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In Vitro: Antibacterial activity of Piper cubeba L. extract against vegetative cells of Bacillus sp.

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Abstract

In the food industry, the Bacillus species, in particular, is known as organisms that cause foodborne diseases and food spoilage. The present study aims to analyse the antibacterial activities of Piper cubeba L. berries extracts on the vegetative cells of Bacillus cereus ATCC33019, B. subtilis ATCC6633, B. pumilus ATCC14884, and B. megaterium ATCC14581. Results showed that exposing of Bacillus sp. to P. cubeba L. extract resulted in an inhibition zone with a large diameter which ranged between 9.50 to 11.40 mm for the extract. The MIC of the extract ranged between 0.156 – 0.313 mg/mL and the MBC at 2.5 mg/mL against Bacillus sp. The time-kill curve plots showed that exposing Bacillus sp. to a concentration of 8× MIC for a period of four hours resulted in the death of all cells. The values of MIC and MBC showed a fluctuating trend when the bacteria were exposed to P. cubeba L. extract treated with different temperature in comparison to untreated extract. Generally, the pH altered extracts caused a variation in the MIC and MBC values of the Bacillus sp. The effect of using varying concentrations of extracts against the Bacillus sp.

Keyword: - Antibacterial activity, Bacillus species, Piper cubeba L., DDA, MIC, MBC.

Introduction

Antimicrobial agents extracted from plants are usually cheap, easily obtainable, very seldom have side effects, and most essentially, the extracts are effective against many common pathogenic bacteria (Durairaj et al., 2009). In addition to plants use in food preparation, Piper cubeba L., from the Piperaceae family is a popular medicinal plant in

many parts of the world. It is used extensively for traditional medicinal purposes by virtue of its therapeutic properties. Apart from its antimicrobial, antiulcer, anticariogenic and antiinflammatory effects, P. cubeba L. known to have a strong antioxidant activity (Chitnis et al., 2007; Singh et al., 2008; Parvez et al., 2010; Raja Mazlan et al., 2018). The high amounts of bioactive compounds including glycosides, alkaloids, tannins, phenolics, and other secondary metabolites, contribute to the antimicrobial activities of P. cubeba L. extract (Mouid et al., 2016).

The antibacterial activity of methanol extract of P. cubeba L. berries against B. pumilus and B. megaterium has never been established. Chitnis et al. (2007) and Singh et al. (2008) have demonstrated the antibacterial activity of P. cubeba L. berries methanol extract against B. cereus and B. subtilis, respectively. Therefore, this study seeks to establish the antibacterial activities of P. cubeba L. berries extract against B. cereus ATCC33019, B. subtilis ATCC6633, B. pumilus ATCC14884, and B. megaterium ATCC14581 vegetative cells in terms of disc diffusion assay (DDA), minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and time-kill curve Plot. The stability of methanol extract of P. cubeba L. at different temperatures and pH conditions were also measured.

Materials and Methods

Sample collection of P. cubeba L.

The dried P. cubeba L. (Piperaceae) berries employed in this study was obtained from a market selling traditional herbs in Pasar Baru Bandung, Indonesia. The P. cubeba L. was collected in April 2015 in a plantation in Jatiroto, Temanggung, Central Java, Indonesia. The Department of Biology, Institut Teknologi Bandung (Indonesia) authenticated the berries on the basis of Flora of Java (Backer & Van de Brink, 1968). A voucher specimen (HBG10PC01) was stored at the Herbarium Bandungense. The procured material was air-dried and put in storage at the Laboratory of Natural Products, Institute of Bioscience (IBS), University Putra Malaysia (UPM). A powerful blender (Waring, model 32 BL 80, New Hartford, USA) was used to pulverized the dried berries into fine power. The powdered P. cubeba L. sample was stored in a airtight polyethylene plastic bag and put in storage in a - 80°C fridge.

Sample preparation

Plant extraction

The extraction of Piper cubeba L. was done utilizing the soaked technique illustrated by Rukayadi et al. (2008). The organic solvent used in the extraction of P. cubeba L. is absolute methanol (R & M Chemicals, 99.8%). One hundred grams of dried P. cubeba L. berries was ground to obtain as a powder. Sample extraction have done once. It was performed using 400 ml room-temperature solvent and 48 hours of conventional shaking. Filtration of the plant extract was done using Whatman filter paper size No. 2 (Whatman International Ltd., Middlesex, England). Following this, the extracts were concentrated using a rotary vacuum evaporator (Heidolph VV2011, Schwabach, Germany) at 40°C for 3-4 hours to obtain a methanol extract of the dried P. cubeba L. berries. The temperature of the rotary evaporator was increased to 85°C for 2 × 30 sec at the end of the extraction process to ensure that the extract is methanol-free (Madiha et al., 2017). Finally, the extracts (19.90 %) were freeze dried for 48 hours to eliminate water.

Preparation of stock extract

The stock extract of methanol was primed by dissolving crude extract of P. cubeba L. in 100% dimethyl sulfoxide (DMSO) (Fisher Scientific, Leicestershire, United Kingdom) to obtain a 100 mg/ml concentration. Further dilution of the solution was done using 1:10 (v/v) sterile deionized distilled water (ddH2O) to produce a 10 mg/ml stock solution. The stock extract was put in storage at 4°C up to the time it is ready for use. The prepared 10% DMSO solution was confirmed not to kill the tested bacteria in this study (Rukayadi et al., 2013).

Preparation of media and reagents

Preparation of Mueller Hinton agar/broth

Preparation of the media was done as per the instruction given by the manufacturer on the media bottle (Oxoid Ltd., United Kingdom). Preparation of Mueller-Hinton agar (MHA) was done in a 500 mL Schott's bottle by putting 10.5 g Mueller-Hinton broth powder (Oxoid Ltd., United Kingdom) and 8.5 g powdered agar in the bottle and dissolving them in 500 mL deionized water. Sterilization was done by autoclaving the solution at 121°C for 15 minutes and the sterilized agar was then transferred aseptically using a laminar safety flow until it covers the surface of sterile petri dishes. The petri

plates were left at room temperature until the agar solidified, and were then stored at 4°C until they are ready for use. Mueller-Hinton broth (MHB) was prepared by dissolving 5.25 g Mueller-Hinton broth powder (Oxoid Ltd., United Kingdom) in 250 mL deionized water in a 250 mL Schott's bottle. Sterilization was done by autoclaving the solution at 121°C for 15 minutes and the sterile solution was put in storage at 4°C.

Preparation of dimethyl sulfoxide (DMSO) and chlorhexidine (CHX)

The present study used a 10% DMSO as the negative control. The DMSO was prepared by dissolving 10 ml DMSO (99.9%) (R& M Marketing, Essex, UK) in 90 ml distilled water. The 10% DMSO was verified to have no harmful effect on all microorganisms tested in this study (Rukayadi et al., 2013). The positive control employed in this study is a commercial antibiotic, chlorhexidine (CHX) (Sigma-Aldrich, USA). CHX was obtained by dissolving 10 mg chlorehexidine in 1 ml distilled water to obtain a 1% of chlorehexidine (stock solution). A final concentration of 0.1% chlorhexidine was obtained by dissolving 100 µL of the stock solution in 900 µL water. Chlorehexidine is able to kill all bacteria. The CHX was stored at 4°C (McDonnell & Russell, 1999).

Preparation of phosphate buffer saline (PBS)

Preparation of 0.85% phosphate buffer saline (pH7.2) was performed by liquefying 27.6 g of sodium phosphate (Oxoid, England) with 85.0 g of sodium chloride (Oxoid, England) in one liter distilled water to make a stock solution. Subsequently, ten mililitre of the stock solution was then diluted in 90 ml distilled water (ratio of 1:10). The phosphate buffer saline was then used in the dilution process to prevent bacteria growth and to obtain an accurate count. Besides that, the buffer is able to inhibit bacterial lysis due to its osmotic pressure (Bacteriological Analytical Manual, 1998).

Antibacterial activity test

Bacterial strains and inoculums preparation

The Bacillus cereus ATCC33019, B. subtilis ATCC6633, B. pumilus ATCC14884 and B. megaterium ATCC14581 used in the present study was supplied by American Type Culture Collection (ATCC) (Rockville, Maryland, United States). Bacillus strains were sub-cultured on fresh media and incubated at 4°C for 24 hours. The colony form of the bacteria can be stored for a couple of weeks on the Nutrient Agar (NA) plates prior to subculture (Addgene, 2014). For the preparation of stock culture, 0.5 mL of the

overnight culture with an appropriate broth media were mixed with 0.5 mL of 50% sterile glycerol. Bacterial culture were mixed using a vortex and stored at -20°C; this preparation can be kept preserved for 6 month to 1 year (Stockinger Lab, 2001). Finally, all the stock cultures were stored in the Microbial Laboratory, Institute of Bioscience, University Putra Malaysia.

The stock culture of the bacteria was grown on MHA at 37°C for 12-24 hours (Rukayadi et al., 2013). A sterile cotton swab was used to transfer 2-3 colonies of strains to 1 mL of MHB and mixed using a vortex for 15 minutes. The bacteria suspension was then grown at 37°C for 12-24 hours. Ten microlitre of the bacteria suspension was transferred into 10 mL of MHB. The turbidity of inoculums were standardized between 105 - 108 CFU/mL before testing by using standard broth microdilution method (Rukayadi et al., 2013) and inoculum quantification (Indira, 2014), which was carried out by plating 20 µL bacteria suspension on MHA and a count of the visible colonies was made after incubation at 37°C for 12-24 hours (CLSI, 2012).

Disc diffusion assay (DDA)

The method suggested by the Clinical and Laboratory Standard Institute (CLSI) (2012) was employed to carry out the disc diffusion assay against Bacillus sp. The inoculum was prepared and immediately spread on an MHA plate as a single uniform colony with a sterile cotton swab. A sterile self-punched disc paper with a diameter of 6 mm was attached to the inoculated MH agar. Each paper disc was imbued with 10 mg/mL P. cubeba L. in the amount of 10 µL extract. The positive and negative controls were prepared using 500 µg/mL and 10% for CHX and DMSO, respectively. After a 24-hour incubation of the plates at 37°C, the diameter of inhibition zone was measured (in millimetres (mm)) and recorded. Analysis was carried out in three times in triplicate data (n = 3 × 3).The handling of all bacteria and the preparation of media were done using aseptic technique in class II biosafety cabinet (CLSI, 2012).

Minimum concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC of the extract against Bacillus sp. were established as suggested by the Clinical and Laboratory Standard Institute (CLSI) (2012). The minimum inhibitory concentration (MIC) of the extract against B. cereus, B. subtilis, B. pumilus, and B. megaterium vegetative cells was established via the broth microdilution method, which

was performed using a 96-well round bottom microtiter plate (Greiner, Germany). The inoculum suspension of all bacteria species in this test range between 106 and 108 CFU/mL. The first column of wells were designated as the negative control growth and were filled with 100 µL MHB. The second column was designated as a positive control growth column and the wells were filled with 100 µL bacterial suspension. Micro twofold dilution of varying concentrations that range from 5 mg/mL in column 12 to 0.019 mg/mL in column 3, and the MIC was established after incubating the plates at 37°C for 24 hours. MIC is the minimum extract concentration which prevent growth in the well.

Minimum bactericidal concentration (MBC) is the minimum concentration of antibacterial agent which prevent growth on the MH agar plates. The suspension from each MIC well was subcultured on MH agar plates in order to establish the MBC. A pipet was used to transfer 10 µL of suspension from columns 1 to 12 of the wells to agar plates. The plates were incubated 37°C for 24 hours, which is the time required for visible growth to be observed on the plates. The handling of all bacteria and the preparation of the media was carried out using an aseptic procedure in biosafety cabinet Class II.

Determination of time-kill curve

The time-kill assay of B. cereus, B. subtilis, B. pumilus, and B. megaterium was performed using methanol extracts of P. cubeba L. according to method described in CLSI (2012), with some modifications. In the first step approximately 106 CFU/mL of inoculum suspension was prepared; the MHB medium containing inoculum were then used to dilute the extracts to obtain final concentrations of 0×, 1/2×, 1×, 2×, 4× and 8× 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 hours), 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 hours. The graph of Log10 CFU/mL against time was plotted. The time-kill assay were carried out two times in triplicate data (n = 2 × 3).

Effect of pH and temperature on antibacterial activity of P. cubeba L. extract

The effect of varying pH (5, 7, 8, 9 and 10) and temperatures (28ºC ± 2, 50ºC ± 2, 80ºC ± 2 and 120°C ± 2) were tested to determine the stability of P. cubeba L extract against Bacillus sp. The method proposed by Durairaj et al. (2009) was modified slightly and utilized to perform the analysis. The preferred pH for the extracts were adjusted using hydrochloric acid 0.1 M (HCl, Merck, Damstadt, Germany) and sodium hydroxide 0.1 M (NaOH, Sigma Aldrich, United States). As for the temperature conditions, the extracts were exposed to the set temperature for 15 minutes in a water bath. The extracts were left to cool at ambient temperature before analysis was carried out. The pH and temperatures of the altered extracts were then tested to determine the value of MICs and MBCs. For each experiment, the analysis was performed in two independent replications and was repeated three times for each experiment (n = 2 × 3).

Statistical analysis

Excel (v. 2010), GraphPad Prism version 6.00 for Windows (v. 6.00, GraphPad Software, San Diego, CA, USA) was employed to perform the statistical analysis. Results are given as a mean of three replicates ± SD. The significant difference at $P < 0.05$ was established by performing ANNOVA.

Results and Discussion

Yield of Piper cubeba L. extract

Dried Piper cubeba L. berries was extracted using absolute methanol 99.9% (v/v) and the yields of the crude extract was presented in Table 3.1. The yield of herbal extracts are influenced by the types of soaking solvent, ratio of soaking solvent, type of extraction technique, and soaking period (Sultana et al., 2009; Abdullah et al., 2015).

The first and important step in utilizing herbal plant is extraction. Extraction generally yields the desired chemical compounds/components which are then subjected to further separation, purification and characterization. The basic steps involved in extraction are pre-washing of plants/parts of medicinal plants, drying, grinding, and combining the samples with extraction solvents, filtration, and finally evaporation of the solvent prior to use/storage. Samples have to be pulverized to ensure their homogeneity. Moreover, this step would produce a much higher surface area of the sample, thereby enhancing the kinetics of analytic extraction. Extraction must be done following appropriate steps

to prevent loss of potential active constituents and to ensure that the components are not destroyed or distorted (Sasidharan et al., 2011).

In this study, the crude extract was obtained using absolute methanol and the yield of crude extract is 19.90%. There is no previous report regarding the yield of P. cubeba L. crude extract that use methanol as a solvent. It is important to emphasised that the use of methanol as a solvent for food applications is not recommended. Even though the present study used methanol for extraction, precaution was taken to ensure that the final crude extract did not contain methanol residue. As the extraction process was almost complete, the temperature of the rotary evaporator was increased to 85°C for 2 × 30 seconds (Madiha et al., 2017). Since the boiling temperature of methanol is 50°C, the methanol is expected to completely evaporate at this temperature. This confirms that the P. cubeba L. crude extract obtained in this study is a methanol-free extract.

Antibacterial activity of P. cubeba L. extract

Disc diffusion assay (DDA)

Disc diffusion assay (DDA) is a preliminary screening for determining the antibacterial activity of selected plants against selected bacteria species. The principle of DDA is greater zone of inhibition is the higher antibacterial activity results.

Table 1. Inhibition zone of P. cubeba L. berries extracts against Bacillus sp.

Bacillus sp.	Inhibition zone (mm)		
	P. cubeba extract [1% (w/v)]	CHX [0.1%(w/v)]	DMSO [10%(v/v)]
B. cereus ATCC33019	11.40 ± 0.60	11.40 ± 0.30	n.a
B. subtilis ATCC6633	10.70 ± 0.60	11.10 ± 0.10	n.a
B. pumilus ATCC14884	9.50 ± 0.50	11.10 ± 0.10	n.a
B. megaterium ATCC14581	10.00 ± 0.00	11.60 ± 0.30	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); n = 3 × 3

The inhibition zones range between 9.50 ± 0.50 and 11.40 ± 0.60 mm. The ability of P. cubeba L. berries extract to inhibit all tested Bacillus sp. 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 11.40 mm whereas the negative control (10% DMSO) did not show any growth suppression. Lau et al. (2014) have shown that the methanol extract of S. polyanthum L. was able to produce inhibition zone of 8.00 and 7.50 mm against B. cereus and B. subtilis, respectively. The previous reports indicated that the leaves of S. polyanthum L. contained essential oils, triterpenoids, saponins, flavonoids, and tannins (Davidson et al., 2005). This inhibition zones are smaller than those obtained in the present study.This due to the high concentrate of essential oil and phenolic content that found in P. cubeba L if compared with other species (Graidist, 2015; Nahak & Sahu 2011). Zhang et al. (2016) reported that these bioactive compounds help the plant to resist against microorganisms, there by providing evidence for anti-infectious properties. Thus, high content of phenols and essential oil indicates antimicrobial properties. These biological activities are related to the molecules structures; through their hydroxyl groups or phenolic rings, phenolic compounds have the capacity to link with proteins and bacterial membranes to form complexes (Cheikna et al., 2011).

Gangoué-Piéboji et al. (2009) stated that a small amount of active compounds could became caught in the pores of the disc and therefore are not be able to move through the disc and reach the inoculated media. As a consequence they are not be able to fulfil their functions. Typically the disc diffusion test is utilised as an initial screening process to ascertain that the active compounds in plant extracts are able to pass through; this is done prior to performing a further determination. However, the data obtained from disc diffusion assay is inadequate as the assay provide only qualitative data and acts as preliminary screening. Moreover, the hydrophobic nature of most EOs and plant extracts prevents the uniform diffusion of these substances through the agar medium (Rios et al., 1988; Janssen et al., 1987), hence cannot be accurately measure the antibacterial activity of the extracts. Therefore, MIC and MBC determination will further confirm the antibacterial activity of the P. cubeba L. extracts.

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

Bacillus contamination in food has always been a problem to the food industry. Cases of B. cereus contamination are often reported and linked to rice and starchy foods (Baron,

1996; Altayar & Sutherland, 2006; Kim et al., 2013), while B. subtilis contamination is associated with dry ingredients, such as herbs and spices, milk powder and rice (Brul et al., 2011; Kim et al., 2013). Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and time kill curve were employed to established the potency of P. cubeba L. extracts against B. cereus, B. subtilis, B. pumilus and B. megaterium.

MIC is the lowest concentration required to prevent not less than 99% bacteria growth (bacteriostatic). MBC is the lowest plant extracts concentration needed to kill not less than 99% of the bacteria (bactericidal) (Rukayadi et al., 2013). Table 2 presents a summary of the MIC and MBC of P. cubeba L. extracts against four strains of Bacillus.

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of P. cubeba L. extracts against Bacillus sp.

Bacillus sp.	MIC		MBC	
	P. cubeba **mg/mL**	**CHX mg/mL**	***P. cubeba*** **mg/mL**	**CHX mg/mL**
B. cereus **ATCC33019**	**0.313**	**0.016**	**2.500**	**0.125**
B. subtilis **ATCC6633**	**0.313**	**0.016**	**2.500**	**0.125**
B. pumilus **ATCC14884**	**0.156**	**0.008**	**2.500**	**0.250**
B. megaterium **ATCC14581**	**0.156**	**0.008**	**2.500**	**0.125**

Very few studies have reported the MIC of P.cubeba L. extracts and there is no reported studies on the MBC of P.cubeba L. extracts. The lack of lipopolysaccharides on the outer membrane of Gram-positive bacteria contributes to the susceptibility of Bacillus sp. to plant extracts; however, the lipopolysaccharides are present on the outer membrane of Gram-negative bacteria (Alzoreky & Nakahara, 2003). Previous literature have reported that plant extracts were suitable for preventing the growth of Gram-positive bacteria (Hendra et al., 2011; Pimentel et al., 2013). Savitha and Louis (2015) have identified the antimicrobial activity of methanolic extract of S. caryophyllatum on Gram-positive bacteria, such as S. aureus, B. cereus, B. subtilis, B. megaterium, and Gram-negative bacteria, such as E. coli, Pseudomonas aeruginosa, Klebsiella sp., Salmonella typhi and Proteus mirabilis. The greatest diameter of inhibition zone of 24.0 mm was obeserved at 40.00 mg/mL concentration against B. subtilis followed by 21.0 mm for E. coli and 20.0 mm for B. cereus, while the MIC value for B. subtilis is 0.125

mg/mL. These results show better antimicrobial activity compared to the methanolic extract used in this research. Additionally, the findings of this research are congruent with those of an earlier study which has demonstrated that the methanol extract of S. polyanthum L. had MIC and MBC of between 0.31 and 2.50 mg/mL (Lau et al., 2014). As reported by Konate and Souza (2010), this S. alba extract contained bioactive compounds such as saponosides, coumarins, steroids, polyphenol compounds and alkaloids. Generally, all extracts show different antibacterial level among different microbes tested. These inconsistencies might be due to the different expression of the bioactive compounds present in the extracts on the microbial cell. As suggested by Cowan (1999), essential oils and polyphenolic compounds exhibited different bacteriostatic and bactericidal effect on bacterial strains. According to Graidist, 2015 the amount of essential oils and polyphenolic compounds in P. cubeba is higher than other Piper sp. The minimum inhibitory concentration (MIC) is the parameter that commonly used to guide the selection on the antimicrobial agent used in treatment by predicting their efficacy at a standard inoculum approximately 105 CFU/mL after an incubation period of 18-24 h. However, MIC only provides limited information on the kinetics of the antimicrobial action. Therefore, time-killing assay was performed in order to find the correlation between the rate of bactericidal activity with the incubation time and concentration of antimicrobial agent (Mueller et al., 2004).

Determination of time-kill assay curve

Time-kill curves show the dependence between concentration and time. Prolonged periods of treatment with higher extract concentration have been found to be directly proportional with the reduction of viable vegetative cell count. According to Pankey and Ashcraft (2009), the bactericidal activity of the antimicrobial agents is a reduction of total count of CFU/mL for 99.9% (≥3 Log10) compared to the original inoculum. The concentrations of P. cubeba L. extract in ½× MIC, 1× MIC, 2×MIC, 4×MIC and 8× MIC are given in Table 3.

Plots of the time–kill curves for vegetative cells of B. cereus ATCC33019, B. subtilis ATCC6633, B. pumilus ATCC14884 and B. megaterium ATCC14581 were constructed to determine the association between MIC and the bactericidal activity of varying concentrations of P. cubeba L. extract between 0× MIC to 8× MIC. The bactericidal

endpoint for B. cereus (Figure 1), B. subtilis (Figure 2), B. pumilus (Figure 3) and B. megaterium (Figure 4) were achieved four hours after incubation with a concentration of 5 mg/mL, 5 mg/mL, 2.5 mg/mL, and 2.5 mg/mL (8× MIC), respectively. The effectiveness of the extract possibly indicate a high quantity of major secondary metabolites such as phenolics, glycosides, alkaloids, tannins and other identified metabolites that have been reported previously for Piper cubeba extracts (Nahak & Sahu, 2011). Secondary metabolites are synthesized as defance mechanism plants from microbial attacks and predatory animals (Razmavar et al., 2014). Asuquo and Udobi (2016) reported that the extract of Musa paradisiaca is believed to have a promising potential in a wide range of applications in the food industry. Furthermore, this effect is the result of the presence of phytochemical constituents such as; tannin, flavonoid and essential oil in the plant. Moreover, the adsorption of this phytochemical constituents is most likely responsible for the antibacterial activity by causing membrane disruption which then causes leakage of cellular contents and, ultimately, the death of cells (Negi, 2012)

Table 3. Concentration of P. cubeba L. extract in ½× MIC, 1× MIC, 2× MIC, 4 × MIC and 8× MIC

Bacillus sp.	½ × MIC (mg/mL)	1 × MIC (mg/mL)	2 × MIC (mg/mL)	4 × MIC (mg/mL)	8 × MIC (mg/mL)
B. cereus ATCC33019	0.313	0.625	1.250	2.500	5.000
B. subtilis ATCC6633	0.313	0.625	1.250	2.500	5.000
B. pumilus ATCC14884	0.156	0.313			
B. megaterium ATCC14581	0.625	1.250	2.500	1.250	2.500

Time-kill curve of B. cereus ATCC33019

The bactericidal endpoint of P. cubeba L. extract against B. cereus ATCC33019 (Figure 1) was reached after four hours of incubation with a concentration of 5 mg/mL (8× MIC). Jobim et al. (2014) reported that the MIC of A. aculeatum extract on B. cereus is 16.00 mg/mL; however, no killing endpoint was observed in the killing curve even after treatment with 66.40 mg/mL and a 10-hour incubation period. This indicates that higher concentration is not required to kill B. cereus. The bactericidal endpoint of Eugenia polyantha for B. cereus ATCC33019 was reached after a four-hour incubation with a concentration of 2.5 mg/mL (Lau et al., 2014). Yusoff et al. (2015) discovered

that C. caudatus extract prevents the growth of B. cereus strain after a two-hour of incubation with a concentration of 50 mg/mL. It can be seen that 5 mg/mL P. cubeba L. extract at a four-hour incubation period has a better bactericidal effect on B. cereus than A. aculeatum and C. caudatus. In addition, P. cubeba L. contains tannin, which is an active compound that has the ability to inhibit the growth of microbes. The activity of tannin depends on the capacity of this compound to selectively inhibit the activity of certain enzymes, or its ability to inhibit the ligagen-binding chain in some receptors (Sumono & Wulan, 2008). Generally, methanol extracts of berries of P. cubeba L. acted as bactericidal extracts. In the present study, it was found that there was a relationship between the time of exposure and the viable count of B. cereus.

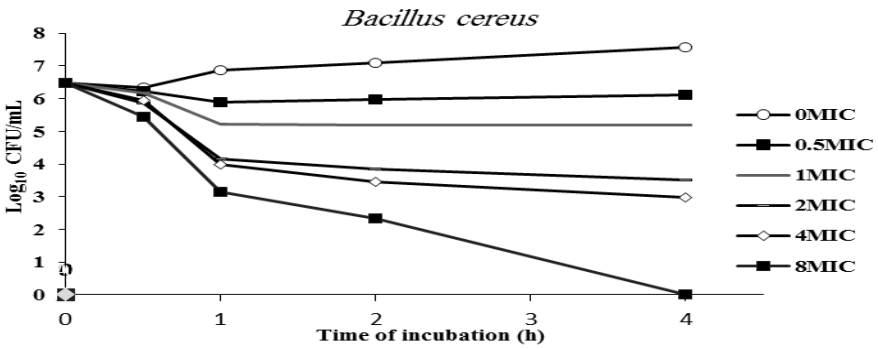

Figure 1. Time-kill curve plots for B. cereus ATCC33019 following exposure to P. cubeba L. extract at 0× MIC (0 mg/mL), 0.5× MIC (0.313 mg/mL), 1× MIC (0.625 mg/mL), 2× MIC (1.250 mg/mL), 4× MIC (2.500 mg/mL), and 8× MIC (5.000 mg/mL).

Time-kill curve of B. subtilis ATCC6633

The bactericidal endpoint of P. cubeba L. extract against B. subtilis ATCC6633 (Figure 2) is 5 mg/mL (8× MIC) for four hours of incubation. Lau et al. (2014) reported that the bactericidal endpoint of E. polyantha extract against B. subtilis ATCC6633 was reached after four hours of incubation at a concentration of 5 mg/mL. The time kill of P. cubeba L. extract against B. subtilis is similar to that of E. polyantha extract. However, Chahardehi et al. (2015) stated that the bacteriostatic effect of ethyl acetate extract of Urtica dioica on B. subtilis at a concentration of 16.66 mg/mL was observed after an incubation period of 16 hours. P. cubeba L. extract has a more potent antimicrobial activity against B. subtilis than U. dioica extract.

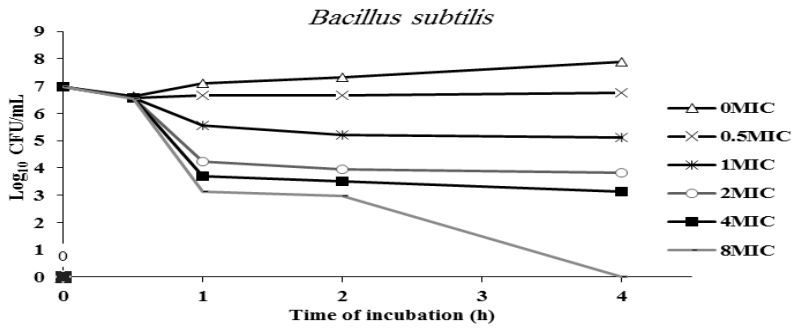

Figure 2. Time-kill curve plots for B. subtilis ATCC6633 following exposure to P. cubeba L. extract at 0× MIC (0 mg/mL), 0.5× MIC (0.313 mg/mL), 1× MIC (0.625 mg/mL), 2× MIC (1.250 mg/mL), 4× MIC (2.500 mg/mL), and 8× MIC (5.000 mg/mL).

Time-kill curve of B. pumilus ATCC14884

The bactericidal activity of P. cubeba L. extracts against B. pumilus ATCC14884 was evaluated using time-kill curves, and the findings of this evaluation are presented in (Figure 3). The figure shows that the time kill endpoint was achieved at 2.5 mg/mL concentration for four hours of incubation. Muniandy et al. (2015) pointed out that a 2.5 mg/mL E. polyantha extract has bacteriostatic effect on B. subtilis. Rasool and Mughal (2014) reported that methanolic extracts of Heliotropium curassavicum, Withania somnifera, Citrullus colocynthis, Ranunculus muricatus, Ricinus communis and Nerium oleander are able to prevent S. aureus, E. coli, B. subtilis, B. pumillus, S. typhimurium, and P. aeroginosa from growing, with MICs 0f between 0.001 and 0.005 mg/mL.

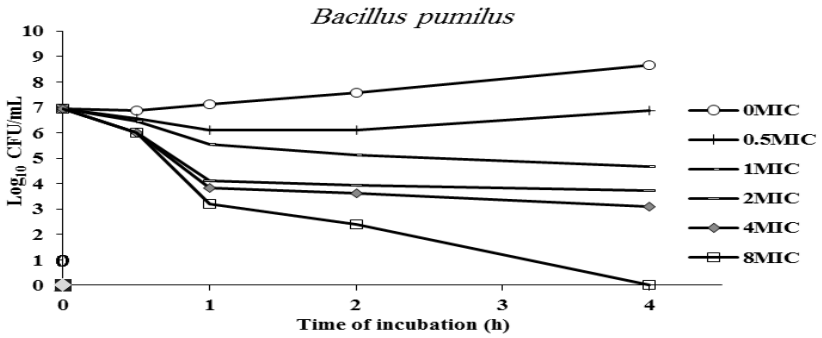

Figure 3. Time-kill curve plots for B. pumilus ATCC14884 following exposure to P. cubeba L. extract at 0× MIC (0 mg/mL), 0.5× MIC (0.156 mg/mL), 1× MIC (0.313 mg/mL), 2× MIC (0.625 µg/mL), 4× MIC (1.250 mg/mL), and 8× MIC (2.500 mg/mL).

Time-kill curve of B. megaterium ATCC14581

Figure 4 shows the time-kill curve of P. cubeba L. extracts against B. megaterium ATCC14581. The curve shows that the 2.5 mg/mL (8× MIC) P. cubeba L. extracts kill virtually all B. megaterium for four hours of incubation. Mahbub et al. (2011) reported that 4.500 mg/mL of Crescentia cujete extract has bacteriostatic effect on B. megaterium after an incubation period of 24 hours. The present study has found that P. cubeba L. extracts are able to kill B. megaterium at a similar concentration, while the bacteriostatic effect of Moringa oleifera extract on B. megaterium is 2.5 mg/mL. P. cubeba L. extract has the same antibacterial agent as Moringa oleifera.

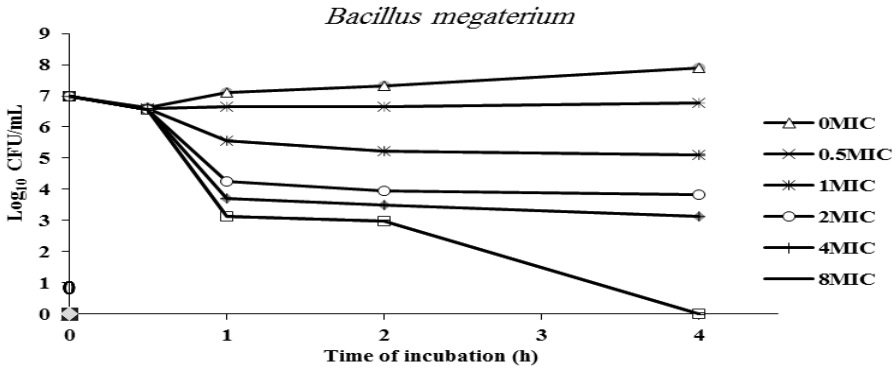

Figure 4. Time-kill curve plots for B. megaterium ATCC14581 following exposure to P. cubeba L. extract at 0× MIC (0 mg/mL), 0.5× MIC (0.156 mg/mL), 1× MIC (0.313 mg/mL), 2× MIC (0.625 mg/mL), 4× MIC (1.250 mg/mL), and 8× MIC (2.500 µg/mL).

Bacillus sp. is a Gram-positive bacteria and in contrast to Gram-negative bacteria is more suseptible to antibacterial agents. Which this probably associated with the structure of cell membrane, where the outer membrane of Bacillus sp. deteriorate rather easily due to its single peptidoglycan layer (Zhang et al., 2016). The activity of selected plant essential oil (EO) combinationsthe could cause damage to both the cell and the cytoplasm external envelope. The hydrophobicity of the main antibacterial compositions of the extract enables the extract to partition in the lipids of the cell membranes and mitochondria, thereby modifying the structure of the cell membranes and mitochondria and altering their functions (Lv et al., 2011). The production of certain macromolecules, such as DNA, RNA, protein, or polysaccharides, can be disrupted by the active components which then resulted in the death of the cells (Rhayour et al., 2003; Wu et al., 2009). Taking into account the whole range of groups

of chemical compounds present in crude extract, their antibacterial activity most probably occur through different mechanisms and a number of targets in the cell (Skandamis & Nychas, 2001; Carson et al., 2002). Crude plant extracts typically include a mixture of particles with varying compositions and chemical structures which could influence their biological actions (Saritha et al., 2015). The antibacterial action of an extract may be limited to membrane disruption. The antimicrobial action of a various proteins and small molecules is believed to cause the formation of pores in the membrane of bacteria and this as a result causes the seepage of cellular contents (Yenugu et al., 2006).

Stability of P. cubeba L. extract at different temperatures

The stability of the bioactive compounds present in plant extracts is significantly affected by determinants such as processing temperature, pH and storage condition (Negi, 2012). There is currently no report on the stability of P. cubeba L. extracts which have been exposed to varying conditions. Hence, the stability of P. cubeba L. extracts due to heat and pH was analyzed with respect to their antimicrobial activity against Bacillus sp. The extracts were exposed to temperatures of 50 ± 2°C, 80 ± 2°C and 121 ± 2°C for 15 minutes prior to analysis. The untreated extracts which have temperatures of 4 ± 2°C and 28 ± 2°C were analyzed as control extracts. Table 5 presents the MIC and MBC of the heat treated extract against the Bacillus sp. The values show a fluctuation in the trend of heat treated P. cubeba L. extracts in comparison to the untreated extracts. The B. cereus and B. pumilus exposed to P. cubeba L. extracts that have been treated at specific temperatures show constant MIC values as compared to the extract with a temperature of 4 ± 2°C. The B. subtilis and B. megaterium exposed to extracts which have been treated at specific temperatures show constant MBC values as compared to the extract with a temperature of 4 ± 2°C (Table 5). The B. cereus and B. pumilus that were exposed to P. cubeba L. extracts which have been heat-treated at 28 ± 2°C and 50 ± 2°C produced similar MIC values. The MIC for B. pumilus subjected to heat treated extract (80 ± 2°C and 121 ± 2°C) are similar to that of the untreated extract. The MBC values for B. pumilus and B. subtilis subjected to extracts that have been heat treated at 50 ± 2°C, 80 ± 2°C and 121 ± 2°C are similar as those subjected to extracts with temperatures of 4 ± 2°C and 28 ± 2°C. These results show the stability of the extract which have undergone heat treatment during rotary

evaporation. The results also show that the extraction process and heat treatment have no significant effect on the antimicrobial activity of extracts.

Table 5. Stability of P. cubeba L. extract at a different temperature

Bacillus sp.	(mg/mL)	4 ± 2°C	28 ± 2°C	50 ± 2°C	80 ± 2°C	121 ± 2°C
B. cereus	MIC	0.313	0.313	0.313	0.625	0.625
ATCC33019	MBC	5.000	2.500	5.000	5.000	5.000
B. subtilis	MIC	0.156	0.156	0.156	0.156	0.156
ATCC6633	MBC	5.000	5.000	5.000	5.000	5.000
B. pumilus	MIC	0.313	0.313	0.313	0.313	0.313
ATCC14884	MBC	5.000	5.000	5.000	5.000	5.000
B. megaterium	MIC	0.313	0.156	0.156	0.156	0.078
ATCC14581	MBC	5.000	2.500	5.000	5.000	5.000

Stability of P. cubeba L. extract at different pH

The stability of P. cubeba L. extract against Bacillus sp. was analyzed under varying pH conditions. The analyses were done at an acidic pH of 5, neutral pH of 7, and alkaline pH of 9. Table 6 presents the MIC and MBC values for Bacillus sp. exposed to different pH of P. cubeba L. extracts. The original extract was alkaline with a pH of 8.58 ± 0.05. Generally, the Bacillus sp. exposed to extracts with altered pHs have varying MIC and MBC values. The MIC values for B. cereus, B. megaterium, and B. pumilus were lower when exposed to alkaline extracts compared to those exposed to acidic extracts. On the contrary, B. subtilis exposed to extracts with pH of 7, 8, and 10 have lower MIC values compared to those exposed to extracts with pH of 5 and 9. All Bacillus sp. were similar MBC values at all pHs.

Table 6. Stability of P. cubeba L. extract at a different pHs

Bacillus sp.	**mg/mL**	**5**	**7**	**8**	**9**	**10**
B. cereus	**MIC**	**0.625**	**0.625**	**0.313**	**0.313**	**0.313**
ATCC33019	**MBC**	**5.000**	**5.000**	**5.000**	**5.000**	**5.000**
B.subtilis	**MIC**	**0.156**	**0.078**	**0.078**	**0.156**	**0.078**
ATCC6633	**MBC**	**5.000**	**5.000**	**5.000**	**5.000**	**5.000**
B. pumilus	**MIC**	**0.625**	**0.625**	**0.313**	**0.313**	**0.313**
ATCC14884	**MBC**	**5.000**	**5.000**	**5.000**	**5.000**	**5.000**
B. megaterium	**MIC**	**0.313**	**0.313**	**0.078**	**0.156**	**0.156**
ATCC14581	**MBC**	**5.000**	**5.000**	**5.000**	**5.000**	**5.000**

Conclusion

P. cubeba L. extract have exceptional antibacillus activities with diameters of inhibition zones of between 9.50 ± 0.50 – 11.40 ± 0.60 mm. MIC and MBC were 0.156 – 0. 313 mg/mL, and 1.25 – 2.5 mg/mL, respectively, against B. cereus, B. subtilis, B. pumilus, and B. megaterium. From time kill assays, eight MIC were found to kill 99.90% of Bacillus sp. after 4 houre treatment. Thus, P. cubeba L. extract might be good to develop as a food preservative. However, the mechanisms of killing require further investigation .B. cereus, B. subtilis, B. pumilus, and B. megaterium showed similar MIC and MBC values when exposed to extratcs that have been treated at different temperatures. Most strains showed stable MBC values when exposed to different pH.

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