, H., Ismail, A. F., Sharif, S. *et al.* (2020). Antioxidant, antimicrobial and antiviral properties of herbal materials. *Antioxidants* **9(12), 1309.**

Pisoschi, A. M., Pop, A., Georgescu, C., Turcuş, V., Olah, N. K. and Mathe, E. (2018). An overview of natural antimicrobials role in food. *European Journal of Medicinal Chemistry* **143, 922-935.**

Raj, A., Menon, V. and Sharma, N. (2020). Phytochemical screening, antimicrobial, antioxidant and cytotoxic potential of different extracts of *Psidium guajava* leaves. *Vegetos* **33(4), 750-758.**

Rukayadi, Y., Lau, K. Y., Zainin, N. S., Zakaria, M. and Abas, F. (2013). Screening of antimicrobial activity of tropical edible medicinal plant extracts against five standard microorganisms for natural food preservatives. *International Food Research Journal* **20(5), 2905-2910.**

Sanches, N. R., Garcia Cortez, D. A., Schiavini, M. S., Nakamura, C. V. and Dias Filho, B. P. (2005). An evaluation of antibacterial activities of *Psidium guajava* (L.). *Brazilian Archives of Biology and Technology* **48, 429-436.**

Scavo, A., Pandino, G., Restuccia, C., Parafati, L., Cirvilleri, G. and Mauromicale, G. (2019). Antimicrobial activity of cultivated cardoon (*Cynara cardunculus* L. var. *altilis* DC.) leaf extracts against bacterial species of agricultural and food interest. *Industrial Crops and Products* **129, 206-211.**

Soliman, F. M., Fathy, M. M., Salama, M. M. and Saber, F. R. (2016). Comparative study of the volatile oil content and antimicrobial activity of *Psidium guajava* L. and *Psidium cattleianum* Sabine leaves. *Bulletin of Faculty of Pharmacy, Cairo University* **54(2), 219-225.**

Yu, H. H., Chin, Y. W. and Paik, H. D. (2021). Application of natural preservatives for meat and meat products against food-borne pathogens and spoilage bacteria: A review. *Foods* **10(10), 2418.**

Zaka, S. M., Zeng, X. N., Holford, P. and Beattie, G. A. C. (2010). Repellent effect of guava leaf volatiles on settlement of adults of citrus psylla, *Diaphorina citri* Kuwayama, on citrus. *Insect Science* **17(1), 39-45.**

Zhao, L., Duan, F., Gong, M., Tian, X., Guo, Y., Jia, L. *et al.* (2021). (+)-Terpinen-4-ol inhibits *Bacillus cereus* biofilm formation by upregulating the interspecies quorum sensing signals diketopiperazines and diffusing signaling factors. *Journal of Agricultural and Food Chemistry* **69(11), 3496-3510.**

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