Antibacterial composition of bioautographic fractions, characteristics, and stability of *Carica papaya* seed extract

[1,2]*Abdullah Sani, M. S., [3,4]Bakar, J., [3,4]Abdul Rahman, R. and [5,6]Abas, F.

[1]*International Institute for Halal Research and Training, Level 3, KICT Building, International Islamic University Malaysia, Jalan Gombak, 53100 Kuala Lumpur, Malaysia*
[2]*Konsortium Institut Halal IPT Malaysia, Ministry of Higher Education, Block E8, Complex E, Federal Government Administrative Centre, 62604 Putrajaya, Malaysia*
[3]*Laboratory of Halal Science Research, Halal Product Research Institute, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*
[4]*Department of Food Technology, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*
[5]*Laboratory of Natural Medicines and Product Research, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*
[6]*Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*

Abstract

The present work aimed to evaluate the potential of *Carica papaya* seed extract (CPSE) as an antibacterial agent against *Salmonella* Enteritidis, *Bacillus cereus, Vibrio vulnificus*, and *Proteus mirabilis*. The bioautography of the CPSE on thin-layer chromatography (TLC) plates was performed, followed by fractionation of the CPSE by column chromatography using hexane:ethyl acetate (1:1) eluent. Minimum Inhibitory Concentration (MIC), toxicity, composition, and stability of the crude, fractions, and sub-fractions of the CPSE were evaluated. The bioautographic fractions of the CPSE at MIC of 5.63 mg/mL had shown that hexane:ethyl acetate (1:1) fraction and its sub-fraction 3C (Rf = 0.94 ± 0.03) demonstrated the equivalent MIC value (5.63 mg/mL) with the crude CPSE. However, the hexane:ethyl acetate (1:1) fraction and sub-fraction 3C had higher toxicity (LC_{50} = 1.797 ± 0.305 and 0.332 ± 0.059 mg/mL, respectively) than the crude CPSE (LC_{50} = 5.505 ± 0.718 mg/mL). Thus, only the crude CPSE was subjected to stability study. The dominant cis-vaccenic acid in sub-fraction 3C demonstrated the lowest MIC against *B. cereus* (1.41 mg/mL), *P. mirabilis* (1.41 mg/mL), and *S.* Enteritidis (0.70 mg/mL) in its pure form. Hence, these results signified the potency of the *cis*-vaccenic acid as an antibacterial compound from the CPSE. The stability study of the crude CPSE solution showed that at MIC of 5.63 mg/mL, the crude CPSE solution acted as a potent antibacterial agent in acidic condition (pH 4), water activity (A_w) < 0.950, and temperature < 40°C.

Introduction

Papaya (*Carica papaya*) is a highly commercialised tropical fruit. Ripe *C. papaya* is mainly consumed with dessert, while papain from the plant's latex is commercialised as meat tenderiser, and is also being used as an enzyme in several enzymatic extraction studies (Song *et al.,* 2020). In 2017, of the 28 million metric tonnes of papaya production worldwide, 5 million metric tonnes of the seeds were discarded (FAOSTAT, 2019). In Central Asia and Middle East countries, the seeds are popularly used to marinate meat, as substitute for black pepper, and added in salad dressings due to its spicy taste (Sani *et al.,* 2020). Apart from its use in the culinary field, antibacterial properties of the *C. papaya* seed extract (CPSE) against *S.* Enteritidis, *V. vulnificus, P. mirabilis*, and *B. cereus* has also been demonstrated (Sani, 2018). Nevertheless, it is unlikely to determine which compounds in the CPSE are responsible for inhibiting the growth of pathogenic bacteria (Sani *et al.,* 2017b). Since the yield and consumption of *C. papaya* seeds are high, it is worth investigating its compositions and potent antibacterial components that potentially inhibit the growth of pathogenic bacteria.

The identification of chemical component in the CPSE is often performed using high-performance liquid chromatography with diode array detector

(HPLC-DAD). This technique has identified phenolic compounds such as campesterol, stigmasterol, and β-sitosterol in CPSE oil (Sani *et al.,* 2020). This analytical technique requires pure standards for retention-time confirmation, which are very costly (Kupska *et al.,* 2016). Due to the sensitivity towards volatile detection, gas chromatography-mass spectrometry (GC/MS) analysis of CPSE only detected 25 compounds (Sani *et al.,* 2017b), mostly representing the non-polar compounds. Furthermore, the compounds that were detected only indicated the overall composition of the CPSE and not the actual antibacterial compounds in the extract. Bioautography of thin-layer chromatography (TLC) (Móricz *et al.,* 2017) and the subsequent column-chromatographic fractionation (Perez *et al.,* 2020) of the plant extract will purify the extract and facilitate the identification of potent antibacterial compounds using temperature-programmed GC/MS. Trimethylsilyl (TMS) derivatising agent was used in confirming the plant metabolites in order to enhance the detection of polar and non-polar compounds that render the inhibitory effects (Sani *et al.,* 2020).

An active antibacterial agent should have low toxicity that imposes potent concentration against pathogens but minimally affects the host while possesses stable chemical and physical properties (Rodríguez-Moyá and Gonzalez, 2015). Thus, crude, partially purified, and purified extracts should be subjected to toxicity test in order to meet this requirement prior to the application, since fractionation and purification may affect the production cost (Pinazo *et al.,* 2016) besides the toxicity towards consumers. However, due to animal rights issue, there are efforts to substitute rats, mice, or rabbits with wax moth larva (Martins *et al.,* 2019) and *Artemia salina* for toxicity tests (Swarnkumar and Osborne, 2020). The present work used *A. salina* for toxicity test because it has a strong correlation coefficient of *in vitro* toxicity against mice. The extracted CPSE may contain residual methanol, although it had been removed before further analysis. Since the presence of methanol may cause toxicity, its residual content should be determined. In the present work, we proposed the utilisation of GC/MS equipped with a headspace unit to measure the residual methanol content.

To date, several studies reported the antibacterial activities of the CPSE. The antibacterial potency of the ethanolic extract of the seeds has been reported against *S.* Enteritidis, *B. subtilis, E. coli,* and *P. aeruginosa* (Sowhini *et al.,* 2020). Another study demonstrated a strong antibacterial capacity of CPSE against *S.* Enteritidis, *V. vulnificus, P. mirabilis,* and *B. cereus* (Sani *et al.,* 2017b), but did not investigate the stability of the antibacterial compounds. The stability of the compounds is a very crucial aspect for any investigation prior to the application of the CPSE in foods, cosmetics, and pharmaceuticals. Therefore, the present work investigated the stability of CPSE as an antibacterial agent against pH, A_w, and temperature. To the best of our knowledge, this is the first study that investigated the antibacterial activity, toxicity, residual methanol content, and stability of the CPSE via bioautographic assay method. Hence, the findings from the present work are expected to bring new insight on CPSE, and propose suitable methods used to study the antibacterial activity of other plant extracts.

Materials and methods

Plant materials

Carica papaya cv. Sekaki fruits at sixth maturity stage were purchased from the D'Lonek Sdn. Bhd. Organic Farm, Rembau, Negeri Sembilan, Malaysia. The *C. papaya* plant, flower, and fruit obtained from this farm were deposited to the Herbarium of Institute of Bioscience, Universiti Putra Malaysia for the issuance of a plant voucher (SK 2368/14). The seeds of *C. papaya* were removed from the fruit, washed thoroughly with distilled water, oven-dried at 40°C for 3 d, stored in airtight amber bottles, and frozen at -20°C until further analyses (Sani *et al.,* 2017a).

Extraction of phytochemicals

The first step involved grinding the dried seeds in 240 W electrical blender (Panasonic MX-337, Malaysia), and sieving them to obtain 125 μm particle size prior to extraction. Then, 500 mL of methanol was added to 50 g of dried ground seed, and was weighed into a conical flask, which was equivalent to 10:1 methanol-to-solid ratio. Upon mixing, extraction was carried out at 30°C for 8 h in a shaker at 100 rpm, followed by filtration through Whatman No. 1 filter paper (GE Healthcare, UK). Next, the filtrate was transferred into pre-weighed flat bottom flasks, and was concentrated using a rotary vacuum evaporator (Eyela N-1001, Japan) at 40°C. Finally, the concentrated CPSE was stored at 4°C until further use (Sani *et al.,* 2017a).

Fractionation and bioautography on TLC plates

The CPSE was fractionated using TLC over 10 × 20 cm silica gel plates with pre-coated fluorescent indicator UV_{254} (Macherey-Nagel, Germany). A list of solvents including 5% ethyl

acetate in petroleum ether, hexane:ethyl acetate (1:3, 1:1, and 3:1), hexane:diethyl ether (1:1), diethyl ether, diethyl ether:methanol (1:1), Milli-Q water, and 0.1% formic acid in acetonitrile were selected as the eluents based on the polarity increment (Abd, 2020). Upon the establishment of the fractionation, the ultraviolet (UV)-active components on the developed TLC plates were identified under UV light, and the retention factor (R_f) of each component was calculated.

The plates were then dried at 30°C for 3 h before being evaluated bioautographically against *S. Enteritidis* (ATCC 13076), *V. vulnificus* (ATCC 27562), *P. mirabilis* (ATCC 12453), and *B. cereus* (ATCC 10875). The suspension of the pathogen containing 10^6 CFU/mL was sprayed onto the developed TLC plates, and incubated at 37°C for 24 h. The plates were then sprayed with a 2 mg/mL solution of iodonitrotetrazolium chloride (INT), and incubated for another 6 h. Post-incubation, inhibition of bacterial growth was indicated by the formation of white spots against a pink background on the TLC plates. The fractionation and bioautography on the TLC plates were performed in triplicates.

Fractionation of Carica papaya seed extract by column chromatography

Approximately, 1 g of crude CPSE was fractionated using activated silica gel 60 (40 g, 0.040 - 0.063 mm; Merck) packed column. The first 30 fractions were collected using the 25 mL collector, whereas the next 25 fractions were collected with the 40 mL collector. Each fraction was subjected to TLC run, and UV-active fractions on the developed TLC plates were identified under UV light, whereby fractions with equal R_f were pooled. Since the hexane:ethyl acetate (1:1) eluent was chosen as the best eluent, three sub-fractions were eluted using hexane:ethyl acetate (1:1); 400 mL (sub-fraction 3A, R_f = 0.44), 1300 mL (sub-fraction 3B, R_f = 0.74), and 50 mL (sub-fraction 3C, R_f = 0.94). Sub-fractions 3A, 3B, and 3C represented the detected component of 3A, 3B, and 3C on the fractionation and bioautography on the TLC plates. These sub-fractions were eluted at 1 mL/min, evaporated at 40°C using a rotary evaporator, weighed, and their compositions were identified using GC/MS. The fractionation method was repeated ten times to collect a sufficient amount of hexane:ethyl acetate (1:1) fraction and sub-fraction 3C for MIC assay, toxicity assay, and GC/MS analysis.

Minimum inhibitory concentration assay

A total of 10 g of crude CPSE was fractioned using a packed column to obtain 0.510 g sub-fraction 3A, 6.023 g sub-fraction 3B, and 0.335 g sub-fraction 3C. However, only sub-fraction 3C along with the crude CPSE and hexane:ethyl acetate (1:1) fraction were subjected to MIC assay since the white spots which indicated antibacterial activity were present in these samples.

A two-fold serial microdilution method of 96 multi-well microtiter plate was used to determine MIC (Sowhini *et al.*, 2020). A 100 μL aliquot of Tryptone Soy Broth (TSB), supplemented with Tween 80 at a concentration of 1% (v/v) was pipetted into each well. A volume of 100 μL of 0.5×10^5 μg/mL crude extracts, hexane:ethyl acetate (1:1) fraction, and sub-fraction 3C in dimethyl sulfoxide (DMSO) was added into individual first well. A serial dilution was performed, whereby a volume of 100 μL of the mixture from the first well was pipetted into the next well of each microtiter row repetitively until the eleventh well. A volume of 100 μL from the eleventh well was discarded to ensure the total volume of all wells was 100 μL.

An aliquot of 90 μL from each well was mixed with 10 μL of 10^6 CFU/mL bacterial suspensions to obtain the final extract concentration range from 22.50 to 0.02 mg/mL. For a positive control growth, 90 μL of TSB was mixed with 10 μL of 10^6 CFU/mL bacterial suspensions in the twelfth well. The optical density was measured at 600 nm in an Ultra Microplate Reader (Biotek Instruments, Winooski, USA) at the pre-incubation period (T_0). The 96 multi-well microtiter plate was incubated at 37°C for 24 h in an incubator (Heidolph, Germany), and then, the optical density was measured at the post-incubation period (T_{24}). The MIC is defined as the lowest concentration of an antibacterial agent demonstrating a complete growth inhibition of the tested bacterial strains. The MIC value is estimated based on the differences in absorbance of T_{24} and T_0, *i.e.,* $T_{24} - T_0$, which could be equal to zero or negative values.

To compare the antibacterial activity of the CPSE with pure compounds, the MIC assay was executed for a standard solution of oleic acid, linolenic acid, hexadecanoic acid, *cis*-vaccenic acid, 4-hydroxy-benzoic acid, *o*-tolunitrile, 2-phenylace-tonitrile, benzyl isothiocyanate, stigmasterol, β-sitosterol, and campesterol. The MIC assay was also executed for tetracycline chloride as a positive control.

Toxicity assay

The crude, hexane:ethyl acetate (1:1) fraction, and sub-fraction 3C of the CPSE were mixed with 1% (v/v) Tween 80, and dissolved in DMSO. The mixtures were then diluted with 0.021 g/mL artificial seawater (Sigma Chemicals Co., UK) in vials to produce a series of concentrations ranging between 0.001 to 10 mg/mL. As for the positive control, vincristine sulphate solutions were prepared with concentrations between 0.001 to 1 mg/mL, while 0.021 g/mL of artificial seawater served as the negative control. Total volume for the final mixture was 5 mL. These mixtures were subjected to toxicity study against *A. salina*.

The *A. salina* eggs (Ocean Star International, Inc., USA) were hatched in artificial seawater, which was prepared by dissolving 25 g of artificial sea salt into 1.2 L of distilled water in a 2 L beaker. The *A. salina* larvae were obtained at 25 - 29°C after 24 h incubation. Approximately, 15 mL of 4.86% yeast solution was added into the 2 L beaker in order to feed the larvae. After 24 h, the larvae were attracted to the surface of the artificial seawater using a light source. A total of 10 larvae were collected using a pipette, and added into each sample and positive control vials. The vials were incubated for another 24 h. Post-incubation, the vials were examined, and the number of dead larvae was counted. The mortality percentage at each sample was determined using Eq. 1:

Mortality percentage (%) = [(sample death – control death) / total *A. salina* in each vial] × 100

(Eq. 1)

The mean percentage of mortality was plotted against the logarithm of concentrations. Lethal concentration (LC_{50}) was derived from the best fit line obtained through linear regression analysis (Sani, 2018).

Gas chromatography-mass spectrometry (GC/MS) analysis of Carica papaya seed extract composition and residual methanol content

Approximately, 0.01 g of dried CPSE was derivatised with 0.5 mL of *N,O*-bis-trimethylsilyl-trifluoroacetamide (BSTFA):trimethylchlorosilane (TMCS) (99:1) (Macherey-Nagel, Germany), to which 0.5 mL anhydrous pyridine was added to produce 0.01 g/mL CPSE concentration. The mixture was then heated at 60°C for 30 min before being injected into model 7890A gas chromatography (GC) system equipped with a model 5975 of mass detector (MS) (Agilent Technologies, USA).

The GC/MS analysis was performed using an HP-5MS column (30 m × 0.25 µm, film thickness of 0.25 µm) via linear temperature programming (LTP). The oven temperature was programmed at 70°C/min, and was linearly raised to 300°C at 4°C/min. The carrier gas used was helium at a flow rate of 1.2 mL/min (Sani *et al.*, 2020).

To measure the content of residual methanol, a range of working standards of methanol from 500 - 50,000 ppm was prepared in headspace bottles. The working standards were spiked with 10,000 ppm isopropanol as an internal standard, and the headspace bottle was tightly capped with a clamp. Approximately 0.1 g of CPSE was re-dissolved with 1 mL methanol in a pre-weighed bottle, and concentrated at 40°C under reduced pressure until a constant weight was achieved. The dried CPSE was also spiked with 10,000 ppm isopropanol prior to GC/MS analysis. The dried CPSE in the headspace bottle was heated at 70°C for 10 min and injected in split mode into a 90°C injector with a 1 mL/min constant flow rate of helium gas. The methanol in the CPSE was separated by a DB-WAX polar fused silica capillary column (30 m × 0.25 mm, 0.85 µm film thickness). The temperature programming of the oven was initiated at 35°C for 2 min, followed by heating to 90°C at 25°C/min, and were held for 5 min.

As for MS detection of the CPSE composition and residual methanol, the electron ionisation mode with ionisation energy of 70 eV was used, with a mass range of *m/z* 20 - 700 units. The MS transfer line and MS quadrupole temperature were set at 230 and 150°C, respectively. The CPSE compounds including their trimethylsilyl (TMS) derivatives were identified by their retention times and 90% similarity match against mass fragmentation patterns of standards according to the National Institute of Standard Mass Spectral 11 library (NIST11). For the content of residual methanol, it was detected using selected ion monitoring (SIM) mode and confirmed using NIST11.

Stability study of Carica papaya seed extract as affected by pH, water activity, and temperature

To investigate the effect of pH towards the crude CPSE, 29.6 mg of nitrogen-blow down extract was mixed with sterilised TSB, 1% Tween 80, and pre-determined volume of 1 M hydrochloric acid (HCl) solution to produce CPSE solutions at pH 4, 5, 6, and 7, with a final volume of 5 mL. These pH values were selected because plant extract has exhibited potent antibacterial activity at low pH (Saliani *et al.*, 2015).

To investigate the effect of A_w towards the crude CPSE, 29.6 mg of nitrogen-blow down extract was mixed with sterilised TSB, 1% Tween 80, and 20% sodium chloride (NaCl) stock solution to prepare a 5 mL final volume of CPSE solutions with Aw of 0.999 (0% NaCl), 0.997 (0.5% NaCl), 0.980 (3.5% NaCl), 0.950 (9.0% NaCl), and 0.909 (15% NaCl).

To investigate the effect of temperature towards the crude CPSE, 29.5 mg of nitrogen-blow down extract was heated at 60, 80, 100, 150, and 200°C for 15 min before mixing with 1% Tween 80 and TSB to a 5 mL final volume of CPSE solutions. The final CPSE solutions with 5.63 mg/mL MIC (Sani *et al.*, 2017b) as affected by pH, A_w, and temperature were subjected to the test of percentage growth inhibitions of the tested pathogens.

A volume of 10 μL of TSB containing 10^6 CFU/mL of the tested pathogens was mixed with 190 μL of individual crude CPSE solution in a 96 multi-well microtiter plate. A volume of 200 μL TSB inoculated with the respective pathogens was prepared as positive control, while 200 μL mixture of crude extract, Tween 80, and TSB without pathogens was prepared as a negative control. The optical density was measured at 600 nm in a microplate reader at the pre-incubation period (T_0). The 96 multi-well microtiter plate was incubated at 37°C for 24 h, and shaken at 210 rpm to prevent adherence and clumping. The optical density was measured at 600 nm in the microplate reader at the post-incubation period (T_{24}). The effect of pH, A_w, and temperature on the crude CPSE was evaluated via the determination of the percentage of growth inhibition using Eq. 2:

Percentage of growth inhibition (PI) (%) = [1 – (OD test well / OD of positive control well)] × 100

(Eq. 2)

Statistical analysis

Data were expressed as mean ± standard deviation of triplicate R_f, LC_{50}, and residual methanol. One-way analysis of variance (ANOVA) with Tukey's test was conducted using XLSTAT-Pro (2017) statistical software (Addinsoft, Paris, France) to determine the significant difference between the means at 95% confidence level ($p < 0.05$) for the bioautography on TLC plates, toxicity assay, and content of residual methanol.

Results and discussion

Fractionation and bioautography on thin-layer chromatography plates

The eluents had developed components on the TLC plates over a wide range of R_f values. Based on Table 1, the R_f values of the components ranged from 0.17 to 0.97. Of the nine different eluents used to separate the components of CPSE (Table 1), hexane:diethyl ether (1:1) and diethyl ether fractionated the highest number of components (four components each), followed by hexane:ethyl acetate (1:1) and hexane:ethyl acetate (3:1) (three components each). The 5% ethyl acetate in petroleum ether and diethyl ether:methanol (1:1) fractionated two components, respectively, and hexane:ethyl acetate (1:3) fractionated one component. However, the water and 0.1% formic acid in acetonitrile did not fractionate any component from the CPSE. Previous studies reported that the 5% ethyl acetate in petroleum ether and hexane:ethyl acetate fractionated component containing benzyl isothiocyanate and fatty acids, respectively. Other eluents such as hexane:diethyl ether (1:1) and diethyl ether have successfully fractionated sterols, while 0.1% formic acid in acetonitrile was a suitable eluent to fractionate sugars and organic acids. On the other hand, diethyl ether was reported to fractionate sterols and terpenes, while methanol successfully fractionated flavonoids (Abd, 2020). Although different eluents fractionated components of specific compounds, the present work focussed on the fractionated components that exhibited antibacterial activities.

The bioautographic method was employed on the developed TLC plates to identify components with antibacterial white spots against the tested pathogens. From all the developed TLC plates, only component 1B from eluent 5% ethyl acetate in petroleum ether, component 2A from hexane:ethyl acetate (1:3), and component 3C from hexane:ethyl acetate (1:1) exhibited antibacterial white spot against *S.* Enteritidis, *B. cereus, V. vulnificus,* and *P. mirabilis*. These antibacterial components had Rf values ranging between 0.88 - 0.94. Of these components, component 3C had the most intense antibacterial white spot as compared to component 1B and 2A. Although the R_f of component 3C ($R_f = 0.94 \pm 0.03$) was insignificantly different ($p < 0.05$) with the R_f of component 1B ($R_f = 0.90 \pm 0.01$), its R_f value was significantly different ($p < 0.05$) with the R_f value of component 2A ($R_f = 0.88 \pm 0.04$). Hence, only hexane:ethyl acetate (1:1) eluent was employed to fractionate component 3C of CPSE, and the component 3C was subjected to further analysis. The components fractionated by hexane:ethyl acetate (3:1), hexane:diethyl ether (1:1), diethyl

Table 1. Retention factor of components on thin-layer chromatography plates.

Eluent	No. of detected component[1]	Component detected	R_f^2	Presence of antibacterial white spot	No. of component with antibacterial activity	Sensitive bacteria
5% ethyl acetate in petroleum ether	2	Component 1A	0.55 ± 0.01^d	No	1	na
		Component 1B	0.90 ± 0.01^{jk}	Yes		S. Enteritidis, B. cereus, V. vulnificus, and P. mirabilis
Hexane:ethyl acetate (1:3)	1	Component 2A	0.88 ± 0.04^j	Yes	1	B. cereus and P. mirabilis
Hexane:ethyl acetate (1:1)	3	Component 3A	0.44 ± 0.02^c	No	1	na
		Component 3B	0.74 ± 0.01^g	No		na
		Component 3C	0.94 ± 0.03^k	Yes		S. Enteritidis, B. cereus, V. vulnificus, and P. mirabilis
Hexane:ethyl acetate (3:1)	3	Component 4A	0.31 ± 0.00^b	No	0	na
		Component 4B	0.78 ± 0.01^h	No		na
		Component 4C	0.95 ± 0.01^k	No		na
Hexane:diethyl ether (1:1)	4	Component 5A	0.17 ± 0.01^a	No	0	na
		Component 5B	0.33 ± 0.01^b	No		na
		Component 5C	0.60 ± 0.01^e	No		na
		Component 5D	0.91 ± 0.01^k	No		na
Diethyl ether	4	Component 6A	0.65 ± 0.02^f	No	0	na
		Component 6B	0.73 ± 0.02^g	No		na
		Component 6C	0.79 ± 0.02^h	No		na
		Component 6D	0.92 ± 0.04^k	No		na
Diethyl ether:methanol (1:1)	2	Component 7A	0.67 ± 0.03^f	No	0	na
		Component 7B	0.85 ± 0.02^i	No		na
Water	0	na	na	na	0	na
0.1% formic acid in acetonitrile	0	na	na	na	0	na

[1]Identified compound produced purple spot under UV-VIS observation; and [2]Means of Rf with different lowercase superscripts are significantly different ($p < 0.05$). Rf = retention factor, and na = not available.

ether, and diethyl ether:methanol (1:1) eluents did not exhibit antibacterial white spot. These findings also indicated that the non-polar eluents were more suitable for fractionating antibacterial components of the CPSE. Also, they suggested that the antibacterial components were of the non-polar group.

Fractionation of Carica papaya seed extract by column chromatography

A total of three sub-fractions of CPSE using hexane:ethyl acetate (1:1) were collected according to their R_f on TLC. The weight and recovery percentage of the sub-fraction 3A were 0.071 g and 7.1%; sub-fraction 3B were 0.603 g and 60.3%; and sub-fraction 3C were 0.033 g and 3.3%, respectively. The total weight and percentage recovery of hexane:ethyl acetate (1:1) fraction were 0.7672 g and 70.2%, respectively. The yield loss was probably due to the compounds binding to active sites on the surface-active suites or decomposed by the silica surface; hence, the compounds could not be eluted from the column packing.

Minimum inhibitory concentration and toxicity assays

Table 2 presents the MIC of the crude CPSE, hexane:ethyl acetate (1:1) fraction, and sub-fraction 3C, together with the pure standard solution of oleic acid, linolenic acid, *o*-tolunitrile, stigmasterol, β-sitosterol, 2-phenyl acetonitrile, benzyl isothiocyanate, hexadecanoic acid, *cis*-vaccenic acid, campesterol, and 4-hydroxy-benzoic acid against the tested pathogens. These pure standards were the identified compounds with the highest relative percentage area from the GC/MS analysis of Sani *et al.* (2017a). From Table 2, pure standard solutions exhibited lower MIC values than the MIC values of the crude CPSE (MIC = 5.63 mg/mL). This result indicated that the pure standard solutions possess stronger inhibitory capacities against the tested pathogens than the crude CPSE. All tested pathogens were sensitive to crude CPSE at 5.63 mg/mL MIC, while only *S.* Enteritidis was resistant to hexane:ethyl acetate (1:1) fraction and sub-fraction 3C at 11.25 mg/mL MIC.

Of the 11 pure standard solutions, *B. cereus* was most sensitive to linoleic, hexadecenoic, *cis*-vaccenic acid, and stigmasterol at 1.41 mg/mL MIC, while *V. vulnificus* was most sensitive to oleic acid at 0.04 mg/mL MIC. Also, *P. mirabilis* was most sensitive to *cis*-vaccenic acid and 4-hydroxy-benzoic acid at 1.41 mg/mL MIC, while *S.* Enteritidis was most sensitive to *cis*-vaccenic acid at 0.70 mg/mL MIC. From these findings,

cis-vaccenic acid had exhibited the highest antibacterial potency against all tested pathogens at 0.70 and 1.41 mg/mL MIC, except for against *V. vulnificus*.

Table 2 lists the LC_{50} for crude extract, hexane:ethyl acetate (1:1) fraction, and sub-fraction 3C of CPSE. The LC_{50} of these samples were determined using the linear regression analysis, as depicted in Figure 1. The coefficient determination (R^2) of the crude CPSE, hexane:ethyl acetate (1:1) fraction, sub-fraction 3C, and vincristine sulphate were 0.8500, 0.9008, 0.9282, and 0.8885, respectively. Among the samples, the sub-fraction 3C demonstrated the lowest LC_{50} (LC_{50} = 0.332 ± 0.059 mg/mL) with significant difference ($p < 0.05$) as compared to the other two samples. The crude CPSE (LC_{50} = 5.505 ± 0.718 mg/mL) and hexane:ethyl acetate (1:1) fraction (LC_{50} = 1.797 ± 0.305 mg/mL) were not toxic against the *A. salina* (Table 2) since the $LC_{50} > 1$ mg/mL (Sani, 2018). Even though crude extract was generally less effective than the purified compound to inhibit pathogen (Yin *et al.,* 2019), the present work had chosen the crude CPSE over the hexane:ethyl acetate (1:1) fraction for further study due to the lower toxicity and MIC than the latter. Also, the production of the hexane:ethyl acetate (1:1) fraction was costly, and the usage of hexane has been associated with toxicity effect to human cells (Pinazo *et al.,* 2016). Besides, sub-fraction 3C was excluded for further study due to higher toxicity (LC_{50} = 0.332 ± 0.059 mg/mL) and higher MIC (11.25 mg/mL) against *S.* Enteritidis.

Composition of sub-fraction 3A, 3B, and 3C of Carica papaya seed extract

Five, 10, and 29 compounds (Table 3) were identified with more than 90% similarity with the standard mass spectra in the NIST 11 library, representing 2.78, 49.03, and 26.55% of the percentage area in the sub-fraction 3A, 3B, and 3C, respectively. The sub-fraction 3A was characterised with the presence of alkane group, namely eicosane (0.88%), having the highest percentage area, followed by heneicosane (0.65%) and hexacosane (0.50%). Fatty acids and fatty acid methyl esters (FAME) groups dominated the sub-fraction 3B composition which consisted of 9-octadecenoic acid (E)-TMS (38.46%), oleic acid-TMS (4.82%), hexadecanoic acid-TMS (2.27%), 9-octadecenoic acid (Z) 2,3-dihydroxypropyl ester (0.59%), 6-octadecenoic acid, (Z)-TMS (0.31%), oleic acid, eicosyl ester (0.26%), and 1,2-benzenedicarboxylic acid butyl 2-ethylhexyl ester (0.01%). The

Table 2. Minimum inhibitory concentration (MIC) and toxicity of crude *Carica papaya* seed extract, hexane:ethyl acetate (1:1) fraction, sub-fraction 3C, and standard solutions.

Solution	Minimum inhibitory concentration (MIC) (mg/mL)				Toxicity (LC$_{50}$) of *A. salina* assay (mg/mL)
	B. cereus	*V. vulnificus*	*P. mirabilis*	*S. Enteritidis*	
C. papaya seed extract					
Crude extract	5.63	5.63	5.63	5.63	5.505 ± 0.718[c]
Hexane:ethyl acetate (1:1) fraction	5.63	5.63	5.63	11.25	1.797 ± 0.305[b]
Sub-fraction C	5.63	5.63	5.63	11.25	0.332 ± 0.059[a]
Fatty acid standard solution					
Oleic acid	5.63	0.04	5.63	5.63	nt
Linolenic acid	1.41	5.63	22.50	5.63	nt
Hexadecanoic acid	1.41	0.35	11.25	5.63	nt
cis-Vaccenic acid	1.41	2.81	1.41	0.70	nt
Organic acid standard solution					
4-Hydoxy-benzoic acid	2.81	0.35	1.41	11.25	nt
Nitrile and heterocyclic nitrogen standard solution					
o-tolunitrile	5.63	11.25	11.25	5.63	nt
2-Phenylacetonitrile	2.81	11.25	5.63	5.63	nt
Benzyl isothiocyanate	11.25	5.63	5.63	5.63	nt
Sterol standard solution					
Stigmasterol	1.41	1.41	>22.50	>22.50	nt
β-Sitosterol	11.25	0.35	5.63	>22.50	nt
Campesterol[1]	>0.05	>0.05	>0.05	>0.05	nt
Positive control					
Tetracycline chloride for MIC assay	<0.02	<0.02	<0.02	<0.02	nt
Vincristine sulphate for *A. salina* assay	nt	nt	nt	nt	0.067 ± 0.000[a]

[1]Concentration of the stock standard solution was 0.1 mg/mL. nt = not tested.

Table 3. Composition of sub-fractions 3A, 3B, and 3C of crude *Carica papaya* seed extract.

No.	Compound[1,2]	Sub-fraction 3A		Sub-fraction 3B		Sub-fraction 3C	
		RT (min)	Area (%)[3]	RT (min)	Area (%)[3]	RT (min)	Area (%)[3]
1.	2-Phenylacetonitrile	nd	nd	7.344	1.33 (2.72)	nd	nd
2.	Hexadecane	nd	nd	nd	nd	18.31	0.47 (1.78)
3.	1-Methylethyl dodecanoic acid ester	nd	nd	nd	nd	19.174	1.30 (4.91)
4.	Tetratetracontane	nd	nd	nd	nd	19.762	0.45 (1.69)
5.	Heptadecane	nd	nd	nd	nd	21.65	1.30 (4.90)
6.	2,6,10,14-Tetramethyl pentadecane	nd	nd	nd	nd	21.879	1.43 (5.37)
7.	2,6-Diisopropylnaphthalene	nd	nd	nd	nd	22.59	0.86 (3.25)
8.	Cyclopentadecane	nd	nd	nd	nd	23.461	0.86 (3.25)
9.	Octadecane	nd	nd	nd	nd	24.309	1.55 (5.84)
10.	Tetracosyl heptafluorobutyrate	nd	nd	nd	nd	24.592	0.56 (2.11)
11.	1,4-Dibromo tetrapentacontane	nd	nd	nd	nd	24.661	0.90 (3.38)
12.	Hexyl undecyl phthalic acid ester	nd	nd	nd	nd	25.387	1.65 (6.23)
13.	Triacontyl pentafluoropropionate	nd	nd	nd	nd	25.471	0.78 (2.95)
14.	Nonadecane	nd	nd	nd	nd	25.715	1.37 (5.16)
15.	Octacosyl trifluoroacetate	nd	nd	nd	nd	25.761	0.51 (1.91)
16.	Tetratriacontyl trifluoroacetate	nd	nd	nd	nd	25.952	0.89 (3.36)
17.	Hexadecanoic acid methyl ester	nd	nd	nd	nd	26.044	1.58 (5.95)
18.	Dotriacontyl heptafluorobutyrate	nd	nd	nd	nd	26.105	0.40 (1.52)
19.	Octatriacontyl pentafluoropropionate	nd	nd	nd	nd	26.166	0.77 (2.90)
20.	Tetratriacontyl pentafluoropropionate	nd	nd	nd	nd	26.342	0.68 (2.58)

No.	Compound	Rt	(%)	Rt	(%)	Rt	(%)
21.	2-Ethylhexylbutyl, 1,2-benzenedicarboxylic acid ester	nd	nd	26.456	0.01 (0.03)	nd	nd
22.	Dotriacontyl pentafluoropropionate	nd	nd	nd	nd	26.533	0.60 (2.27)
23.	Hexadecanoic acid-TMS	nd	nd	26.632	2.27 (4.63)	nd	nd
24.	Hexacosyl heptafluorobutyrate	nd	nd	nd	nd	26.823	1.36 (5.13)
25.	Octacosyl heptafluorobutyrate	nd	nd	nd	nd	27.29	0.33 (1.23)
26.	cis-Vaccenic acid-TMS	nd	nd	27.633	0.62 (1.26)	27.633	3.30 (12.41)
27.	Methyl stearate	nd	nd	nd	nd	27.824	1.09 (4.12)
28.	17-Pentatriacontene	nd	nd	nd	nd	28.176	0.36 (1.37)
29.	trans-9-Octadecenoic acid-TMS	nd	nd	28.206	38.46 (78.43)	nd	nd
30.	Oleic acid-TMS	nd	nd	28.963	4.82 (9.82)	nd	nd
31.	Heptacosane	29.559	0.32 (11.67)	nd	nd	29.559	0.22 (0.82)
32.	2-Hydroxy-1-(hydroxymethyl)ethyl cis-9-octadecenoic acid ester	nd	nd	nd	nd	30.041	0.28 (1.06)
33.	Bis(2-ethylhexyl)phthalate	30.453	0.43 (15.35)	nd	nd	nd	nd
34.	Oleic acid eicosyl ester	nd	nd	30.637	0.26 (0.52)	30.637	0.11 (0.43)
35.	Hexacosane	30.117	0.50 (17.93)	nd	nd	nd	nd
36.	2,3-Dihydroxypropyl cis-9-octadecenoic acid ester	nd	nd	31.187	0.59 (1.21)	nd	nd
37.	Squalene	nd	nd	nd	nd	31.814	0.55 (2.07)
38.	cis-6-Octadecenoic acid-TMS	nd	nd	31.393	0.31 (0.64)	nd	nd
39.	β-Sitosterol-TMS	nd	nd	35.015	0.37 (0.75)	nd	nd
40.	Heneicosane	30.637	0.65 (23.45)	nd	nd	nd	nd
41.	Eicosane	31.615	0.88 (31.59)	nd	nd	nd	nd
	Total area (%)		2.78 (100)		49.03 (100)		26.55 (100)

[1]Compounds identified at more than 90% similarity with the standard mass spectra in NIST 11 library; [2]Compounds identified in trimethylsilyl derivative (TMS) form; and [3]Value in parenthesis is relative percentage area of detected compound in respective sub-fraction. Rt: Retention time (min), and nd = not detected.
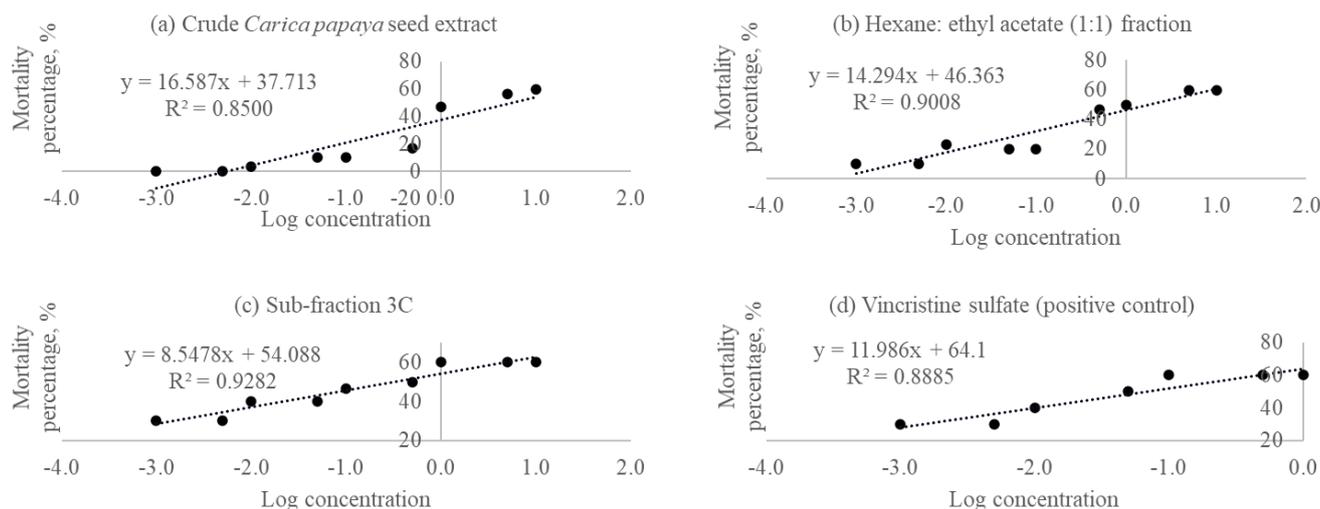
Figure 1. Calibration curve of *Artemia salina* percentage mortality for (a) crude *Carica papaya* seed extract, (b) hexane:ethyl acetate (1:1) fraction, (c) sub-fraction 3C, and (d) vincristine sulphate (positive control).

compounds with TMS nomenclature were detected when the silicon atom of the silyl donor rendered nucleophilic attack to the compounds containing -OH, -SH, and -NH groups, and replaced these groups to produce volatile TMS-form compounds. The compounds in this form have higher volatility characteristics than their natural forms to facilitate the detection in the GC/MS (Sani *et al.*, 2020).

Based on previous studies, the unsaturated fatty acid, 9-octadecenoic acid, was reported to exhibit a broad-spectrum antibacterial capacity against pathogenic bacteria such as *E. coli, P. aeruginosa, B. subtilis,* and *S. aureus* (Abubakar and Majinda, 2016). On the other hand, hexadecanoic acid of *Labisia pumila* was effective against *B. cereus* (Karimi *et al.*, 2015). The β-sitosterol, which was found in minute quantity in the CPSE, has also been reported to exhibit antibacterial activity (Ododo *et al.*, 2016). Even though sub-fraction 3B had the highest percentage area, the result of bioautography on TLC plate did not exhibit antibacterial activity.

Sub-fraction 3C, which was the most potent sub-fraction, was dominated by fatty acids, FAMES, and alkanes with the highest percentage area, which includes phthalic acid hexyl undecyl ester (1.65%), hexadecanoic acid methyl ester (1.58%), dodecanoic acid together with 1-methyl ethyl ester (1.30%) from FAMES group. In addition, octadecane (1.55%), 2,6,10,14-tetramethyl-pentadecane (1.43%), nonadecane (1.37%), hexacosyl heptafluorobutyrate (1.36%), heptadecane (1.30%), and methyl stearate (1.09%) were also detected. Among these compounds, *cis*-vaccenic acid-TMS, which was dominant at 3.30%, supported the antibacterial inhibitory capability (Gupta and Kumar, 2017) of the

component 3C of hexane:ethyl acetate (1:1) on TLC plates (Table 3). It was also the most potent pure standard solution against *B. cereus, P. mirabilis,* and *S. Enteritidis* (Table 2). Based on these findings by Sani *et al.* (2017b) on the compounds in sub-fraction 3C, the fatty acids and FAMES of the CPSE were very likely to render antibacterial inhibitory effects which contradicted the suggested phenolic compounds such as campesterol, stigmasterol, and β-sitosterol in CPSE.

Residual methanol content

The calibration curve of the methanol indicated coefficient determination (R^2) of 0.9820 and linear equation of y = 32.42x + 12.78. The residual methanol concentration was detected at 132.90 ± 22 µg/g, further substantiating the findings of a previous report (Lee and Kim, 2015), where residual methanol which was dried under reduced pressure is very likely to be lower than the allowable limit (3,000 µg/g). However, this limit is established explicitly for pharmaceuticals for human consumption. Since methanol is not listed as generally recognised as safe (GRAS) (Armenta *et al.*, 2020) or green solvent (Häckl and Kunz, 2018), any methanolic plant extract should undergo proper pre-treatments including re-dissolving in water (Lee and Kim, 2015), freeze-drying, ultrafiltration (Davidson *et al.*, 2015), or mild heat treatment for a short period (blanching) (Zhang and Ma, 2016) to degrade the residual methanol content prior to application as an antibacterial agent.

Effects of pH, water activity, and temperature on antibacterial activity of Carica papaya seed extract
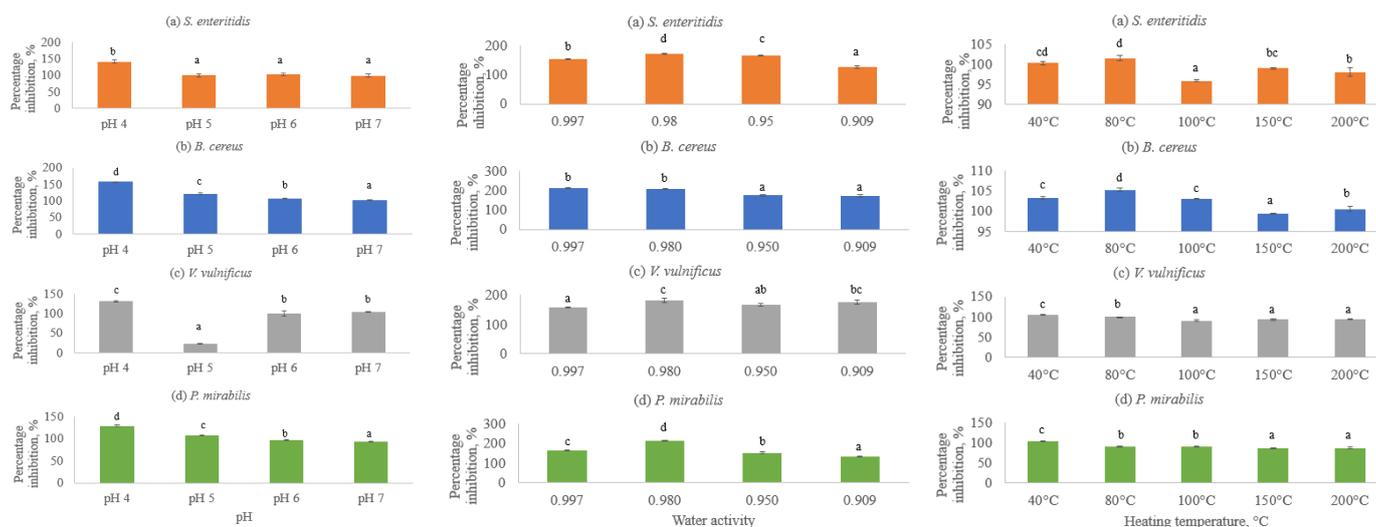
Figure 2. Effect of pH, water activity, and temperature on antibacterial activity of *Carica papaya* seed extract solution against (a) *S. Enteritidis*, (b) *B. cereus*, (c) *V. vulnificus*, and (d) *P. mirabilis* growths.

The effects of pH, A_w, and temperature on the antibacterial activity of crude CPSE was evaluated by determining the percentage of growth inhibition (PI) of the tested pathogens (Figure 2). PI that exceeds 100% suggests high inhibitory efficiency, which is equivalent to MIC (Sowhini *et al.*, 2020). However, inhibition still occurred at a concentration lower than the MIC.

All tested pathogens were sensitive to the crude CPSE solution at pH 4, where the PI in this pH exceeded 100%. Only pH 4 and 6 of TSB yielded PI > 100% for *S. Enteritidis*. As for *B. cereus,* the PI had reduced as the pH increased, indicating that *B. cereus* was sensitive to the extract in acidic condition. In addition, all crude CPSE solutions at different pH exhibited PI > 100%. Only pH 4 and 7 yielded PI > 100% for *V. vulnificus*, while crude CPSE solution at pH 5 exhibited the lowest inhibitory efficacy (22.72%). Also, *V. vulnificus* was most sensitive to the crude CPSE solution at pH 4. The susceptibility of *P. mirabilis* against crude CPSE solution at pH 4 and 5 exhibited PI > 100%, signifying that the crude CPSE solution was potent against *P. mirabilis* in acidic conditions. Although the crude CPSE solution was potent at the acidic condition as evidenced by high inhibitory activity at pH 5, the PI > 100% were observed against *B. cereus* and *V. vulnificus* at pH 7. These results inferred that the inhibitory effects of the crude CPSE solution varied against different tested pathogens.

In addition, the growth of all tested pathogens was inhibited at a different A_w of the crude CPSE solution (PI > 100%). At A_w of 0.980, *S. Enteritidis* and *P. mirabilis* scored the highest PI, and the PI of these pathogens demonstrated a decrease as the A_w decreased to 0.909. Similarly, *B. cereus* was most sensitive to the crude CPSE solution at A_w of 0.997, where the PI decreased as the A_w of the crude CPSE solution decreased. From these results, the crude CPSE solution could be incorporated in food with $A_w < 0.950$ such as noodle, fresh meat, fish, and etc. (Lang *et al.*, 2017).

The potency of the crude CPSE solution against *S. Enteritidis* and *V. vulnificus* exhibited the same pattern (PI < 100%) when the temperature of the crude CPSE solution > 100°C. The PI of *B. cereus* demonstrated 99.45% value when the crude CPSE solution reached 150°C. These results inferred that the crude CPSE solution was inactive against (1) *S. Enteritidis* and *V. vulnificus* when the temperature of the crude CPSE solution exceeded 100°C, and (2) *B. cereus* when the temperature of crude CPSE solution exceeded 150°C, thus supporting the claim of Saliani *et al.* (2015) that most antibacterial agents are inactive at high temperature. As for *P. mirabilis*, the crude CPSE solution exhibited strong inhibitory effect towards this pathogen (PI > 100%) at 40°C. For all tested pathogens, the crude CPSE solution acts as an effective antibacterial agent at < 40°C; hence crude CPSE-treated product should be handled below this temperature before consumption.

In short, even though the crude CPSE solution exhibited MIC of 5.63 mg/mL (Sani *et al.,* 2017b), the inhibition capability of the same or higher concentration may be compromised in solution form at different pH, A_w, and temperature. From these outcomes, the crude CPSE solution was stable and could completely function as an antibacterial agent at acidic condition with $A_w < 0.950$ and temperature < 40°C.

Conclusion

In conclusion, the CPSE demonstrated antibacterial activity against *S.* Enteritidis, *V. vulnificus, P. mirabilis,* and *B. cereus*. The crude CPSE exhibited the lowest toxicity (LC_{50} = 5.505 ± 0.718 mg/mL) as compared to hexane:ethyl acetate (1:1) and sub-fraction 3C. However, sub-fraction 3C which was assessed on bioautography of TLC plates contained the highest relative percentage area of *cis*-vaccenic acid (12.41%). *Cis*-vaccenic acid also had individually demonstrated the lowest MIC against *B. cereus* (1.41 mg/mL), *P. mirabilis* (1.41 mg/mL), and *S.* Enteritidis (0.70 mg/mL). Although the CPSE had achieved a stagnant weight after the methanol removal during rotary evaporation, residual methanol at 132.90 ± 22 µg/g was still detected. The crude CPSE was adequate for antibacterial application study since fractionation and purification of the crude CPSE revealed inhibitory effects but was highly toxic against *A. salina*. Therefore, the crude CPSE was identified as the most viable than the fractionated extract. At 5.63 mg/mL MIC, the crude CPSE solution was stable, and could completely function as an antibacterial agent at acidic conditions with A_w < 0.950 and temperature < 40°C.

Acknowledgement

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