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Isolation and identification of linoleic acid and cubebin mixture from Tailed Pepper (Piper cubeba L.) extract and its anti-Bacillus agent

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عزل وتعريف linoleic acid و cubebin mixture من مستخلص الكحولي لنبات الفلفل الاسود واختبار فعاليتها ضد بكتيريا الباسيلس العصوية

الملخص

يعتبر نبات الفلفل الاسود *Piper cubeba* L. من النباتات الطبية الأكثر استخدامًا في العالم، وهومن عائلة Piperaceae. الهدف من هذه الدراسة هو عزل وتنقية المركبات البيولوجية الفعالة من مستخلصات نبات *Piper cubeba* L . بالإضافة إلى ذلك، تم اختبار المركبات المعزولة من حيث فعاليتها ضد الخلايا الخضرية وجراثيم كلا من *Bacillus cereus* ATCC33019 و *B. subtilis* ATCC6633 و *B. pumilus* ATCC14884 و *B. megaterium* ATCC14581. هذه هي الدراسة الاولي التي يتم فيها عزل هذه المركبات من ثنائي كلورو ميثان (DCM) و ومستخلص n–hexane من المستخلص الميثانولي لنبات الفلفل *P. cubeba* L.. تم عزل هذه المركبات من خلال عدة تقنيات كروماتوجرافية وبيانات قياس الطيف الكتلي (MS). هذه المركبات هي حمض اللينوليك **1** ومزيج الكوبيبين **2**. تم اختبار تأثير المركبات المعزولة على اربع سلالات من بكتيريا باسيلس عصوية. أظهرت النتائج أن تعريض الخلايا الخضرية لـ *Bacillus* sp. إلى المركب (1) نتج عنه منطقة تثبيط بقطر كبير يتراوح بين 9.31 إلى

9.61 ملم. كان نطاق الحد الأدنى للتركيز المثبط 63.0 (MIC) ميكروغرام / مل وكان له تركيز قاتل للجراثيم أدنى (MBC) عند 250.0 ميكروغرام / مل. بعد فترة حضانة مدتها أربع ساعات، وقتلت جميع الجراثيم بتركيز 0.1٪. أظهر المركب **1** نشاطًا جيدًا مضادًا للبكتيريا. ومع ذلك، لم يظهر المركب **2** أي نشاط ضد *Bacillus* sp. قد يمتلك المركب (**1**) تأثيرات مثبطة لنمو البكتيريا العصوية. للمساعدة في زيادة مدة صلاحية الطعام، يمكن إضافته إلى المنتجات الغذائية وكذلك كمادة حافظة.

الكلمات المفتاحية: النشاط المضاد للعصويات، Bacillus sp.، خليط الكوبيبين، حمض اللينوليك ، P. cubeba L.

Abstract

One of the most widely used medicinal plants in the world is *Piper cubeba* L., which is a member of the Piperaceae family. Identifying and isolating the bioactive components from *P. cubeba* L. fractions were the aims of this study. Additionally, *Bacillus cereus* ATCC33019, *B. subtilis* ATCC6633, *B. pumilus* ATCC14884, and *B. megaterium* ATCC14581 vegetative cells and spores were used to assess the isolated compounds' antibacterial and antispore properties. The separation of these compounds from the dichloromethane (DCM) and n-hexane fractions of the methanolic extract of *P. cubeba* L berries is described in this report for the first time. The n-hexane fraction yielded compound (**1**), which was separated, and the DCM soluble fraction yielded compound (**2**). These compounds were isolated using a variety of sequential chromatographic methods. The first compound is linoleic acid **1** while the second one **is** cubebin mixture **2**. Anti-*Bacillus* activity of the obtained compounds were evaluated. The results demonstrated that exposure to compound (**1**) caused a wide inhibition zone with a diameter ranging from 9.31 to 9.61 mm in *Bacillus* sp. vegetative cells. The minimal bactericidal concentration (MBC) for *Bacillus* sp. was 250.0 g/mL, and the minimum inhibitory concentration (MIC) was 63.0 g/mL. After four hours of incubation, isolated chemical (**1**) at a concentration of 0.05% inactivated more than 3-Log10 (90.99%) of the *Bacillus* sp. spores, and at a concentration of 0.1%, all the spores were destroyed. Compound **1** demonstrated

good antibacterial activity against vegetative cells and spores of *Bacillus* sp. However, compound **2** did not show any activity against *Bacillus* sp. Compound (**1**) may possess anti-*Bacillus* effects through its actions. To help increase food shelf life, it might be added to food products as well as preservatives.
Keywords: anti-*Bacillus* activity, *Bacillus* sp., cubebin mixture, linoleic acid, *P. cubeba* L.

Introduction

Antimicrobial agents extracted from plants are generally cheap, easily available, 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 used 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 anti-inflammatory 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)

According to Fatimah *et al*., (2019), the antibacterial activity of methanol extract of *P. cubeba* L. berries against *B. pumilus* and *B. megaterium* has 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 isolated compounds from *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 antispore activity.

Materials and Methods

Sample collection of *P. cubeba* L.

The dried *P. cubeba* L. (Piperaceae) berries utilized in this study were purchased from an Indonesian market selling traditional herbs in Pasar Baru Bandung. The *P. cubeba* L. was gathered in April 2015 at a plantation in Jatiroto, Temanggung, Central Java, Indonesia. Based on the Flora of Java, the Department of Biology at Institute Technology Bandung (Indonesia) verified the authenticity of the berries (Backer & Van de Brink, 1968). The Herbarium Bandungense had a voucher specimen (HBG10PC01) that was kept there. The material was obtained, air-dried, and stored at the Institute of Bioscience (IBS Laboratory)'s of Natural Products, University Putra Malaysia (UPM). The dried berries were pulverized into a fine powder using a strong blender (Waring, model 32 BL 80, New Hartford, USA). The ground *P. cubeba* L. sample was kept in an airtight plastic bag made of polyethylene and kept in a refrigerator set to -80°C.

Solvent-solvent fractionation of the crude extracts

The fractionation methods were performed as described by Teshima *et al*. (1998) with a slight modification. Concentrated crude methanol extract (18.84 g) was dissolved in a ratio of 1:2 methanol/water solution and partitioned into *n*-hexane, dichloromethane (DCM), ethyl acetate (EtOAc), *n*-butanol (*n*-BuOH) and finally into an aqueous methanol. The resulting soluble fractions of *P. cubeba* L. were evaporated to yield 12.86 g n-hexane (68.25%), 1.12 g DCM (5.94%), 0.674 g EtOAc (3.57%), 1.16 g *n*-BuOH (6.81%) and 1.263 g the aqueous methanol (6.70%) fractions.

Nuclear Magnetic Resonance Spectroscopy (NMR).

The sample for NMR measurement was prepared as described by Mediani *et al*., 2012, with some modifications. Ten milligrams of samples were mixed with 0.750 mL of $CDCl_3$ solvent. The mixture was vortexed for 1 min followed by ultra-sonication for 15 min at room temperature (25 °C). Next, the mixture was centrifuged at 13000 rpm for 10 min. The solution was then transferred to NMR tubes. ^{1}H and ^{13}C- NMR spectra were measured at 500 MHz and 125 MHz using Varian UNITY INOVA 500 MHz spectrometer,

respectively, and were recorded. Chemical shifts (δ) were recorded in parts per million (ppm) relative to the tetramethylsilane (TMS) signal used as an internal standard at δ = 0 ppm for ^{1}H and ^{13}C- NMR measurement. The coupling constant in Hertz (Hz) and multiplicity were abbreviated as follows: *J* = coupling constant, s = singlet, d = doublet, br s = broad singlet, t = triplet, q = quartet, dd = double doublet, ddd = doublet of double doublet, and m = multiplet. The two-dimensional NMR spectra, Heteronuclear Single-Quantum Correlation (HSQC), Heteronuclear Multiple-Bond Correlation (HMBC) and Homonuclear Correlation Experiments (^{1}H- ^{1}H COSY) were recorded with the regular pulse sequences. The chloroform solvent peaks were observed at ($^{\delta}H^{1}$ 7.27 and $^{\delta}C^{13}$ 77.23 ppm).

Isolation of compounds from *n*-hexane fraction of *P. cubeba* L.

The A portion of the *n*-hexane fraction (4.9 g) was subjected to normal phase (NP) silica gel column chromatography (Merck Kieselgel 60 Art No. 1.9385.1000 of particle size 0.063 – 0.200 mm). The column was prepared by introducing the slurry of adsorbent (130 g) with 100% *n*-hexane into a glass column. The packed column (23.50 cm diameter × 12.50 cm height) was conditioned with a mobile phase consisting of *n*-hexane: EtOAc at a ratio 9:1. The soluble fraction was dissolved in methanol, coated with silica, and loaded on top of the packed column.

The separation of components in the fraction was carried out by eluting with a mixture of *n*-hexane and ethyl acetate with increasing polarity. Each 100 mL fraction was examined using TLC and similar fractions were combined to produced nine major fractions: H_1 (0.005 g), H_{2-10} (0.003 g), H_{11-20} (0.98 g), H_{21-33} (0.42 g), H_{34-45} (0.71 g), H_{46-50} (01.03 g), H_{51-55} (0.90 g), H_{56-60} (0.68 g) and H_{61-69} (0.245 g). Fraction H_{61-69} (0.245 g) was separated into three sub-fractions ($SH_{61-69/1}$ -- $SH_{61-69/3}$) by column chromatography over silica gel (30 g, 9835; 3 cm x 20 cm) with *n*-hexane: EtOAc, starting with a low polarity and increasing to a higher polarity gradient system and finally with ethyl acetate. The eluents were collected at every 50 mL to produce three sub-fractions, $SH_{61-69/1}$ (0.03 g), $SH_{61-69/2}$ (0.51 g) and $SH_{61-69/3}$ (0.05 g) based on the TLC profile, and were

evaporated to dryness. Sub-fraction $SH_{61\text{-}69/2}$ (0.51 g) was chromatographed on silica and eluted with *n*-hexane: EtOAc to produced 10.8 mg **linoleic acid** (**1**), which is a colourless oil.

Compound (1) (10.8 mg) (colourless oil): MW $[M+H]^+$: $C_{18}H_{32}O_2$ *m/z* 280 ; ^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.26 (1H, brs, OH), 5.36 (4H, m, H-9, H-10, H-12 and H-13), 2.78 (2H, t, *J* = 6.0 Hz, H-11), 2.34 (2H, t, *J* = 7.5 Hz, H-2), 2.06 (4H, m, H-8 and H-14), 1.63 (2H, m, H-3), 1.33-1.26 (12H, m, H-3 to H-7 and H-15), 0.90 (3H, m, H-18); ^{13}C NMR (125 MHz, $CDCl_3$): δ 180.9 (COOH), 130.2 (C-9), 130.0 (C-13), 128.0 (C-10), 127.9 (C-12), 34.0 (C-2), 31.9 (C-16), 27.2 (2C, C-8, C-14), 29.6-29.2 (5C, C-4 to C-7 and C-15), 25.6 (C-11), 24.7 (C-3), 22.7 (C-17), 14.1 (C-18).

Isolation of compounds from dichloromethane fraction of *P. cubeba* L.

One gram of dried dichloromethane fraction was subjected to column chromatography (CC) over normal phase (NP) silica gel (Merck Kieselgel 60 Art No. 1.09385.1000 of particle size 0.040-0.063 mm). The column was prepared by introducing the slurry of adsorbent (69 g) with *n*-hexane: EtOAc from 100:0 (v/v) to 0:100 (v/v) into a glass column, which was half filled with the solvent. The packed column (2.50 cm diameter × 30 cm height) was conditioned with the mobile phase comprising *n*-hexane: EtOAc at a ratio of 9:1. The dichloromethane soluble fraction was dissolved in methanol and mixed with silica gel at a ratio of (1:1). The resulting slurry was evaporated and loaded on top of the packed column. The separation of compounds in the mixture was done by eluting with the above solvents in increasing polarity until 100% EtOAc. About 100 mL of the eluent was collected for each fraction and their TLC profile were obtained.

Similar fractions from the analysis were pooled together and evaporated to dryness to produce thirteen major fractions namely $D_{1—7}$ (0.1041 g), $D_{8—18}$ (0.2714 g), $D_{19—30}$ (0.0841 g), $D_{31—36}$ (0.0958 g), $D_{37\text{--}55}$ (0.3925 g), $D_{56\text{--}69}$ (0.119 g), $D_{70—74}$ (0.073 g), $D_{75—79}$ (0.0346 g), $D_{80—87}$ (0.2412 g), $D_{88—90}$ (0.129 g), $D_{91—94}$ (0.0307 g), D_{95} (0.0291 g) and $D_{96—106}$ (0.0413 g). Fraction $D_{31—36}$ (0.0958 g) was purified by using a mini silica gel column with 100% chloroform to produced two sub-fraction pools, $SD_{31—36/1}$

(0.050 g) and SD $_{31—36/2}$ (0.045 g) . Sub-fraction D $_{31—36/1}$ (0.050 g) was further recrystallized from *n*-hexane-acetone to produce 25 mg cubebin mixture (2) white crystal.

Compound (**2**) (25 mg) (white crystal needles): Melting point: 130-131°C, MW $[M+H]^+$: $C_{20}H_{20}O_6$ *m/z* 356. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 6.60 - 6.73 (6 H, m, H-2', H-5', H-2, H-5, H-6, H-6'), 5.92 (2 H, s, H-10, H-10'), 5.22 (1 H, s, H-9'), 4.11 (1 H, t, *J* = 7.4 Hz), 3.58 (1 H, t, *J* = 7.5Hz) for H-9(2a), 3.82 (1 H, t, *J* = 8.1 Hz), 4.01 (1H, t, *J*= 7.4 Hz) for H-9 (2b) , 2.01 – 2.75 (4 H, m, H-7, H-8, H-7' and H-8'). ^{13}C-NMR (125 MHz, $CDCl_3$): Aromatic rings carbons at δ 121.59 until δ 108.07 (C-6, C-6', C-5, C-5', C-2, C-2'), δ 103.34 (C-9'), δ 100.79 and δ 100.85 (–O-CH_2-O for C-10 and C-10-1), δ 98.82 (C-9), δ 72.23 (C-9) of cubebin (2a) and δ 72,62 (C-9) to epicubebin (2b), δ 42.86, δ 45.88, 52.03 and δ 53.08 [C-8(2a), C-8'(2b), C-8'(2a) and C-8(2b)], 33.61 (C-7'(2a)), 38.88 (C-7(2a)), δ 29.69 (C-7'(2b)), δ 39.20 (C-7(2b)).

Preparation of anti-*Bacillus* agents.

The compounds (**1** and **2)** were dissolved in 100% dimethyl sulfoxide (DMSO, Gibco) to obtain a concentration of 10 mg/mL= 10,000 µg/mL (1%), and the solution was further diluted in 1:100 (v/v) distilled water to obtain 1000 µg / mL (0.1%) stock solution. In the present research, the final concentration of 10% DMSO in stock solution did not result in the death of *B. subtilis*, *B. cereus*, *B. pumilus*, and *B. megaterium*; this finding is consistent with the result obtained by Rukayadi *et al*. (2008). 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 chlorohexidine in 1 ml distilled water to obtain a 1% of chlorohexidine (stock solution). A final concentration of 0.1% chlorhexidine was obtained by dissolving 100 µL of the stock solution in 900 µL water. Chlorohexidine is able to kill all bacteria. The CHX was stored at 4°C (McDonnell & Russell, 1999).

Bacterial strains and inoculums preparation

The *Bacillus cereus* ATCC33019, *B. subtilis* ATCC6633, *B. pumilus* ATCC14884 and *B. megaterium* ATCC14581 used in the current study were provided 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 was mixed using a vortex and stored at -20°C; this preparation can be kept preserved for 6 months to 1 year (Stockinger Lab, 2001). Finally, all the stock cultures were stored in the Microbial Laboratory, Institute of Bioscience, University Putra Malaysia.

Bacterial stock culture was cultured on MHA at 37°C for 12–24 hours (Rukayadi et al., 2013). 2-3 colonies of the strains were transferred to 1 mL of MHB using a sterile cotton swab, and the mixture was vortexed for 15 minutes. After that, the bacteria suspension was cultured for 12 to 24 hours at 37°C. The bacteria suspension was diluted by 10 microliters and added to 10 mL of MHB. Before testing, the turbidity of inoculums was calibrated between 10^5 and 10^8 CFU/mL using the standard broth micro dilution method (Rukayadi et al., 2013) and the inoculum quantification method (Indira, 2014), which involved plating a 20 µL bacteria solution on MHA and counting the visible colonies after incubation at 37°C for 12 to 24 hours (CLSI, 2012).

Disc diffusion assay (DDA).

To conduct the disc diffusion experiment against *Bacillus* sp., the Clinical and Laboratory Standard Institute (CLSI) (2012) method was used. A sterile cotton swab was utilized to spread the inoculum as a single homogenous colony as soon as it had been prepared onto an MHA plate. To the inoculated MH agar, a sterile self-punched disc paper with a 6 mm diameter was placed. Each paper disc was filled with 10 mg/mL *P. cubeba* L. in the amount of 10 µL extract and 10 µL of 1 mg/mL (w/v).

Compounds were loaded on the paper discs. 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 inhibitory 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 micro dilution 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 10^6 and 10^8 CFU/mL. The first column of wells was 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. One hundred microliters of extract (10 mg/mL = 10000 µg/mL) and compounds stock solution (1 mg/mL = 1000 µg/mL) was mixed and diluted in two-folds with the test organism in MHB (100 µL). Micro two-fold 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 sub cultured 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.

Antispore activity assay

A minor modification was made to the technique illustrated by Kida *et al*. (2004) and Rukayadi *et al.* (2009) and it was then used to establish antispore activity. To obtain initial *B. cereus*, *B. subtilis*, *B. pumilus*, and *B. megaterium* spores' suspension of 2×10^6, 3×10^6, 2.5×10^6 and 1.6×10^6 spores/mL, respectively, the prepared spore suspension was thawed and diluted at 1:100 in 0.85% NaCl solution (pH 6.6). In order to acquire the final concentrations of extract (0.030, 0.060, 0.125, 0.250, and 0.500%), the 1% stock extract was diluted in adjusted spore suspension. As a positive control, the standard commercially available 25% glutaraldehyde solution (Merck Darmstadt, Germany) was utilized to assess the sporicidal activity. To obtain a 1% concentration, glutaraldehyde was diluted in distilled water at a ratio of 1:25. The pH of the test solutions did not change when extract or glutaraldehyde were added. One milliliter of each concentration was incubated in a 30°C water bath for varying periods of 0, 1, 2, 3 and 4 hours.

To get bacterial-free spore and avoid the impact of vegetative cells residual, an aliquot in the quantity of 100 mL was transferred to micro centrifuge tubes and centrifuged at (12, 000 g, 4°C, for 5 minutes). It was then rinsed twice with 0.85% NaCl solution (pH 6.6). The pellets were serially diluted, placed onto NA plates, and incubated at 30°C for at least 24 hours after being suspended in 100 L of 0.85% NaCl solution (pH 6.6). (until the colonies are visible on the plates). We counted the colonies on the duplicate plates and computed the mean colony-forming unit (CFU/mL). By dividing the log CFU/mL value of the test solution by that of the control, the differences were calculated (no antibiotic added). The reduction of spore cells in CFU is expressed as sporicidal activity; the determination of sporicidal activity was repeated twice in triplicate ($n = 2 \times 3$).

Statistical Analyses

Excel (v. 2010), Graph Pad Prism version 6.00 for Windows (v. 6.00, Graph Pad 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

Structural elucidation of linoleic acid isolated from *n*-hexane fraction.

Compound (**1**) has been previously identified in various plants, for instance in leaves of *Helichrysum pedunculatum* (Dilika *et al*., 2000), methanol extract of chaste-tree berry (*Vitex agnus-castus* L.) (Liu *et al*., 2004), whole plant of *C. colocynthis* (Rahuman *et al*., 2008), and *Lepidium sativum* seed (Abdel Karim *et al*., 2017). Compound (**1**) was obtained as a colourless oil. (Fig. 1). The mass spectra for compounds **1** (Fig 2) was measured on direct injection probe mass spectrometer (DIP-MS) on GC-MS Shimadzu Qp-2010 (Kyoto, Japan). The mass spectrum for this compound shows a molecular ion peak at m/z 280, which suggest molecular formula $C_{18}H_{32}O_2$ and is identified as 9, 12-octadecadienoic acid (linoleic acid).

The ^{1}H-NMR spectrum of compound (**1**) shows deshielded resonances for olefinic protons at δ 5.36 (4H, m), bis-allylic protons at δ 2.78 (2H, t, J = 6.0, 13.0 Hz), allylic protons at δ 2.06 (4H, m), and a group of methylene protons at δ 2.34 (2H, t, J = 7.5, 15.0 Hz) which correspond with the protons attached to C-2. Other shielded resonances are at δ 0.90 (3H, m) for terminal methyl protons, δ 1.63 (2H, m) which correspond with the methylene protons attached to C-3, and δ 1.33-1.26 (14H, m) which correspond with the methylene protons attached to C-4 to C-8 and C-15-C-16. The ^{13}C-NMR spectrum displays four signals for the olefinic carbons δ 130.2 (C-9), 130.0 (C-13), 128.0(C-10), and 127.9 (C-12), as well as signals for methylene carbons at δ 34 - 22.7. The terminal methyl signal (C-18) resonates at δ 18.1, while the carbonyl signal (C-1) is observed at δ 179.9. Therefore, compound 2 is identified as 9,12-octadecadienoic acid. This compound has been previously isolated from the fruits *Vitrex agnus-castus* (chast-berry) as an estrogenic compound (Liu *et al*., 2004). This is the first report of this compound being isolated from *P. cubeba* berries.

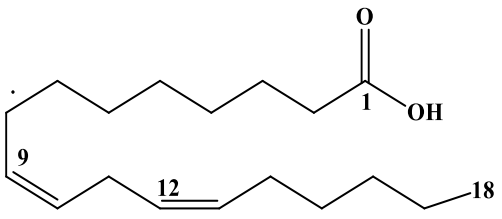

Figure 1. Structure of linoleic acid (9,12-octadecadienoic acid)

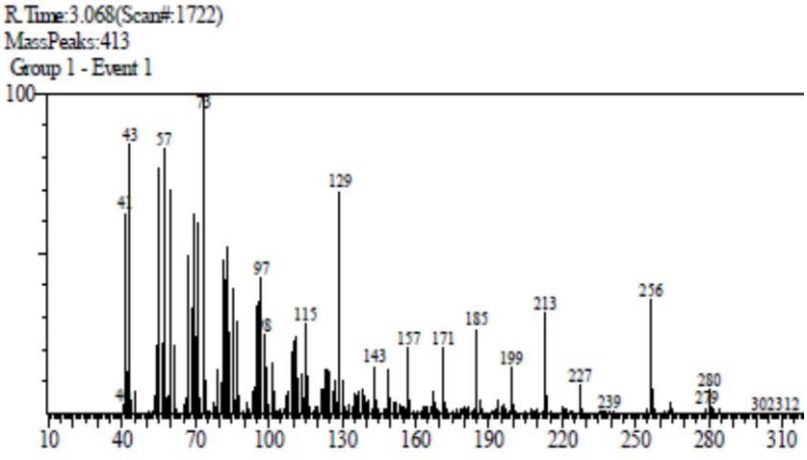

Figure 2. DIP-MS spectrum of linoleic acid

Structural elucidation of cubebin mixture isolated from DCM fraction.

One compound was isolated from the bioactive dichloromethane fraction. The structures of the compounds (Fig. 3), was elucidated using ^{1}H, ^{13}C NMR and mass spectral data as well as by making a comparison with values published in the literature. The identified compound is cubebin mixture (**2**) (Mulik & Laddha, 2015). The mass spectra for compound **2** (Fig 4) was measured on direct injection probe mass spectrometer (DIP-MS) on GC-MS Shimadzu Qp-2010 (Kyoto, Japan).

Isolation and characterization of compound (**2**) cubebin mixture. Problems occur when cubebin ($C_{20}H_{20}O_6$) is mixed with its epimer, making it difficult to identify the structure by using one-dimensional NMR spectroscopy (^{1}H-NMR). Therefore, further isolation of

cubebin and structure elucidation was carried out using two-dimensional NMR (^{13}C-NMR). This method is able to differentiate between this compound and its epimer, which is known as epicubebin. This compound appeared as white crystal needles having a melting point of 133-134 C, similar to that reported by Wei Ming *et al*. (1987) (m.p 131-133 °-C), and it is insoluble or partially soluble in ethanol and soluble in n-hexane. The ^{1}H-NMR spectra of 2a (cubebin) and 2b (epicubebin) indicate the presence of 24 protons. The spectrum shows the presence of methylenedioxy at δ 5.92 (2H, s, H-10 H-10'), a signal at δ 5.22 (1H, d, H-9'), and signals between δ 2.01 – 2.75 (4H, m, H-7, H-8, H-7' and H-8'). The signals of the aromatic region are at δ 6.60 - δ 6.73 (6H, m, H-2', H-5', H-2, H-5, H-6, H-6'). The presence of epicubebin (2b) was confirmed by the signals at δ 3.82 (1H, t, *J*= 8.1 Hz) and δ 4.01 (1H, dd, *J* = 7.4 Hz) for H-9 (2b), and cubebin at δ 3.58 (1 H, t, *J* = 7.1 Hz) and δ 4.11 (1H, dd, *J* = 7.4 Hz) for H-9(2a). The ^{13}C-NMR spectrum in shows the presence of 26 peaks which correspond with the 26 carbon atoms in the molecule. The spectrum shows the presence of 12 methine, 8 methylene and 6 quarternairy carbons. The signal at 72.23 is designated as belonging to C-9 of cubebin, whereas the signal at 72.62 is designated as belonging to epicubebin. In both cubebin (2a) and epicubebin(2b), the signal at 100.79 and 100.85 was the pair -O-CH2-O for C-10 and C-10-1.

Moreover, C-9 and C-9' resonances can be found at 98.82 and 103.34, respectively. The aromatic ring carbons C-6, C-6', C-5, C-5', and C-2, C-2' resonate between 121.59 and 108.07. Other quarternary carbons, designated C-8(2a), C-8'(2b), C-8'(2a), and C-8(2b), can be found at positions 42.86, 45.88, 52.03, and 53.08. Methylene carbon signals can be seen at the following positions: 33.61 for C-7'(2a), 38.88 for C-7(2a), 29.69 for C-7'(2b), and 39.20 for C-7 (2b). Through the use of COSY, HMQC, and HMBC correlation spectra, the structure of cubebin and epicubebin was further supported.

By analysing the extended COSY spectra, it was possible to validate the chemical shifts (ppm) and proton-proton couplings of cubebin and epicubebin. The coupling interactions between H-9 of cubebin and H-9 of epicubebin are confirmed by the^{1}H-^{1}H COSY

spectra. H-8 of the epicubebin and H-9 of the cubebin are involved in another coupling contact. The signals in the HMQC spectrum from 3.58 to 4.11 (H-9) are connected to carbon resonances at 72.23 for C-9(2a) and 72.62 for C-9 (2b). The signals at 100.79 and 100.85 show the link between the proton H-10 (5.92) and the carbons in C-10, C-10'.

According to the HMBC correlation spectrum, the carbon signals at 42.86 for C-8, 98.82 for C-9, 52.03 for C-8', and 38.88 for C-7' are all associated to the C-9 proton signals at 3.82 and 4.01, respectively. The mass spectrum of cubebin (Fig. 4), which shows the molecular ion peak [M]+ at m/z 356 (30), 356 (13), 203 (10), 161 (11), 136 (51), 135 (100), 131 (15), and 77, provided more evidence for the structure's elucidation (Wei-Ming et al., 1987).The percentage of cubebin and epicubebin in the mixture were measured by calculating the integral of δ 3.80; 4.00 for cubebin and 3.57; 4.10 for epicubebin in the ^{1}H-NMR spectrum; these signals do not overlap with another signals. The ratio is 60.61% for cubebin and 39.39% for epicubebin.

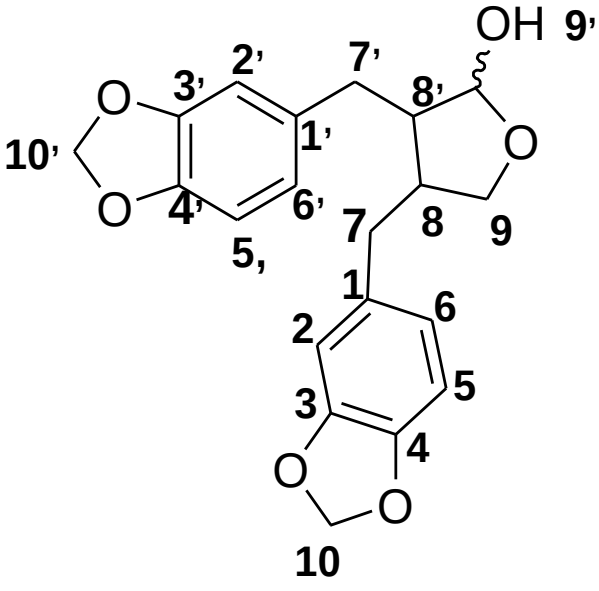

Figure 3. Structure of cubebin mixture

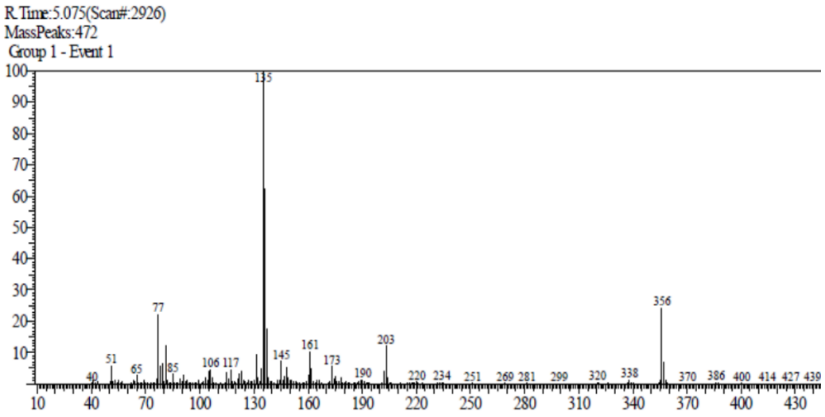

Figure 4. DIP-MS spectrum of cubebin mixture

In vitro activity of linoleic acid and cubebin mixture.

Disc diffusion assay (DDA)

The susceptibility of *B. cereus, B. subtilis, B. pumilus*, and *B. megaterium* to a single antibacterial component isolated from this plant has received few reporting. Bioactive substances were evaluated using the disc diffusion assay (DDA) against Bacillus species. Table 1 provides a summary of the DDA statistics. According to the table, linoleic acid extracted from *P. cubeba* L. exhibits much more antibacterial activity against the Bacillus strain than cubebin mixture at a concentration of 0.1%. Linoleic acid isolated from *P. cubeba* L demonstrate a broad-spectrum activity against all tested bacteria. The inhibition zone range between 9.3 ± 0.5 to 9.6 ± 0.5 mm. In contrast to cubebin mixture, the results of the disc diffusion assay (DDA) showed that linoleic acid had the largest inhibitory zone.

The inhibition zone formed by positive control (chlorhexidine (CHX) (10 mg/ml)) was 11.00 mm and DMSO 10%, which is the negative control, did not inhibit the growth of the tested strain. Linoleic acid, on the other hand, produced inhibition zones of 9.31 ± 0.50, 9.61 ± 0.50, 9.31 ± 0.53 and 9.51 ± 0.53 mm forn *B. cereus, B. subtilis, B. pumilus,* and *B. megaterium*, respectively. However,

cubebin mixture did not show any inhibition zone against all tested *Bacillus* strains.

Table 1. Disc diffusion of isolated compounds against *Bacillus* sp .

***Bacillus* sp.**	**Inhibition zone (mm) ±SD**				
	Methanol extract	**Linoleic acid**	**CHX**	**Cubebin mixture**	**DMSO**
***B. cereus* ATCC33019**	**11.40 ± 0.60**	**9.31 ± 0.50**	**11.4 ± 0.31**	**n. a**	**n. a**
***B. subtilis* ATCC6633**	**10.70 ± 0.60**	**9.61 ± 0.50**	**11.1 ± 0.10**	**n. a**	**n. a**
***B. poilus* ATCC14884**	**9.50 ± 0.50**	**9.31 ± 0.53**	**11.1 ± 0.10**	**n. a**	**n. a**
***B. megaterium* ATCC14581**	**10.00 ± 0.00**	**9.51 ± 0.53**	**11.6 ± 0.30**	**n. a**	**n. a**

N. a: no activity, Diameter of inhibition zones in mm (including disc). Positive control (Chlorohexidine: CHX; 0.1%); Negative control (DMSO; 10%). Results were expressed as means ± standard deviation (SD); n = 2 × 3.

According to Fatimah *et al*., (2019), the exposing vegetative cells of *Bacillus* sp. to β-asarone, and asaronaldehyde isolated from DCM fraction of *P. cubeba* L. resulted in an inhibition zone with a large diameter ranging between 7.21 to 8.31 mm which is lower than the results obtained in the present study. Generally, bacteria species exhibit larger inhibition zone of between 11.1 ± 0.11 mm and 11.6 ± 0.30 mm. linoleic acid showed lower inhibition zone in the range of 9.31 ± 0.50 to 9.61 ± 0.50 mm compared to the control positive. Tippayatum and Chonhenchob (2007) stated that thymol, eugenol and nisin presented the minimum inhibition zones of 8.0 ± 0.8, 7.0 ± 0.4 and 7.2 ± 0.4 mm for *B. cereus*, which is lower than the results obtained in the current study.

MIC and MBC of the isolated compounds.

The minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of bioactive isolated compounds against *B. cereus* , *B. subtilis*, *B. pumilus*, and *B. megaterium* were calculated. A sensitivity to linoleic acid was present in all strains. The compounds had lower MIC and MBC

values than *P. cubeba* L. extract. This discovery offers compelling evidence that the antibacterial properties of *P. cubeba* L. extract is due to linoleic acid. The MIC values of the compounds are presented in (Table 2). Linoleic acid has a lower MIC value of 62.5 µg/mL against *B. subtilis*, *B. cereus*, *B. megaterium*, and *B. pumilus*. All MBC values are larger than the MIC value at 62.5 µg/mL. All tested *Bacillus* sp. showed higher MBC at 250 µg/ml compared to those of the MIC.

Table 2. Minimum inhibitory concentration (MICs) Minimum bactericidal concentration (MBCs) of methanol extract and linoleic acid against *B. cereus*, *B. subtilis*, *B.* megaterium and *B. Pumilus.*

***Bacillus* sp.**	**Methanol extract (µg/ml)**		**Linoleic acid (µg/ml)**		**Cubebin mixture (µg/ml)**		**CHX (µg/ml)**	
	MICs	**MBCs**	**MICs**	**MBCs**	**MICs**	**MBCs**	**MICs**	**MBCs**
***B. cereus* ATCC33019**	**313**	**2500**	**63**	**250**	n. a	n. a	**16**	**125**
***B. subtilis* ATCC6633**	**313**	**2500**	**63**	**250**	n. a	n. a	**16**	**125**
***B. pumilus* ATCC14884**	**156**	**2500**	**63**	**250**	n. a	n. a	**8**	**125**
***B. megaterium* ATCC14581**	**156**	**2500**	**63**	**250**	n. a	n. a	**8**	**125**

Positive control (Chlorohexidine: CHX; 0.1%).

The separated chemicals' antibacterial activity was less potent than that of the crude extract and its fractions. The previous bioassay also revealed the presence of other substances with antibacterial activity in the DCM and n-hexane fractions of the crude extract. The main constituent of the crude extract appears to be a combination of many chemicals. However, it would be assumed to have a significantly lower MIC value if it were the main antibacterial agent. Since this was not the case, other small chemicals may very well have much stronger antibacterial activity, making up a larger share of the overall activity even though they are not as abundantly available. Unfortunately, it is not possible to quantify antibacterial

activity from a bio autography assay, where the inhibition spot is by nature a positive/negative response, and does not reveal detail about the degree of activity of a compound (McGaw *et al.*, 2002).

There are several reports concerning the susceptibility of *B. cereus* to a single antimicrobial compound isolated from plants. Rukayadi *et al*. (2013) stated that macelignan significantly prevent the growth of vegetative cells of *B. cereus* with much smaller MIC of 4 µg/mL, and also has strong bacterial static (MBC) activity against vegetative cells of *B. cereus* with MBC of 8 µg/mL. Furthermore, Mokbel and Hashinaga (2005) stated that β-sitosterol and oleic acid at concentrate of 300 and 250 µg/mL, inhibited the growth of *B. cereus* vegetative cells*;* these concentrations are much higher than the values obtained in the current study.

Similar results were stated for crude extracts of different plants containing β-sitoterol against numerous microorganisms (Beltrame *et al.*, 2002; Bayor *et al.*, 2009; Bumrela & Naik, 2011). Though, the concentrations used in these studies are much higher than the concentration used in the present results. Moreover, Fatimah *et al*., (2019) reported that β-asarone, and asaronaldehyde that isolated from DCM fraction of *P. cubeba* L. berries showed minimum inhibitory concentration (MIC) ranged between 63.0 to 125.0 µg/mL and had minimum bactericidal concentration (MBC) at 250.0 to 500.0 µg/mL against vegetative cells *Bacillus* sp, These MIC were higher than the current study. The leaf and rhizome of *Acorus calamus* have been shown to have antibacterial activity. *A. calamus* rhizomes exhibited strong antibacterial activity against *P. aeruginosa*, *S. aureus*, and *B. subtilis* with MIC of 0.25 (Sabitha *et al*., 2003). *Mycobacterium* sp. and *B. subtilis* are both susceptible to calamus oil (Radusiene *et al*., 2006).

According to Dilika *et al*. (2000) the linoleic acid isolated from the leaves of *Helichrysum pedunculatum* has ability to inhibit the growth of all the Gram-positive bacteria such as *B. cereus* and *B. subtilis* with MIC of 10 and 10 µg/mL, respectively, These MIC were higher than the present study. Ultee *et al*. (2002) tested the effect of carvacrol on the cells of food-borne pathogen *B. cereus* spores, and found that the bactericidal activity of carvacrol is influenced by duration of exposure and concentration. The

mechanism carvacrol's action on *B. cereus* may be attributed to reduced ATP production. Usually, the degree to which the bioactive compounds used in the current study reduced the number *B. cereus, B. subtilis, B. pumilus,* and *B. megaterium* spores is influenced by the concentration of the compounds and the duration of exposure.

Sporicidal activities of linoleic acid against spores of *Bacillus* sp.

The antispore activities of varying concentrations of linoleic acid (62.5, 125, 250, 500 and 1000 µg/mL) against *B. cereus, B. subtilis, B. pumilus,* and *B. megaterium* are shown in (Tables 3, 4, 5 and 6). A marked reduction in the number of spore/mL of > 2 Log10 units for all *Bacillus* was achieved when the spores were exposed to 500 µg/mL linoleic acid. Linoleic acid at a concentration of 500 µg/mL reduced the number of viable *B. cereus* and *B. megaterium* spores to 3.40 ± 0.21 and 3.90 ± 0.08, respectively, after one hour of exposure. It reduced the number of viable *B. subtilis* and *B. pumilus* spores to 3.58 ± 0.07, 3.60 ± 0.6, respectively, after a four-hour exposure to a concentration of 500 µg/mL.

Higher concentration of the compound resulted in smaller logarithm of remaining spores. Complete inhibition of the growth of all spores of *B. cereus*, *B. pumilus*, *B. megaterium*, and *B. subtilis* were achieved when treated with 1000 µg/mL linoleic acid for 1, 2, 3 and 4 hours, respectively. There is no significant difference in results when 62.5, 125 and 250 µg/mL of linoleic acid were used.

β-asarone and asaronaldehyde isolated from *P. cubeba* L. extract inactivated more than 3-Log10 (90.99%) of the spores of *Bacillus cereus* ATCC33019, *B. subtilis* ATCC6633, *B. pumilus* ATCC14884, and *B. megaterium* ATCC14581 after an incubation period of four hours at a concentration of 0.05%, and all the spores were killed at a concentration of 0.1% (Fatimah *et al*., 2019).

Table 3. Sporicidal activity of linoleic acid against spores of *B. cereus* ATCC33019

Concentration (µg/mL(%w/v))	1 (hr.)	2(hr.)	3(hr.)	4(hr.)
0.00	^{a} 5.70 ± 0.13	^{a} 5.70 ± 0.13	^{a} 5.70 ± 0.13	^{a} 5.70 ± 0.13
62.5	^{b} 4.58 ± 0.08	^{b} 4.53 ± 0.07	^{b} 4.45 ± 0.09	^{b} 4.32 ± 0.05

125	[b] 4.52 ± 0.09	[b] 4.41 ± 0.15	[b] 4.29 ± 0.09	[b] 4.18 ± 0.12
250	[b] 4.47 ± 0.15	[b] 4.35 ± 0.19	[b] 4.23 ± 0.17	[b] 4.05 ± 0.15
500	[c] 3.40 ± 0.21	[c] 3.20 ± 0.09	[c] 3.00 ± 0.90	[c] 0.00 ± 0.00
1000	[d] 0.00 ± 0.00	[d] 0.00 ± 0.00	[d] 0.00 ± 0.00	[c] 0.00± 0.00

Results are presented as mean ± standard deviation. Significant differences in mean (n=2× 3) within the same column is indicated with different letters ($p < 0.05$).

Friedman. (2014) stated that oregano oil, thyme oil, cinnamon oil, carvacrol, (S)-perillaldehyde, 3,4-dihydroxyphenethylamine and 3,4-dihydroxybenzoic acid (b-resorcylic acid) had excellent activities against *E. coli* NCTC1186, *S. aureus* ATCC12715 vegetative cells and *B. cereus* vegetative cells and spores. Earlier studies have reported the same findings where macelignan significantly inhibited growth the vegetative cells of *B. cereus* with a MIC of 4µg/mL and completely killed them at an MBC of 8 µg/mL. Macelignan has the ability to inactivate more than 3-Log10 of spore/mL of *B. cereus* spores (Rukayadi *et al*., 2009). These results support the findings of the current results.

Table 4. Sporicidal activity of linoleic acid against spores of *B. subtilis* ATCC3366

Concentration (µg/mL(%w/v))	1(hr.)	2(hr.)	3(hr.)	4(hr.)
0.00	[a] 6.39 ± 0.12	[a] 6.39 ± 0.12	[a] 6.39 ± 0.12	[a] 6.39 ± 0.12
62.5	[b] 4.66 ± 0.020	[b] 4.58 ± 0.02	[b] 4.49 ± 0.06	[b] 4.26 ± 0.10
125	[b] 4.61 ± 0.09	[b] 4.54 ± 0.07	[b] 4.50 ± 0.12	[b] 4.37 ± 0.05
250	[b] 4.58 ± 0.07	[b] 4.33 ± 0.04	[b] 4.14 ± 0.04	[c] 3.93 ± 0.05
500	[b] 4.30 ± 0.08	[c] 3.80 ± 0.11	[c] 3.60 ± 0.09	[c] 3.58 ± 0.07
1000	[c] 3.83 ± 0.32	[c] 3.40 ± 0.09	[c] 3.10 ± 0.08	[d] 0.00 ± 0.00

Results are presented as mean ± standard deviation. Significant differences in mean. (n=2×3) within the same column is indicated with different letters ($p < 0.05$).

Table 5. Sporicidal activity of linoleic acid against spores of *B. pumilus* ATCC14884

Concentration (µg/mL(%w/v))	1(hr.)	2(hr.)	3(hr.)	4(hr.)
0.00	[a] 5.48 ± 0.04	[a] 5.48 ± 0.04	[a] 5.48 ± 0.04	[a] 5.48 ± 0.04
62.5	[b] 4.77 ± 0.11	[b] 4.69 ± 0.06	[b] 4.59± 0.07	[b] 4.47 ± 0.08
125	[b] 4.72 ± 0.06	[b] 4.63 ± 0.05	[b] 4.55 ± 0.05	[b] 4.38 ± 0.04
250	[b] 4.63 ± 0.05	[b] 4.52 ± 0.04	[b] 4.40 ± 0.04	[b] 4.28 ± 0.10
500	[b] 4.00 ± 0.80	[c] 3.90 ± 0.09	[c] 3.75 ± 0.50	[c] 3.60 ± 0.60
1000	[c] 3.30 ± 0.80	[c] 3.00 ± 0.00	[d] 0.00 ± 0.00	[d] 0.00 ± 0.00

Results are presented as mean ± standard deviation. Significant differences in mean (n=2×3) within the same column is indicated with different letters ($p < 0.05$).

Table 6. Sporicidal activity by linoleic acid against spores of *B. megaterium* ATCC14581

Concentration (µg/mL(%w/v))	1(hr.)	2(hr.)	3(hr.)	4(hr.)
0.00	[a] 5.31 ± 0.04	[a] 5.31 ± 0.04	[a] 5.31 ± 0.04	[a] 5.31 ± 0.04
62.5	[b] 4.50 ± 0.17	[b] 4.41 ± 0.18	[b] 4.34 ± 0.18	[b] 4.24 ± 0.21
125	[b] 4.43 ± 0.10	[b] 4.33 ± 0.15	[b] 4.23 ± 0.22	[b] 4.15 ± 0.20
250	[b] 4.39 ± 0.09	[b] 4.24 ± 0.18	[b] 4.10 ± 0.18	[c] 3.94 ± 0.28
500	[c] 3.90 ± 0.08	[c] 3.80 ± 0.09	[c] 3.58 ± 0.12	[c] 3.36 ± 0.14
1000	[c] 3.20 ± 0.90	[c] 3.00 ± 0.00	[d] 0.00 ± 0.00	[d] 0.00 ± 0.00

Results are presented as mean ± standard deviation. Significant differences in mean (n=2×3) within the same column is indicated with different letters ($p < 0.05$).

Conclusion

In summary, two compounds were discovered; one was found in the n-hexane fraction and the other was found in the DCM soluble fraction. These compounds were isolated using a variety of sequential chromatographic procedures, and ^{1}H-NMR, ^{13}C-NMR,

2D NMR, and MS data were used to determine their structures. Linoleic acid **1** and cubebin mixture **2** are these substances. The obtained compounds had their antibacterial and antispore properties evaluated. **Linoleic acid**, the first compound, showed effective antibacterial and antispore activity against *B. cereus* ATTC33019, *B. subtilis* ATCC6633, *B. pumilus* ATCC14884, and *B. megaterium* ATCC14581. However, second compound **(cubebin mixture**) did not show any activity against *Bacillus* strains. The activity of the compound **1 is** possible for antimicrobial properties. They might be used as food additives and preservatives to help extend the shelf life of foods.

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