Evaluation of phenolic constituent, antioxidant and antibacterial activities of sugarcane molasses towards foodborne pathogens


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Abstract

The employment of chemical synthetic as an antimicrobial agent in inhibiting microbial growth has become a major concern due to adverse health impact, food safety crisis and the pressure on food manufacturers. Essential bioactive compound in sugarcane molasses, a by-product from a sugar refinery process could be effective as an alternative antimicrobial substance. However, their antimicrobial properties are not understandable. This study aimed 1) to detect the total phenolic compounds present in sugarcane molasses extract and 2) to determine the antioxidants and antibacterial activities of sugarcane molasses extract towards foodborne pathogens. The phenolic compounds of sugarcane molasses extract were determined by UHPLC-MSMS. Antioxidant activities were estimated by a total phenolic compound assay and a 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. Meanwhile, antibacterial activities were carried out via disc diffusion, minimum inhibition concentrations (MICs) and minimum bactericidal concentrations (MBCs) assays. In this study, several extracted compounds were identified in sugarcane molasses extract and included gallic acid, phenylvaleric acids, quinic acid, tannic acid and 6-C-glucosyl-8-C-arabinosyl apigenin or arabinoysl-glucosylapigenin. The sugarcane molasses extract showed high total phenolic compounds with values of 7.6 mg GAE/g extract. Meanwhile, antioxidant activities of sugarcane molasses extract were also found high and the 50% inhibitory concentration (IC_{50} value) was about 0.79 mg QE/g. The inhibition zone against four foodborne pathogens, Staphylococcus aureus, Listeria monocytogenes, Escherichia coli and Salmonella enterica serovar Typhimurium ranged from 8.82±0.3 mm to 25.05±1.6 mm. Meanwhile, the MICs of sugarcane molasses extract ranged from 3.125% to 6.25% v/v and MBCs were 6.25% to >12.5% v/v. In conclusion, sugarcane molasses extract is rich in phenolic compounds and has the potential to be applied as the natural antioxidant and antibacterial compounds.

1. Introduction

Since the last decade, synthetic chemical preservative has been used as an antimicrobial agent to control microbial food spoilage and extend the product shelf life (Saeed et al., 2019). However, the application of synthetic preservative has increased adverse health impact, food safety crisis and the pressure on food manufacturers. The abuse and misuse of synthetic preservative caused the rising of potential toxicity in food product and led to the development of antimicrobial resistance (Nychas, 1995; Bilal et al., 2017). With consumer awareness of food safety, synthetic-free preservative has become a high demand in the food market (Arshad and Batool, 2017). This reveals the need for an alternative antibacterial as one of the strategies to preserve food from spoilage and pathogenic microorganism.

Thus, extensive research to extract natural antibacterial from the plant is employed. This ‘greener’ technology is introduced as they were potent biochemical factories with phytomedicine component (Mohanraj, 2014). Plant materials which initially used as a traditional healer had been a precious resource for the medicinal and pharmaceutical field nowadays. This is
because plant-originated medicine is more economical, environmental, and health-friendly compared to the synthetic antibacterial (Dahiya and Purkayastha, 2012). Phytoconstituent or phytobiotic in plant material such as alkaloid, tannins, phenolic acid, etc., are potentially effective as natural antibacterial therapy to many diseases (Edeoga et al., 2005; Dhama et al., 2014).

Sugarcane molasses is thick, dark, and sweet syrup derived from the sugar refining process (Arimi et al., 2014). It is composed by numerous essential elements; sugar, nitrogenous materials, organic acid and trace compounds provide multi-beneficial health consequence (Olbrich, 1963; Saska and Chou, 2002; Zhao et al., 2015). Besides, the composition of sugar cane molasses contain phenolic compounds that posing antioxidant (Saska and Chou, 2002; Guimarães et al., 2007; Valli et al., 2012), antibacterial (Takara et al., 2007; Chandra et al., 2008; Zhao et al., 2015), and DNA-damaging-protective activities (Guimarães et al., 2007; Abbas et al., 2014). Even though many researchers have carried out studies to evaluate different epidemiological of sugarcane molasses, only a little research reported on its antioxidant and antibacterial properties. Therefore, the objective of this work was to detect the total phenolic compounds present in sugarcane molasses extract, analyse the antioxidant activities of sugarcane molasses extract and explore its potent antibacterial activities against foodborne pathogens.

2. Materials and methods

2.1 Sample preparation

In this study, a sugarcane by-product, which is known as molasses, was used. The sugarcane molasses was received from Matahari Sdn. Bhd., Selangor, Malaysia.

2.2 Extraction of sugarcane molasses

A total of 60 mL of acidic ethanol solvent (Systerm, Selangor, Malaysia) and 2 g of sugar cane molasses were added into 100 mL beaker. The mixture was sonicated using ultrasound-assisted extraction machine for 90 mins. Next, the treatment was centrifuged at 1000 x g for 10 mins. The supernatant was concentrated to 10 mL in a vacuum and freeze-dried at 45°C in a vacuum and freeze-dried torr. The extract was re-dissolved in deionised water for next uses.

2.3 Total polyphenol content

The total phenolic content of sugarcane molasses was evaluated using the Folin-Ciocalteu method of Abas et al. (2014) with slight modifications. Briefly, 20 µL of sugarcane molasses extract in DMSO, and 100 µL of Folin-Ciocalteu reagent was transferred to 96-well microplate. The mixture was mixed well and stayed in the dark for 5 mins. Then, 80 µL of 75% sodium carbonate (2%) was added to the filled well. The microplate was immediately placed in a microplate reader. The absorbance was measured at 765 nm. Total phenolic content was calculated from a calibration curve of gallic acid and expressed as milligrams of gallic acid equivalent (GAE) per gram of sugarcane molasses extract.

2.4 Radical scavenging activity

Radical scavenging activity of sugarcane molasses extract was evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) method described by Mohamad et al. (2004) with slight modifications. Briefly, 50 µL of sugarcane molasses extract in DMSO, and 100 µL of DPPH reagent was transferred to 96-well microplate. Then, 50 µL of sugarcane molasses extract was prepared in 100 µl MeOH as the sample standard in the same microplate. 100 µL of DPPH reagent was added into all well and kept in the dark for 30 mins. The absorbance was then measured at 517 nm. The percentage inhibition of DPPH activity was calculated based on the following formula:

\[
\frac{(OD_{control} - OD_{sample}) \times 100}{OD_{control}}
\]

2.5 UHPLC-MS/MS analysis

Phenolic constituents of sugarcane molasses extract were identified using the Ultra-High Performance Liquid Chromatography-Mass Spectrometry (UHPLC-MS/MS) method. The column used was a Phenomenex Synergy RP C18 column (100A x 100 mm x 3µm x 2.0 mm) at a flow rate of 400 µL/min and using 20 µL injections. The Flexar UHPLC system was coupled to a Sciex 3200 hybrid trap triple quad tandem mass spectrometer. The mobile phase comprised (A) water and 0.1% formic acid; and (B) acetonitrile and 0.1% formic acid. Phenolic compounds were monitored and identified by comparing the chromatographic retention times and total intensity measured. Sciex internal natural product database was used as the mass spectral library for the identification of phenolic compounds. All assays were carried out in triplicate.

2.6 Test microorganisms

Four bacterial strains used were two Gram-positive Staphylococcus aureus ATCC 29737, and Listeria monocytogenes ATCC 19112, and two Gram-negative Escherichia coli ATCC 10536 and S. enterica serovar Typhimurium ATCC 13311. The stock cultures of all four bacteria were obtained from the culture collection of...
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2.7 Preparation of test microorganisms

The stock cultures of the bacteria were sub-cultured on Nutrient agar (Oxoid, UK) at 37°C for 24 hrs. Colonies of fresh cultures of the different microorganisms from overnight growth were picked and suspended in 3 mL of Nutrient broth (Oxoid, UK) in falcon tubes and incubated for 24 hrs at 37°C. Then, the inoculum was diluted in 0.1M Phosphate-buffered saline (PBS) to standardise density for use in the next assay.

2.8 Disc diffusion assay

Antibacterial activities of sugarcane molasses extract were measured by using a standard disc diffusion method (Bauer et al., 1966). Four selected foodborne pathogens; S. aureus ATCC 29737, L. monocytogenes ATCC 19112, E. coli ATCC 10536 and S. enterica serovar Typhimurium ATCC 13311 were prepared in broth dilution series to 10⁵ CFU/mL. 0.1 mL of the diluted inocula were uniformly spread on Mueller-Hinton (MH) agar (Oxoid, UK) using a sterile cotton swab. Then, the 6 mm discs filled with different concentrations of diluted molasses extract (6.25%, 12.5% and 25% w/v) were placed on the inoculated MH agar. Sterile distilled water was used as a negative control, and standard ampicillin disc was used as a positive control. The plates were incubated at 37°C for 24 hrs. The results were obtained by measuring the diameter of the inhibition zone (DIZ).

2.9 Minimum Inhibitory Concentrations (MICs)

The minimum inhibitory concentrations (MICs) were measured by the micro dilutions using a 96-well microplate with two-fold serial dilutions (0.049, 0.098, 0.195, 0.391, 0.781, 1.562, 3.125, 6.25, 12.5, 25% w/v). All the inhibited bacteria by the treatment of sugarcane molasses extract were used and prepared in a broth dilution series to 10⁵ CFU/mL. 0.1 mL of inocula were injected into microplate wells 3 to 12. Then, two-folded serially diluted sugarcane molasses extract was pipetted into the inoculated wells from wells 12 to 3. Fresh uninoculated MH broth was used as a positive control and inoculated MH broth was used as a negative control. The microplates were incubated overnight. The turbidity was checked through the Benchmark Plus microplate spectrophotometer (Scopic Resources, Pulau Pinang, Malaysia) to determine the MICs.

2.10 Minimum Bactericidal Concentrations (MBCs)

MBC test was performed following the MIC test via a streak plate method. Each sugarcane molasses dilutions with no bacterial growth (light turbidity) from the MIC test was assayed. A loop-full of bacterial strain was inoculated into sterile Mueller-Hinton plates. The plates were then incubated at 37°C for 24 hrs. The least concentration that did not show any growth of tested organisms was considered as the MBC value of the tested sugarcane molasses extract against the tested bacterial species (Balouiri et al., 2016).

2.11 Statistical analysis

The experimental results were statistically analysed using Microsoft Excel 2013. All experiments were performed in triplicate and the results were expressed as average values.

3. Results and discussion

3.1 Total phenolic content of sugarcane molasses extract

Phenolic compounds are secondary metabolites that can be found in plant tissues which responsible as the bioactive compound. They are made up of hydroxylated aromatic rings attached to the phenyl or aryl group compound (Balasundram et al., 2006). Phenolic compounds are the most abundant structures in plants that possess antioxidant activity through their redox reaction (Johari and Khong, 2019). Total phenolic content is an analysis to measure the amount of phenolic content in one sample. In order to calculate the total phenolic content of sugarcane molasses extract, a gallic acid standard graph was developed (Figure 1) and standard curve \( y = 1.506 \ln (x) + 0.801 \), where \( R^2 \) = 0.987. Based on the standard graph, the total phenolic content was expressed as 7.60 mg GAE/g extract. This was slightly higher compared with the study by Zhao et al. (2015), 4.32 mg GAE/g. The result was in agreement with the previous research by Iqbal et al. (2017) in which sugarcane molasses extract exhibited a higher total phenolic compound than other sugarcane by-products due to the high coloured component as a source of phenolic compounds. The high amount of phenolic content in sugarcane molasses extract can be an aid for bioactivity. Thus, the sugarcane molasses extract was expected to have some antioxidant and antibacterial activities.

3.2 Antioxidant activities of sugarcane molasses extract

Phenolic compounds in the plant have the ability to scavenge free radical because they contain hydroxyl groups. They can easily donate an atom to the unstable free radical; hence the importance of their antioxidant activity (Kaurinovic and Vastag, 2019). In this study, the DPPH assay was used to evaluate antioxidant properties by estimating free radical scavenging activity. The DPPH free radical has the ability to accept an electron from an antioxidant compound (Aksoy et al., 2013).
Figure 2 shows the scavenging of radicals was found high and the 50% inhibitory concentration (IC\textsubscript{50} value) was about 0.79 mg QE/g sugarcane molasses extract. The concentration for IC\textsubscript{50} detected in the extract was notable and resulted in high antioxidant activities. In agreement with the previous study, sugarcane molasses exhibited high antioxidant effect activity with value of 1.9 mg TE/g extract (Ali \textit{et al}., 2019). High antioxidant activity of sugarcane molasses extract has a positive relationship with the total phenolic content. Commonly, the total phenolic compound is correlated to the antioxidant activity. This is because they have the capability to destroy free radicals by transferring their electrons to react with the free radicals (Pior \textit{et al}., 2005). The antioxidant properties of sugarcane molasses extract might be attributed to the higher phenolic compounds present. The total phenolic compounds and antioxidant properties in sugarcane molasses extract can be an essential parameter response to the presence of antimicrobial activities.

### 3.3 Presence of phenolic constituent in sugarcane molasses extract

UHPLC-MSMS is an analytical technique used as a qualitative analysis of non-volatile compounds such as phenolics, terpenoids and alkaloids (Kumar, 2017). This analysis was employed to identify the major phenolic constituent present in sugarcane molasses extract. This provides separation of compounds into stationary and mobile phases. In the present study, UHPLC was coupled to a mass spectrometer (MS) to improve and facilitate the detection of metabolites in a better resolution and fast run time. The combination of UHPLC-MS provides excellent selectivity and sensitivity (Lei \textit{et al}., 2018). Figure 3 represents the major compounds in sugarcane molasses that have been separated through chromatography. Retention time (RT) is responsible for measuring the time taken for a compound to pass chromatography from injection time until the detection time of the particular compounds.

In sugarcane molasses extract, several major phenolic constituents were identified. These were phenylvaleric acid, quinic acid, tannic acid, apigenin and gallic acid as recorded in Table 1. However, there were still several unknown compounds present that not is able to identify in this study. Thus, more work is required to identify these compounds. In UHPLC-MSMS separation and detection, the compound detected with m/z value of 175.03 (RT=0.993min) was phenylvaleric acid. The identities of quinic acid, tannic acid, apigenin and gallic acid as recorded in Table 1.
Phenolic compounds with less-complex structures possess antimicrobial activities and play essential roles in killing antibiotic-resistant pathogens. Snoch et al. (2019) suggested phenylvaleric acid, which is one of the phenolic acids, has a strong antibacterial effect towards S. aureus, C. albicans and E. coli. Quinic acid also has strong antibacterial properties where it can easily attack the intracellular cell of bacteria. Quinic acid has the ability to reduce the succinate dehydrogenase activity and DNA content of bacteria (Bai et al., 2018). Studies by Slobodníková et al. (2016) demonstrated tannic acid which represents one of the most common polyphenols, possess antibacterial against both Gram-positive and Gram-negative bacteria by penetrating the lipid bilayers and damaging their membrane. In short, the phenolic constituents that have been identified in sugarcane molasses extract might have high biological and pharmacological activity hence can be employed as antimicrobial agents in food.

### 3.4 Diameter of inhibition zone

The agar diffusion assay was developed as a primary test to determine the antibacterial activities of an organic or inorganic agent. It showed if the antibacterial agents can inhibit or kill the bacteria. Evaluations of antibacterial activities of sugarcane molasses extracts against four foodborne pathogens are recorded in Table 2. The results showed that sugarcane molasses extract exhibited significant inhibitory effect towards all tested food-borne pathogens. The mean inhibition zone diameter against L. monocytogenes ATCC 19112, E. coli ATCC 10536 and S. enterica serovar Typhimurium ATCC 13311 in 6.25% sugarcane molasses extract produced no significant effect, however, Staphylococcus aureus ATCC 29737 showed an inhibition effect with diameter zone 11.67±1.6 mm. At a concentration of 12.5% sugarcane molasses extract, both Gram-positive S. aureus ATCC 2973 and L. monocytogenes ATCC 19112 had a zone of inhibition of 20.47±1.6 mm and 7.60±1.1 mm, respectively. Meanwhile, for Gram-negative E. coli ATCC 10536 and S. enterica serovar Typhimurium ATCC 13311, the inhibition zones were 6.89±0.7 mm and 6.67±0.4, respectively. At a concentration of 25%, clear inhibition zones were seen for S. aureus ATCC 2973, L. monocytogenes ATCC 19112, E. coli ATCC 10536 and S. enterica serovar Typhimurium ATCC 13311 at 25.05±1.6, 9.97±0.2, 9,01±0.7, and 8.82±0.3 mm, diameter respectively. The antibacterial effect of sugarcane molasses extract was higher for Gram-positive bacteria compared to Gram-negative bacteria. S. aureus ATCC 2973 was found to be the most sensitive to the antibacterial effect of sugarcane molasses extract. This study showed Gram-positive bacteria were easier to be inhibited by the sugarcane molasses extract compared to Gram-negative bacteria. The previous study also reported the antibacterial effects of sugarcane bagasse extract, a by-product of sugar manufacturing, were significantly higher in Gram-positive bacteria compared to Gram-negative bacteria (Zhao et al., 2015). This is in contrast to Chen et al. (2017) reported that Gram-negative bacteria was the one that can be inhibited more easily by the molasses compared to Gram-positive bacteria.

The different results observed in antibacterial activity may be due to the differences in susceptibility of each species of bacterium rather than merely a difference between Gram-positive and Gram-negative bacteria. Gram-negative bacteria are highly resistant to the sugarcane molasses extract may be due to their complex membrane that blocks the diffusion of foreign materials (Dahl et al., 1989). Besides, the inconsistency of results

<table>
<thead>
<tr>
<th>Peak number</th>
<th>RT (min)</th>
<th>Measured values (m/z)</th>
<th>Tentative Identity of Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.993</td>
<td>175.03</td>
<td>Phenylvaleric acid</td>
</tr>
<tr>
<td>2</td>
<td>1.298</td>
<td>191.22</td>
<td>Quinic acid</td>
</tr>
<tr>
<td>3</td>
<td>4.441</td>
<td>183.19</td>
<td>Tannic acid</td>
</tr>
<tr>
<td>4</td>
<td>8.205</td>
<td>563.46</td>
<td>Apigenin</td>
</tr>
<tr>
<td>5</td>
<td>5.666</td>
<td>169.2</td>
<td>Gallic acid</td>
</tr>
</tbody>
</table>

### Table 2. Antibacterial properties of sugarcane molasses extract against selected foodborne pathogens using disc diffusion assay

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>The diameter of the inhibition zone, DIZ (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sugarcane molasses extract, w/v</td>
</tr>
<tr>
<td></td>
<td>6.25%</td>
</tr>
<tr>
<td>S. aureus ATCC 29737</td>
<td>11.67±1.6</td>
</tr>
<tr>
<td>L. monocytogenes ATCC 19112</td>
<td>NIZ</td>
</tr>
<tr>
<td>E. coli ATCC 10536</td>
<td>NIZ</td>
</tr>
<tr>
<td>S. enterica ser. Typhimurium ATCC 13311</td>
<td>NIZ</td>
</tr>
</tbody>
</table>

*: The inhibition zones (in diameter) were included 6 mm of the disc; : (NIZ) means no inhibition zone.
between our results and those of others may relate to the cultivation of sugarcane and the effect of geographical area (Tumin et al., 2005).

3.5 MIC and MBC of sugarcane molasses extract

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests were used to observe the inhibition of growth and eradication of bacteria, respectively. This study refers to MIC as the lowest concentration required to inhibit 99% of bacterial growth. Meanwhile, MBC is defined as the lowest concentration needed to kill at least 99% of the bacteria.

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of sugarcane molasses extract towards four food-borne pathogens; S. aureus ATCC 29737, L. monocytogenes ATCC 19112, E. coli ATCC 10536 and S. enterica serovar Typhimurium ATCC 13311 are detailed in Table 3. The results showed MIC values lie between 3.125% and 12.5% respectively, for the four bacteria. The lowest MIC values (3.125%) were observed on the sugarcane molasses extract towards both Gram-positive bacteria, S. aureus ATCC 29737 and L. monocytogenes ATCC. Meanwhile, the extract against both Gram-negative bacteria, E. coli ATCC 10536 and S. enterica serovar Typhimurium ATCC 13311 was one fold more than the inhibition of the other bacteria with values of 6.25%.

Table 3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of sugarcane molasses extract against selected foodborne pathogens

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>MICs (v/v)</th>
<th>MBCs (v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus ATCC 29737</td>
<td>3.13%</td>
<td>6.25%</td>
</tr>
<tr>
<td>L. monocytogenes ATCC 19112</td>
<td>3.13%</td>
<td>6.25%</td>
</tr>
<tr>
<td>E. coli ATCC 10536</td>
<td>6.25%</td>
<td>12.50%</td>
</tr>
<tr>
<td>S. enterica ser. Typhimurium</td>
<td>6.25%</td>
<td>&gt;12.5%</td>
</tr>
</tbody>
</table>

Minimum bactericidal concentrations can be affected by ethanol extraction. These effects were also observed against four microorganisms tests which are S. aureus ATCC 29737, L. monocytogenes ATCC 19112, E. coli ATCC 10536 and S. enterica serovar Typhimurium ATCC 13311 for sugarcane molasses extract. Based on Kang et al. (2011), the value of the lowest MBC obtained was not more four times higher than that of MIC’s for a particular pathogen. The lowest MBC (6.25%) was obtained for S. aureus ATCC 29737 and L. monocytogenes ATCC 19112 in this study. Meanwhile, the highest MBC values were (>12.5%) obtained from S. Typhimurium ATCC 13311. The values were not more than four times greater than that of the MIC’s for the corresponding microorganism (Table 3). From this study, we conclude that Gram-positive bacteria were more sensitive to sugarcane molasses extract compared to the Gram-negative bacteria.

Higher inhibition against Gram-positive bacteria may be due to the composition and the structure of the cell wall (Delgado-Adámez et al., 2012; Zhao et al., 2015). The thick peptidoglycan with a sensitive layer in Gram-positive, bacteria can be penetrated by foreign materials and absorbed into easily. This characteristic provides a high opportunity for the sugarcane molasses extract to disrupt the cell wall synthesis of bacteria, thus causing death, (Guerra-Rosas et al., 2017). The lower bacteriostatic activity was found with Gram-negative bacteria. The less susceptibility of these isolates towards sugarcane molasses extracts may be due to the presence of the lipopolysaccharide layer which limits the diffusion of hydrophobic compounds (Dahl et al., 1989; Adamez et al., 2012; Zhao et al., 2015; Chen et al., 2017). Gram-negative bacteria also have an outer membrane with an external monolayer of lipopolysaccharide which was robust and highly impermeable to toxins and antibacterial agents (Arumunnee et al., 2016).

4. Conclusion

In conclusion, the results obtained in the present study indicate that sugarcane molasses extract is a valuable by-product, which contains various polyphenol compounds that contribute to the antioxidant and antibacterial properties. The total phenolic content in this extract was evaluated and found to be high in comparison to previous studies. The extract also showed antioxidant capabilities and had high antibacterial activity towards selected foodborne pathogens. Sugarcane molasses may be applied as an alternative antibacterial compound. However, this needs to be confirmed by the application of sugarcane molasses extract in the food system.

Conflict of Interest

The authors declare no conflict of interest.

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