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Chemometrics-based evaluation on the effect of sonication, contact time and solid-to-solvent ratio on total phenolics and flavonoids, free fatty acids and antibacterial potency of *Carica papaya* seed against *S. enteritidis, B. cereus, V. vulnificus* and *P. mirabilis*

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ABSTRACT

This study was aimed at extraction optimization of antibacterial agents from *Carica papaya* seed against *S. enteritidis, B. cereus, V. vulnificus* and *P. mirabilis* as affected by sonication-assisted extraction (SAE), contact time (CT) and solid-to-solvent ratio (SSR). The principal component analysis (PCA) and individual evaluation approaches identified that no SAE, 8 CT and 1:10 SSR were the best treatments with the highest antibacterial potency. The PCA identified no SAE, 8 CT, and 1:5 SSR as the second-beat treatment. The yield, total phenolic compound (TPC), C18:1n9t and C16:1 free fatty acids (FAs) in no SAE, 8 CT and 1:10 SSR treatment inhibited *B. cereus, V. vulnificus* and *P. mirabilis* growths while C21:0 and C15:0 in 30 min SAE, 8 CT and 1:2 SSR inhibited *S. enteritidis* growth. The yield, TPC, C18:1n9t and C16:1 FAs, and C6:0 and C24:1n9, C20:1, C4:0 and C20:0 FAs had antagonistic effects on *B. cereus, V. vulnificus* and *P. mirabilis* growths. The C21:0, C15:0, C6:0 and C13:0, and C23:0, C20:0 and C11:0 FAs had antagonistic effects on *S. enteritidis* growth. The PCA also denoted that the MIC_{50} and MIC_0 had a higher variation than MIC; hence, the former variables were better to use in PCA.

1. Introduction

The antibacterial compounds had been searched from various plant by-products originating from tropical fruits such as pineapple, *Carica papaya*, and mango. The *Carica papaya* cv. Sekaki or Hong Kong, Eksotika I and Eksotika II are varieties in Malaysia that provide an abundance of seed as a cheap source for the antibacterial product. They can also be sprinkled on salads or soups or added to any dish to substitute for black pepper (Rosa et al., 2021). Reports on antibacterial activities from *Carica papaya* seeds are still scanty, as evident by the recent review on the agro-industrial potential of exotic fruit by-products (Sani et al., 2017b). Currently, Sani et al. (2021a) found that *S. enteritidis, B. cereus, V. vulnificus* and *P. mirabilis* were sensitive against a methanolic extract from the seed of riped *Carica papaya*. However, the optimization information on extraction treatment to obtain the antibacterial potency from the *Carica papaya* seed extract is still negligible.

Generally, direct extraction without manipulating the extraction treatments is applied to study the antibacterial properties of natural by-products. Recently, new techniques such as microwave-assisted extraction (Boukhatem et al., 2022) and supercritical CO_2 extraction (Zhao & Zhang, 2013) have been utilized to extract bioactive compounds from plants and their by-products. However, relative to those techniques, sonication-assisted extraction (SAE) is more convenient, affordable, environmentally friendly and industrially employed in local companies, by which the active compounds could be extracted in a shorter time and higher efficiency (Sagar & Pareek, 2020). Likewise, time of contact (CT) is a necessary treatment to be optimised to minimize the process's energy cost. Longer contact time exposed active sites of the solid area, im-

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proved sample homogeneity and increased extraction yield (Raks et al., 2018). The solid-to-solvent ratio (SSR) was also a significant variation in the extraction of natural by-products as it allowed maximum surface contact between solid and solvent and increased mass transfer rate. Both treatments also affected phenolic and flavonoid contents (Djemaa-Landri et al., 2020). Studies on contact time affecting antioxidative properties for plants and its by-products such as basil (da Silva et al., 2022) and SSR on grape (Ryu et al., 2020) have been reported; nevertheless, the SAE, CT and SSR had never been carried out to investigate their effect on antibacterial activities of *Carica papaya* seed.

A common approach to optimize the extraction of antibacterial compounds is by carrying out extraction treatment and evaluating the treatment effect individually, where only minimum inhibitory concentration (MIC) was considered. This approach brings minimal information for researchers to investigate the antibacterial activity of *Carica papaya* seed extract. Sani et al., (2017a) proposed including total phenolic compound and flavonoid compounds since these phytochemicals were reported to render antibacterial activities (Alonso-Esteban et al., 2019). However, to gain more information on the variables contributing to the antibacterial potency, free fatty acids in their ester forms were included since 86.05% of *Carica papaya* seeds are dominated by the free fatty acids and their esters (Sani et al., 2020). Therefore, our study quantified 37 free fatty acids in their ester form and evaluated the effect of treatments and these variables using a chemometric-based approach, i.e., principal component analysis (PCA) suitable for a multivariate dataset (Sani et al., 2021b). Very negligible report employed the chemometric-based approach to evaluate the antibacterial potency, especially on *Carica papaya* seed extract to date. It is also recommended to add MIC_{50} and MIC_0 as additional antibacterial variables since they have a variation that could render a more meaningful insight into the PCA results.

It is anticipated that this study could provide new insight into the antibacterial activities of *Carica papaya* seed extract, promote the application of cheap and ubiquitous by-products, and provide economic extraction procedures. This study will indirectly accelerate the quest for natural antibacterials where other works could adopt this approach to optimize the extraction method of plant by-products that could render antibacterial activities.

2. Material and method

2.1. Experimental design

The experiment was designed as depicted in Fig. 1. The experiment was divided into three effects which involved the effect of sonication-assisted extraction (SAE), contact time (CT) and solid-to-solvent ratio (SSR). The best treatment from each effect was applied in the following effect. For instance, the best treatment for the SAE effect was used in the CT effect, and both the best treatment from SAE and CT effect was then employed in the SSR effect. For each effect, the yield, total phenolic content (TPC) and total flavonoid content (TFC) of the extract ware determined as well as the antibacterial activities, consisting of minimum inhibitory concentration (MIC), MIC_{50}, and MIC_0 of *S. enteritidis, B. cereus, V. vulnificus* and *P. mirabilis*. Evaluation on the (1) individual treatment and variable and (2) treatments and multivariable via chemometrics were carried out and compared. The correlations among the variables were assessed, and the variables that characterised the treatment were identified.

2.2. Plant material

Carica papaya cv. Sekaki fruit was bought from D'Lonek Sdn. Bhd. Organic Farm, Rembau, Negri Sembilan, Malaysia and numbered as SK 2368/14 voucher by Herbarium of Institute of Bioscience, Universiti Putra Malaysia. *Carica papaya* seeds were taken out from the fruit and treated as described by Sani et al. (2017a).

2.3. Extraction of phytochemicals

Methanol (MeOH) was used as solvents in the extraction, according to Sani et al. (2020). No SAE (0 min SAE), 8 h CT and 1:2 SSR were standard extraction treatments initially carried out by Sani et al. (2017b). Briefly, 50 g of dried ground *Carica papaya* cv. Sekaki seed was weighed into a conical flask, and 100 mL of methanol was added. Extraction was carried out at 30 °C in a shaker (100 rpm) followed by filtration through Whatman No.1 filter paper (GE Healthcare, UK). The filtrate was transferred into pre-weighed flat bottom flasks and concentrated using a rotary vacuum evaporator (Eyela N-1001, Japan) at 40 °C.

To study the effect of SAE, extraction was carried out without SAE, and at 15 min, 30 min and 60 min sonication at 60 Hz with 550 W power. To study the effect of CT, extractions were carried out at 2, 4 and 8 h. To study the effect of SSR, extractions were carried out at 1:2, 1:5 and 1:10 SSR for an investigation in a laboratory scaling. Each extraction treatment was done in triplicate.

2.4. Minimum inhibitory concentration (MIC), MIC_{50} and MIC_0

A two-fold serial microdilution method of 96 multi-well microtiter plates was used for MIC determination with modifications. Briefly, 100 μL of tryptone soy broth (TSB) was added to each well. A volume of 100 μL of 0.5×10^5 μg/mL crude extracts in dimethyl sulfoxide (DMSO) was added to the first well. A volume of 100 μl from the first test well was pipetted into the second well of each microtiter row, and then 100 μl of serial dilution was transferred from the second to the third and followed through until the eleventh well. A volume of 100 μL from the last well was discarded. An aliquote of 90 μL from each well was pipetted, mixed with 10 μL of 106 CFU/mL bacterial suspensions, and then added back into each well. This preparation will make up 22.5–0.02 mg/mL extract concentrations from the first to the eleventh well. The microtiter plate was incubated at 37 °C for 24 h on a Heidolph Inkubator and Titramax 1000 (Germany) at 210 rpm to prevent adherence and clumping, after which the optical density was measured at 600 nm in Tecan Infinite® 200 Microplate Reader (Switzerland) before (T_0) and after (T_{24}) incubation. A TSB medium incubated with a target bacterium (without an antibacterial agent) was used as a positive control of growth in the twelfth well in each row.

The MIC was defined as the lowest concentration of antibacterial agent showing a complete growth inhibition of the tested bacterial strains, which was related to a difference absorbance of T_{24} and T_0 (T_{24}-T_0) equal to zero or negative values.

The graphs of percentage inhibition for each extraction treatment were plotted, and the MIC was compared to the percentage inhibition where MIC had 100% bacterial inhibition. MIC_{50} was determined by calculating the concentration that gave 50% inhibition by using linear regression ($y = mx + c$), where $y = 50\%$, m = slope of regression, c = intercept of regression and x = concentration of extract at 50% inhibition. From the percentage inhibition graphs, the concentration of extract which gave 0% inhibition was also determined as MIC_0. All determinations were performed in triplicate (Sowhini et al., 2020).

2.5. Quantitation of total phenolic content (TPC)

Total phenolic contents of *Carica papaya* crude extracts were determined by colorimetry assay with Folin-Ciocalteu according to Sani et al. (2017a). An amount of 0.05 g *Carica papaya* seed extract was diluted to 100 mL in a volumetric flask, and 1 mL of the diluted extract was mixed with 1 mL of 1:10 diluted Folin-Ciocalteu reagent (Sigma-Aldrich, Switzerland) in a 5 mL volumetric flask wrapped with aluminum foil and vortexed for 10 s. The mixture was then incubated at 30 °C for 5 min, mixed with 1 mL sodium carbonate (10%, w/v) solution (Sigma-Aldrich, Switzerland) and marked up to the volume. Then, the mixture was vortexed (VTX-3000 L, Copens Scientific, Germany) for another 10 s and incubated in the dark at 30 °C for 30 min. The mixture

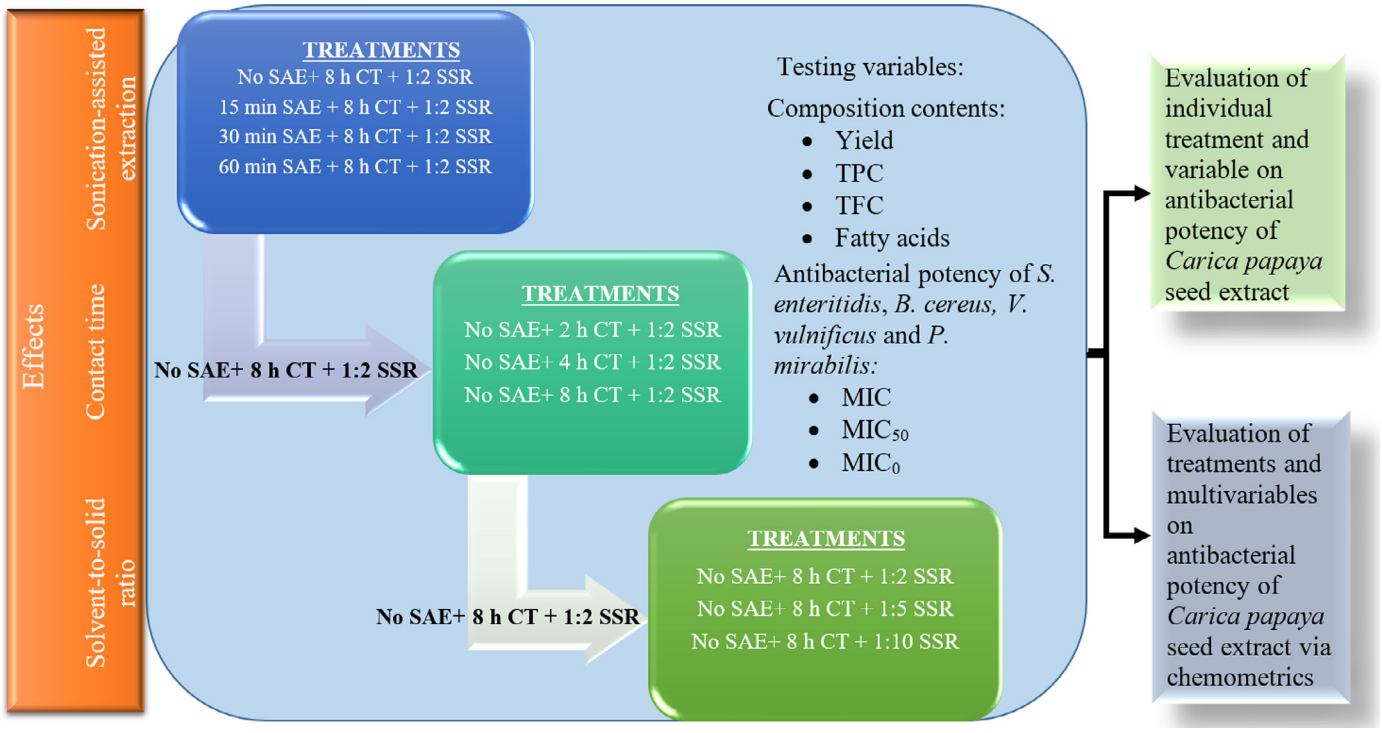

Fig. 1. Experimental design of effect of sonication-assisted extraction, contact time and solvent-to-solid ratio on the antibacterial potency of *Carica papaya* seed against *S. enteritidis, B. cereus, V. vulnificus* and *P. mirabilis*.

produced a blue aqueous layer, and its absorbance was measured using a spectrophotometer (U-2810 Hitachi, Japan) at 747 nm against methanol as a blank in triplications. The exact incubation procedure was employed to prepare 0–10 mg/L gallic acid standard (Sigma-Aldrich, Switzerland) solutions in methanol. A calibration curve of gallic acid absorbance versus concentration was plotted, and the concentration of *Carica papaya* seed extract was computed from the calibration equation. Results were expressed as gallic acid equivalent (GAE) in mg/g of dry weight (DW) of the sample (mg GAE/g DW).

2.6. Quantitation of total flavonoid content (TFC)

A series of quercetin working standards at 0–12.5 mg/L, including ethanol as blank, was measured spectrophotometrically against ethanol at 438 nm, and a calibration curve was established. Total flavonoid contents of *Carica papaya* crude extracts were determined following Sani et al. (2017b). An amount of 0.05 g *Carica papaya* seed extract was diluted in a 100 mL volumetric flask with ethanol. A volume of 1.25 mL the diluted extract was mixed with 0.5 mL of 0.1 g/mL aluminum chloride solution (Sigma-Aldrich, Switzerland) and 0.5 mL of 1 M sodium acetate solution in 5 mL volumetric flask wrapped with aluminum foil, marked up to volume with ethanol and vortexed for 10 s. Then the solution mixture was incubated at 30 °C for 15 min. After incubation, the absorbance of a yellow color solution indicated the presence of the flavonoids was measured spectrophotometrically against ethanol. The measurements were carried out in triplicate, and results were expressed as quercetin equivalent (QE) per gram dry weight (DW) of the sample (mg QE/g DW).

2.7. Analysis of free fatty acids methyl esters by gas chromatography-mass spectrometer

A series of working FAMEs standard in hexane ranging from 0.0005 to 3 mg/mL was prepared in a 1 mL volumetric flask and injected into gas chromatography-mass spectrometry (GC/MS). A concentration of 0.01 g/mL *Carica papaya* seed extract was esterified by mixing the extract with 0.6 mL of hexane and 0.4 mL of 1 M solution of sodium methoxide. Then, the mixture was vortexed for 30 s. A volume of 0.6 mL of top layer contaning hexane was analyzed by GC/MS for FAME quantification.

Separation and detection of FAMEs was carried out on an Agilent-Technologies 7890A gas chromatography (GC) system equipped with an Agilent-Technologies 5975 mass spectrometer (MS) system (Agilent Technologies, USA). The working standards and top hexane layer of the *Carica papaya* seed extracts were injected into an injector temperature maintained at 260 °C. A volume of 1 µL of the standard and extracts was split at 1:10 ratio and eluted into the GC system by helium at 1 mL/min flow rate. The FAMEs were separated by an HP-88 capillary column (100 m x 0.25 mm, film thickness 0.20 µm) with an oven temperature program at (1) 150 °C for 5 min, (2) heated to 240 °C at 4 °C/min and (3) held for 15 min. The separated FAMEs were eluted through MS transfer line and mass quadrupole set at 230 °C and 150 °C, respectively, ionised at 70 eV and detected by MS system at a mass range of m/z 20–700 units. The FAMEs detection and quantification were operated in scan and selected ion monitoring (SIM) modes.

A retention-time-lock mode of stable palmitic acid (C16:0) was executed to avoid changes in retention times in calibration curves due to column maintenance or column change. The FAMEs were identified by their retention times, comparison of their mass fragmentation patterns with standards from the National Institute of Standard (NIST) Mass Spectral 11 library and confirmation with the working standards. The linearity of the calibration curve was established and assessed, where the correlation coefficient $R^2 > 0.98$ indicated an acceptable identification (Sani et al., 2021b).

2.8. Statistical analysis

Data were expressed as mean ± standard deviation of triplicate analysis of extraction yield, TPC and TFC and MIC_{50}. One-way analysis of variance (ANOVA) with Tukey's test was conducted using XLSTAT-

Table 1
Extraction yield, total phenolic and total flavonoid contents of *Carica papaya* seed extracts.

Treatment[1]	Extraction yield[3] (mg/g)	TPC[3] (mg GAE / g DW)	TFC[3] (mg QE / g DW)
15 min SAE	20.73 ± 0.15^{b}	18.39 ± 0.81^{d}	2.31 ± 0.11^{d}
30 min SAE	19.12 ± 0.15^{b}	18.03 ± 0.71^{d}	2.70 ± 0.03^{c}
1 h SAE	21.57 ± 0.34^{b}	19.38 ± 0.74^{cd}	4.94 ± 0.18^{a}
2 h CT	11.95 ± 0.07^{c}	9.85 ± 0.51^{f}	4.43 ± 0.31^{b}
4 h CT	15.75 ± 0.08^{c}	13.89 ± 0.75^{e}	2.86 ± 0.15^{c}
8 h CT[2]	21.59 ± 0.07^{b}	20.10 ± 0.74^{c}	1.91 ± 0.04^{e}
1:5 SSR	76.02 ± 0.13^{a}	51.05 ± 1.99^{b}	2.32 ± 0.09^{d}
1:10 SSR	81.15 ± 0.48^{a}	62.78 ± 1.51^{a}	2.20 ± 0.07^{d}

[1] 15 min SAE - 15 min sonication assisted extraction; 30 min SAE - 30 min sonication assisted extraction; 1 h SAE - 1 h sonication assisted extraction; 2 h CT - 2 h contact time; 4 h CT - 4 h contact time; 8 h CT - 8 h contact time; 1:5 SSR - 1:5 solid-to-solvent ratio; 1:10 SSR - 1:10 solid-to-solvent ratio.
[2] Extract was prepared without sonication at 1:2 solid-to-solvent ratio.
[3] Means ± S.D. are from triplicate measurements.

Pro (2019) statistical software (Addinsoft, Paris, France) to determine the significant difference between the means at 95% confidence level ($p < 0.05$) for extraction yield, TPC and TFC and MIC_{50}.

In this study, a principal component analysis (PCA) of Pearson correlation at α of 0.05 was employed to describe the correlation and distribution of significant chemical constituents and fatty acids towards the antibacterial potency of *Carica papaya* seed extract affected by the sonication, contact time and solid-to-solvent ratio. For the antibacterial variables, the MIC, MIC_{50} and MIC_0 for *S. enteritidis, B. cereus, V. vulnificus* and *P. mirabilis* were assigned as MICSE, MICBC, MICVV, MICPM, $MIC_{50}SE$, $MIC_{50}BC$, $MIC_{50}VV$, $MIC_{50}PM$, MIC_0SE, MIC_0BC, MIC_0VV and MIC_0PM, respectively. The dataset was transformed into independent variables known as principal components (PCs). Cumulative variability (CV) of two dimensional PCs entailing PC1 and PC2 were computed for *Carica papaya* seed extract profiling. The variables with strong, moderate and weak factor loading (FL) were identified. Based on these FLs, the variable correlations and their contributions to extraction treatments were assessed.

3. Results and discussion

3.1. Yield, TPC and TFC as affected by sonication, contact time and solid-to-solvent ratio of Carica papaya seed extract

Extraction yields by different extraction treatments are shown in Table 1. The application of SAE resulted insignificant different ($p < 0.05$) in the extraction yields (19.12–21.57 mg/g) as compared to no SAE yield. This occurrence was possibly due to the extraction reaching equilibrium before 15 min SAE contact time, reducing solvent's permeability into cell structures on account of insoluble lipids existence on the ruptured cell or re-adsorption of active components because of a large specific area of the ruptured cells (Şahin et al., 2020). The extraction yield of *Carica papaya* seed extract also increased as contact time increased (Table 1). Among the treatments, 8 h CT had produced the significant ($p < 0.05$) highest yield (21.59 mg/g). The yield of *Carica papaya* seed extract increased as SSR increased (Table 1), where 1:5 and 1:10 SSR showed significant yield ($p < 0.05$) as compared to 1:2 SSR. The high SSR increased the concentration gradient between the solid and the solvent, enhanced diffusion rate, and allowed greater extraction of solids by solvent (Djemaa-Landri et al., 2020). Hence, no SAE, 8 CT and 1:10 SSR were the best treatments due to the highest yield.

A calibration curve of TPC for *Carica papaya* seed extracts was established to obtain calibration equation $y = 0.0827x + 0.0007$ with coefficient determination (R2) of 0.9999. All SAE time Table 1 showed lower TPC (18.03–19.38 mg GAE/g DW) than no SAE (20.10 ± 0.74 mg GAE/g DW) and 60 min SAE. These treatments also exhibited insignificant TPC differences ($p < 0.05$) compared to no SAE, due to small energy generation during sonication that caused low cavitation bubbles in the cell wall. This result was in agreement with work on *Mentha aquatica*, where sonication had an insignificant impact on the TPC (Safaiee et al., 2019). Özcan et al. (2021) also found insignificant TPC of almond (*Prunus amygdalus*) varieties due to thermal dissociation of TPC during SAE.

The TPC of *Carica papaya* seed extract increased as contact time increased (Table 1) (Djemaa-Landri et al., 2020). Among the treatments, 8 h CT had produced the significant ($p < 0.05$) highest yield (21.59 mg/g). The 8 h CT also exhibited the highest amount of TPC (20.10 mg/g) with a significant difference ($p < 0.05$) value as compared to other treatments due to longer contact time had improved surface area and slurry homogeneity of the sample; hence, provided a positive influence on phenolics by allowing the progressive release of phenolics from solid matrix to solvent (Ben-Ali et al., 2018). Thus, the 8 h CT was the best CT treatment since it gave the highest values of yield and TPC. Of the SSR treatments, the 1:10 SSR also had the highest (62.78 mg/g) and significant amount of TPC ($p < 0.05$) compared to the 1:5 SSR (51.05 mg/g) and 1:2 SSR (20.10 mg/g).

The calibration equation of TFC for *Carica papaya* seed extracts was established to obtain calibration equation $y = 0.0762x - 0.0126$ with an R^2 of 0.9994. The SAE gave a significant difference ($p < 0.05$) of TFC (2.31–4.94 mg QE/g DW) in Table 1 as compared to no SAE treatment TFC (1.91 mg QE/g DW), where the 60 min SAE demonstrated the highest value, possibly due to flavonoids in the *Carica papaya* seed were in the form of flavonoid glycosides, which have thermal stability. Since the *Carica papaya* seed used in this study did not undergo an acid hydrolysis process, only flavonoid glycosides were extracted. This finding was also evident in *Ferula persica* extract, where the TFC in the form of glycosides were higher in the SAE method compared to no SAE (Taghinia et al., 2019). Pan et al. (2012) claimed that the highest recovery of TFC in hawthorn seed was obtained at 91 °C, which exceeded the temperature observed in this study (70 °C), indicating the increment of temperature during SAE had not destabilized flavonoid glycosides. The increment of temperature during SAE was also reported to enhance solubility, increase the diffusion coefficient, and increase the extraction rate of TFC (Safaiee et al., 2019). From this finding, the no SAE was the best treatment compared to other SAE treatments.

On the other hand, the CT effect on TFC exhibited an inverse trend, where 2 h CT yielded the highest (4.43 mg/g) with a significant amount ($p < 0.05$). Even though Efthymiopoulos et al. (2019) found that the optimum contact time in coffee extraction was 2 h, our result showed that 8 h was the best CT to obtain the highest TPC.

The 1:5 SSR produced the highest TFC (2.32 mg/g) with insignificant difference ($p < 0.05$) compared to the 1:10 SSR (2.20 mg/g). Although the TFC generally increased significantly ($p < 0.05$) for 1:10 and 1:5 SSR than the 1:2 SSR in Table 1, the TFC increment may not be directly proportional since the TFC increment was halted once reaching the solid and solvent equilibrium regardless of the SSR (Şahin et al., 2020). Hence, the 1:10 SSR was the best SSR treatment compared to the 1:5 SSR and 1:2 SSR due to the TFC.

Although the 1:10 SSR had the TPC and TFC at 62.78 mg/g and 2.20 mg/g, respectively, the TPC was equivalent to 0.0628 g in 1 g of dried extract or 6.28% of the dried *Carica papaya* seed extract. Like-

wise, the TFC represented only 0.22% of the *Carica papaya* seed extract. Hence, phenolics and flavonoids were not dominant in the *Carica papaya* seed extract, which supported the finding of Sani et al. (2020). They found that the *Carica papaya* seed extract was dominated by 86.05% fatty acids and fatty acid methyl esters, followed by 5.59% amides, 1.19% nitriles, 0.51% sterols and 0.03% organic acids. To obtain this composition, Sani et al. (2020) derivatised the *Carica papaya* seed extract with N, O-bis-trimethylsilyl-trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) at a 99:1 ratio to ensure both polar and non-polar compounds were volatile for the detection by GC/MS. The identified compounds were confirmed by comparing the mass spectra with the National Institute of Standard (NIST) Mass Spectral Library. The results suggested that the sterols and organic acids may contribute to the TPC and TFC values.

3.2. MIC, MIC_{50} and MIC_0 as affected by sonication, contact time and the solid-to-solvent ratio of Carica papaya seed extract

The MIC test, a descriptive antibacterial method, had only given limited information on bacterial inhibition and inadequate comparison between extraction treatments. Thus, Sowhini et al. (2020) proposed to apply MIC, MIC_{50} (minimum concentration which gave 50% inhibition) estimation and MIC_0 (minimum concentration, which gave 0% inhibition) information from the percentage inhibition to investigate the effect of extraction treatments on antibacterial properties of extracts.

The MIC, MIC_{50} and MIC_0 of *Carica papaya* methanolic seed extract as affected by SAE against *S. enteritidis, B. cereus, V. vulnificus* and *P. mirabilis* growths were shown in Table 2. The *Carica papaya* seed concentration range used for all tested pathogens was 22.5–0.02 mg/mL. Among the tested pathogens, the *S. enteritidis, V. vulnificus and P. mirabilis* gave the lowest MIC (5.63 mg/mL). *B. cereus* had a MIC of 11.25 mg/mL as affected by 15, 30 and 60 min SAE and MIC of 5.63 mg/mL as affected by no SAE.

The lowest MIC_{50} value for *S. enteritidis* and *P. mirabilis* was obtained from 15 min SAE and 60 min SAE, respectively, while 30 min SAE rendered the lowest MIC_{50} for *B. cereus* and *V. vulnificus* (Table 2). However, as we performed the significant test on these data, the MIC_{50} from these treatments were not significantly different from the no SAE treatment. The no SAE treatment gave the lowest MIC_0 than SAE treatments for all tested pathogens (Table 2). Based on MIC, MIC_{50} and MIC_0 comparisons among treatments, no SAE was the best treatment compared to other SAE treatments.

Table 2 exhibited the CT effect on percentage inhibition, MIC, MIC_{50} and MIC_0 of *S. enteritidis, B. cereus, V. vulnificus* and *P. mirabilis*. The MIC values were higher at 2 h and 4 h contact time for *B. cereus, V. vulnificus* and *P. mirabilis* than 8 h CT, indicating that longer contact time produced a higher concentration of antibacterial compounds due to larger surface contact area between solvent and solute. The MIC of these microorganisms also exhibited a strong correlation with contact time (Table 2). However, the 8 h extraction did not inhibit *S. enteritidis*.

The lowest MIC_{50} (Table 2) values for *S. enteritidis, B. cereus* and *V. vulnificus* were obtained from 8 h contact time, where significant differences of inhibitions were shown against *S. enteritidis* and *V. vulnificus* only. At 4 h contact time, *P. mirabilis* attained an insignificant difference of MIC_{50} compared to 8 h contact time.

All contact time treatments provided < 0.02 mg/mL of MIC_0 against *B. cereus* and *P. mirabilis. S. enteritidis* was the most sensitive against 2, 4 and 8 h contact time treatments where this microorganism showed MIC_0 at 1.41 mg/mL for both 2 h and 4 h contact time, while at 8 h contact time, the MIC_0 reduced to 0.70 mg/mL (Table 2). The 8 h CT also affected the *V. vulnificus* inhibition by reducing the MIC_0 from 0.35 mg/mL to < 0.02 mg/mL. This study concluded that the 8 h CT was the best CT treatment since it gave MIC, MIC_{50} and MIC_0.

The MIC, MIC_{50} and MIC_0 (Table 2) were tabulated from the percentage inhibition of, respectively tested pathogens. All SSR treatments had not affected the MIC since all the MIC values of *S. enteritidis, B.*

Table 2
MIC, MIC_{50} and MIC_0 of *Carica papaya* methanolic seed extracts against *S. enteritidis, B. cereus, V. vulnificus* and *P. mirabilis*.

Treatment[1]	MIC (mg/mL)				MIC_{50}[3] (mg/mL)				MIC_0 (mg/mL)			
	S. enteritidis	*B. cereus*	*V. vulnificus*	*P. mirabilis*	*S. enteritidis*	*B. cereus*	*V. vulnificus*	*P. mirabilis*	*S. enteritidis*	*B. cereus*	*V. vulnificus*	*P. mirabilis*
15 min SAE	5.63	11.25	5.63	5.63	3.66 ± 0.06^{c}	3.94 ± 0.05^{a}	3.78 ± 0.04^{c}	3.99 ± 0.06^{a}	1.41	1.41	0.02	0.70
30 min SAE	5.63	11.25	5.63	5.63	3.80 ± 0.06^{b}	3.40 ± 0.11^{de}	3.61 ± 0.16^{c}	2.90 ± 0.20^{b}	1.41	< 0.02	< 0.02	0.02
1 h SAE	5.63	11.25	5.63	5.63	4.05 ± 0.01^{a}	3.64 ± 0.13^{b}	3.74 ± 0.16^{c}	2.37 ± 0.34^{c}	1.41	< 0.02	0.02	< 0.02
2 h CT	5.63	11.25	11.25	11.25	4.04 ± 0.03^{a}	3.85 ± 0.20^{a}	4.45 ± 0.24^{a}	2.48 ± 0.24^{bc}	1.41	< 0.02	0.35	< 0.02
4 h CT	5.63	11.25	11.25	11.25	4.02 ± 0.08^{a}	3.56 ± 0.09^{bc}	4.01 ± 0.10^{b}	2.41 ± 0.31^{c}	1.41	< 0.02	< 0.02	< 0.02
8 h CT[2]	5.63	5.63	5.63	5.63	3.67 ± 0.01^{c}	3.43 ± 0.07^{cd}	3.65 ± 0.08^{c}	2.45 ± 0.37^{c}	0.70	< 0.02	< 0.02	< 0.02
1:5 SSR	5.63	5.63	5.63	5.63	3.07 ± 0.05^{d}	3.22 ± 0.06^{f}	3.35 ± 0.06^{d}	1.84 ± 0.45^{d}	0.35	< 0.02	< 0.02	< 0.02
1:10 SSR	5.63	5.63	5.63	5.63	2.87 ± 0.10^{e}	3.27 ± 0.10^{ef}	1.87 ± 0.25^{e}	2.37 ± 0.22^{c}	< 0.02	< 0.02	< 0.02	< 0.02

[1] 15 min SAE - 15 min sonication assisted extraction; 30 min SAE - 30 min sonication assisted extraction; 1 h SAE - 1 h sonication assisted extraction; 2 h CT - 2 h contact time; 4 h CT - 4 h contact time; 8 h CT - 8 h contact time; 1:5 SSR - 1:5 solid-to-solvent ratio; 1:10 SSR - 1:10 solid-to-solvent ratio.
[2] Extract was prepared without sonication at 1:2 solid-to-solvent ratio.
[3] Means ± S.D. are from triplicate measurements.

Table 3
Characteristics of analytical curves for fatty acid methyl esters.

No.	Fatty acid methyl ester	Assignment	Mass	Retention time, min	Coefficient determination (R^2)	Linearity equation
1.	Butyric acid	C4:0	102	9.690	0.9974	$y = 0.3406x + 14.727$
2.	Hexanoic acid	C6:0	130	10.144	0.9966	$y = 0.0923x - 15.233$
3.	Octanoic acid	C8:0	158	10.940	0.9952	$y = 0.3662x + 59.885$
4.	Decanoic acid	C10:0	186	12.304	0.9969	$y = 2.0091x - 437.51$
5.	Undecanoic acid	C11:0	200	13.253	0.9945	$y = 1.5095x - 323.16$
6.	Dodecanic acid	C12:0	214	14.399	0.9966	$y = 4.0352x - 1227.9$
7.	Tridecanoic acid	C13:0	228	15.689	0.9962	$y = 1.481x + 26.513$
8.	Myristic acid	C14:0	242	17.148	0.9964	$y = 4.2624x - 1108$
9.	Myristoleic acid	C14:1	240	18.327	0.9962	$y = 0.4265x + 230.14$
10.	Pentadecanic acid	C15:0	256	18.670	0.9961	$y = 2.5957x - 136.37$
11.	Cis-10-pentadecenoic	C15:1	254	19.912	0.9946	$y = 0.4751x + 182.81$
12.	Palmitic acid	C16:0	270	20.285	0.9964	$y = 13.148x - 3445.9$
13.	Palmitoleic acid	C16:1	268	21.295	0.9885	$y = 0.4777x + 666.03$
14.	Heptadecanic acid	C17:0	284	21.822	0.9960	$y = 2.8421x + 105.16$
15.	Cis-10-heptadecenic acid	C17:1	282	22.877	0.9863	$y = 0.5708x + 420.79$
16.	Stearic acid	C18:0	299	23.418	0.9972	$y = 2.6297x - 466.87$
17.	Elaidic acid	C18:1n9t	296	24.007	0.9903	$y = 0.7292x + 523.17$
18.	Oleic acid	C18:1n9c	296	24.329	0.9908	$y = 1.3187x + 894.16$
19.	Linolelaidic acid	C18:2n6t	294	24.947	0.9916	$y = 1.7255x + 495.27$
20.	Linoleic acid	C18:2n6c	294	25.631	0.9947	$y = 1.7667x - 902.56$
21.	Arachidic acid	C20:0	327	26.430	0.9911	$y = 504.82x - 542,642$
22.	γ-linolenic acid	C18:3n6	292	26.580	0.9923	$y = 155.49x + 166,488$
23.	Linolenic acid	C18:3n3	292	27.142	0.9959	$y = 163.93x - 36,714$
24.	Cis-11-eicosenoic acid	C20:1	325	27.259	0.9975	$y = 210.9x - 23,947$
25.	Heneicosanoic acid	C21:0	341	27.835	0.9833	$y = 278.58x - 425,782$
26.	Cis-11,14-eicosadienoic acid	C20:2	323	28.547	0.9950	$y = 177.5x - 51,134$
27.	Behenic acid	C22:0	355	29.305	0.9904	$y = 670.73x - 984,863$
28.	Cis-8,11,14-eicosatrienoic acid	C20:3n6	321	29.505	0.9973	$y = 164.72x - 23,659$
29.	Cis-11,14,17-eicosatrienoic acid	C20:3n3	321	30.146	0.9918	$y = 261.24x - 230,243$
30.	Erucic acid	C22:1n9	353	30.147	0.9965	$y = 187.08x + 42,244$
31.	Arachidoic acid	C20:4n6	318	30.245	0.9926	$y = 152.73x + 32,586$
32.	Tricosanic acid	C23:0	369	30.713	0.9900	$y = 2.1268x - 1870.7$
33.	Cis-13,16-docosadienoic acid	C22:2n6	351	31.517	0.9970	$y = 1.2291x - 1040.4$
34.	Cis-5,8,11,14,17-eicosapentaenoic acid (EPA)	C20:5n3	316	31.901	0.9917	$y = 122.77x + 6381.4$
35.	Tetracosanoic acid	C24:0	383	32.281	0.9842	$y = 8.0799x - 11,217$
36.	Cis-15-tetracosenic acid	C24:1n9	381	33.234	0.9947	$y = 232.97x - 73,520$
37.	Cis-4,7,10,13,16,19-docosahexaenoic acid (DHA)	C22:6n3	343	36.354	0.9908	$y = 101.36x + 62,011$

cereus, V. vulnificus, and *P. mirabilis* remained unchanged as per the SSR increment.

The insignificant difference of MIC_{50} of *B. cereus* was given by 1:10 SSR (3.27 ± 0.10 mg/mL) as compared to 1:5 SSR (3.22 ± 0.06 mg/mL) (Table 2). The 1:5 SSR had positive effect against *P. mirabilis*, by rendering significant difference ($p < 0.05$) of MIC_{50} (1.84 ± 0.45 mg/mL). For *S. enteritidis* and *V. vulnificus*, the significant differences ($p < 0.05$) of lowest MIC_{50} (2.87 ± 0.10 mg/mL) and (1.87 ± 0.25 mg/mL), respectively were obtained from 1:10 SSR, where both MIC_{50} of these microorganisms indicated strong correlation against SSR.

However, all SSR treatments did not influence *B. cereus, V. vulnificus* and *P. mirabilis,* where the MIC_0 for these microorganisms remained at < 0.02 mg/mL. The MIC_0 from 1:10 SSR also had presented the lowest value for all microorganisms (< 0.02 mg/mL). The 1:10 SSR had lowered the MIC_0 of *S. enteritidis* to < 0.02 mg/mL from 0.70 mg/mL of 1:2 SSR (Table 2). The 1:10 SSR was chosen as the best SSR treatment compared to the 1:5 SSR and 1:2 SSR due to the lowest MIC_{50} and MIC_0.

3.3. Correlation of total phenolic and flavonoids, fatty acids and antibacterial potency of Carica papaya seed extract on the extraction treatments

The purposes of using PCA in this study were to describe the correlation and distribution of yield, TPC, TFC and free fatty acid methyl esters (FAMEs) on the antibacterial potency of *Carica papaya* seed extracts as affected by the SAE, CT and SSR. The free FAMEs are more volatile than free fatty acids, and due to this characteristic, the FAMEs are preferable for the detection by GC/MS (Dorni et al., 2018).

Table 3 shows the characteristics of the analytical curves with the R^2 values. The $R^2 > 0.98$ indicated that the analytical curve values had established linear regression models, which were adequate for the FAMEs determination in the *Carica papaya* seed extract (Sani et al., 2021b). By analyzing the FAMEs that take about 80.23% of the *Carica papaya* seed extract (Sani et al., 2020), we could investigate their influences on the yield, TPC and TFC on the pathogens inhibition via a chemometric technique such as PCA.

The PCA exhibited two principal components (PCs) entailing 36 variables that represent cumulative variability of 46% with an eigenvalue (EV) of 6.57 in Fig. 2(a) for the whole dataset. In principle, variables far away from the axes F1 and F2 had a strong factor loading (FL). Of the 36 variables, the yield, TPC, C18:1n9t, C15:0, C16:0, C18:2n6c and C21:0, MIC_{50}SE, MIC_{50}VV, MIC_0SE, had strong FL (FL ≥ |0.75|) that were dominant in this study. Besides, the TFC, C14:0, C16:0, C16:1, C18:0, C18:1n9c, C24:1n9, C6:0, C14:0, C18:0, and C23:0, MICBC, MICVV, MICPM, MIC_{50}BC, MICBC, MIC_0SE had moderate FL (|0.500| < FL < |0.749|) while the other variables had a weak FL (FL ≤ |0.499|).

Fig. 2(a) of the variable plot also depicts the positive correlations among yield, TPC, C18:1n9t and C16:1, since these variables were positioned together. Although free fatty acids and FAMEs were dominant in *Carica papaya* seed extract, other compounds such as organic acids and sterols with a phenolic backbone may render the antibacterial potency (Sani et al., 2020); therefore, the TPC may of an influencing factor in this study. The yield, TPC, C18:1n9t and C16:1 also had a negative correlation with the antibacterial variables except for MICSE on the opposite side of Fig. 1(a), i.e., MIC_{50}SE, MIC_{50}VV, MIC_0SE, MIC_{50}PM, MIC_0PM, MIC_0BC, MICBC, MIC_{50}BC, MICVV, MICPM and MIC_0VV. This correlation signified that higher yield, TPC, C18:1n9t and C16:1 possibly

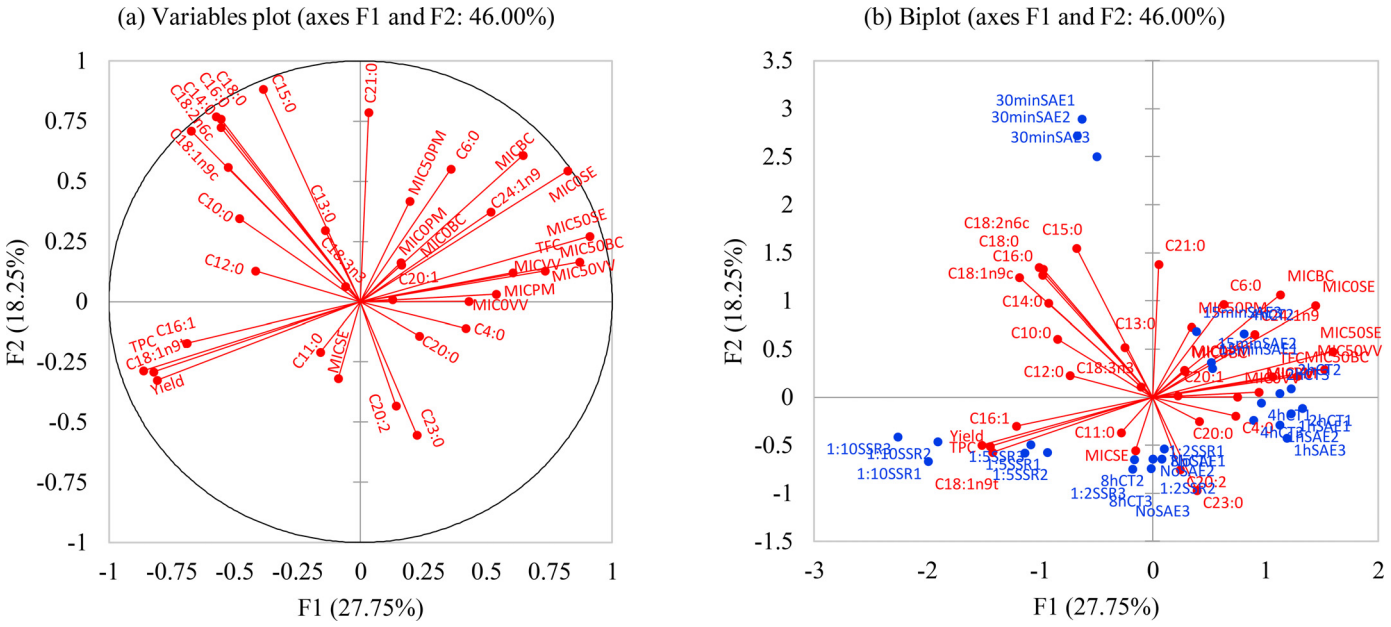

Fig. 2. (a) Variable plot and (b) biplot of correlation among the yield, TPC, TFC, and fatty acids and antibacterial potency of *Carica papaya* seed extract.

lead to lower MIC, MIC_{50} and MIC_0; hence, inhibited the growth of *B. cereus, V. vulnificus* and *P. mirabilis*. Likewise, since the C6:0 and C24:1n9 were located in the same quadrant of these antibacterial variables, they were moderately rendered increment growth of the pathogens. Also, the C20:1, C4:0 and C20:0 had weak effects on the increment of pathogens' growth. The opposite direction of the yield, TPC, C18:1n9t and C16:1 against C6:0 and C24:1n9, C20:1, C4:0 and C20:0 also indicated that these variables had antagonistic effects on the potency of *Carica papaya* seed extract. Hence, partially purified *Carica papaya* seed extract or a mixture of phenolics and purified C18:1n9t and C16:1 shall be employed instead of the crude extract to enhance the antibacterial potency of the *Carica papaya* seed extract against *B. cereus, V. vulnificus*, and *P. mirabilis*. This was evident since organic acids and sterols may have rendered MIC at 2.81 mg/mL, 0.35 mg/mL, and 1.41 mg/mL towards *B. cereus, V. vulnificus*, and *P. mirabilis*, respectively (Sani et al., 2021a). An interesting observation showed that C15:0, C18:0, C16:0, C14:0, C18:2n6c, C18:1n9c, C23:0 and C20:2 did not affect the inhibition of *B. cereus, V. vulnificus* and *P. mirabilis*. This finding was due to their 90° direction towards the MIC, MIC_{50} and MIC_0 of these pathogens.

Further investigation on the antibacterial variables, only MIC_{50}SE, MIC_{50}VV and MIC_0SE had the strong FL (Fig. 2(a)) due to PCA measures variances in the dataset. This variance measurement fitted for continuous variables compared to the end-point MIC value of pathogens (Sowhini et al., 2020). Hence, recording the antibacterial activity of *Carica papaya* seed extract would be better in MIC_{50} and MIC_0 instead of MIC.

To note that C21:0 and C15:0 had strongly inhibited the *S. enteritidis* growth, while C6:0 and C13:0 had moderate effects in Fig. 2(a). On the contrary, C23:0 had moderately enhanced the growth of this pathogen, while C20:0 and C11:0 had weak influences. Also, the C10:0, C12:0, C4:0 and C20:0 had no inhibition effect on this pathogen. These variables also denoted the synergistic effect among the free fatty acids and antagonistic reactions between C21:0, C15:0, C6:0 and C13:0, and C23:0, C20:0 and C11:0. This finding may propose that although individual short, medium, and long-chain free fatty acids have antibacterial activity (Bae & Lee, 2017), their combination may also facilitate pathogen inhibition.

The biplot in Fig. 2(b) exhibited four specific clusters, i.e., 1:10 SSR, 1:5 SSR, 30 min SAE and 15 min SAE, and two mixed clusters. The 1:10 SSR was positioned at the most left of the F1 axes, followed by the 1:5 SSR where the yield, TPC, C18:1n9t and C16:1 were high while C6:0, C24:1n9 and C20:1 were low in these clusters. The extraction of *Carica papaya* seed through no SAE, 8 h CT and 1:10 SSR would be the best treatments to exert the highest antibacterial potency because of their opposite position of the antibacterial variables except MICSE. Likewise, the no SAE, 8 h CT and 1:5 SSR extraction treatments could be the second-best option.

The 30 min SAE cluster was also dominant as it is located to the left side of the F2 axes in Fig. 2(b). High C21:0 and C15:0 and low C20:2 and C23:0 characterised this cluster which also denoted that the extraction treatments 30 min SAE, 8 h CT and 1:2 SSR could inhibit the *S. enteritidis* as depicted with the opposite position of this cluster against the MICSE.

Nevertheless, the 15 min SAE cluster was positioned with the antibacterial variables except for MICSE, indicating 15 min SAE, 8 CT, and 1:2 SSR could not enhance the antibacterial potency of *Carica papaya* seed extract (Fig. 2(b)). This result was due to high C6:0, C24:1n9 and C20:1 and low yield, TPC, C18:1n9t, and C16:1 may render antagonistic effects and had facilitated the pathogens' growth. One mixed cluster consisting of 1 h SAE, 2 h CT and 4 h CT also had indicated they could not inhibit the pathogens' growth. In the same position, the C4:0 and C20:0 were dominant in this cluster. Another mixed cluster had the extracts produced from the standard extraction treatments, i.e., no SAE, 8 h CT and 1:2 SSR. The C23:0, C20:2, and C11:0 were high in this cluster, but they could not inhibit the *S. enteritidis*. To note, the two mixed clusters and 15 min SAE cluster were located at the center of the F1 and F2 axes; therefore, they were the least effective extraction treatment in this study.

From these evaluations, the no SAE, 8 h CT and 1:10 SSR would be the best treatments to inhibit the *B. cereus, V. vulnificus*, and *P. mirabilis* growths while 30 min SAE, 8 h CT and 1:2 SSR could be the best extraction treatments to inhibit the *S. enteritidis*. It is also recommended to apply PCA to facilitate the interpretation of multivariable datasets instead of evaluating individual variables (Sani et al., 2021b). Besides providing information on the best extraction treatments, the PCA presents in-depth information on the variable correlations and proposes variables with synergistic and antagonistic effects that the evaluation of individual variables could not provide.

4. Conclusion

The chemometrics-based evaluation via PCA was superior over the individual evaluation treatment on antibacterial potency of *Carica pa-*

paya seed extract. The no SAE, 8 h CT and 1:10 SSR were the most efficient treatments for extracting antibacterial compounds from *Carica papaya* seed than other SAE and CT treatments. These extraction parameters also rendered the highest antibacterial activities against the *S. enteritidis, B. cereus, V. vulnificus* and *P. mirabilis*. To note, this study was carried out in a laboratory scaling which showed that high CT and low SSR might increase the antibacterial activity; hence, other or future research may choose a high CT and low SSR in their antibacterial studies based on our finding. Also, this study did not identify individual phenolics and flavonoids since the analysis of these compounds is in the pipeline and will be reported in the subsequent report. Besides identifying the best treatment, the PCA had successfully identified the characterizing variables on the treatments and synergistic and antagonistic effects of the variables. Although the PCA could delineate this information, only 46% of the dataset was explained in this study; hence, more variables and samples will be included in future research. The results and methods described here could help optimize extraction procedures through antibacterial test assessment and thus help reveal novel antibacterial compounds, which are expected to serve as an antibacterial agent food preservative. By adopting the chemometric-based approach, further research on the antibacterial activity of plant by-products could identify the significant antibacterial compounds that have synergistic and antagonistic effects without carrying out extract purification. Hence, the cost of the analysis could be reduced.

Research involving human participants and/or animals

We declare no human and/or animals involved in this study.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Muhamad Shirwan Abdullah Sani reports financial support was provided by the Islamic Development Bank and the Ministry of Higher Education Malaysia. Muhamad Shirwan Abdullah Sani reports a relationship with the and Malaysia Ministry of Higher Education that includes: funding grants.

CRediT authorship contribution statement

Muhamad Shirwan Abdullah Sani: Conceptualization, Data curation, Writing – original draft. **Jamilah Bakar:** Methodology. **Azman Azid:** Software, Validation. **Muhammad Javid Iqbal:** Visualization, Investigation.

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