

CHAPTER 8 :DISCUSSION

It can be summarized from the research that:

- 1) The antibacterial activity of Ajwa date extracts increased with heat-treatment, however longer heating durations at a higher temperature would decrease the antibacterial activity in Ajwa date. The antibacterial activity of Ajwa date extracts was dose-dependent, in which higher extract concentration showed higher bacterial inhibition zone. The methanol extract exhibited more potent antibacterial activity, with higher bacterial inhibition zone and lower MIC/MBC values against all tested bacteria compared to aqueous extracts.
- 2) Antimicrobial activity against all tested bacteria was recorded from the Ajwa dates-infused water prepared according to the Prophet Muhammad's (PBUH) practice. The antibacterial activity showed a positive correlation with the phytochemical content in Ajwa dates-infused water. Therefore, the antibacterial properties of Ajwa dates were possibly due to the presence of phenolic, tannin, flavonoid, or synergy of the active compounds.
- 3) Comparison between different date varieties showed that Ajwa, Medjool and Mariami date extracts showed antibacterial and anti-adhesion activities against all tested bacteria; with Ajwa dates hot aqueous extracts showed significantly higher inhibition zone and higher anti-adhesion activity against all tested bacteria compared to Medjool and Mariami date extracts. Phytochemical analysis showed that Ajwa date extracts contained the highest phytochemical content, with 78

compound peaks being detected compared to Mariami and Medjool dates. Date fruits extracts contained various phytochemicals, including phenolic acid, flavonoids, organic acids, fatty acids, and terpenoids. It was found from the phytochemical quantification that Ajwa dates contained significantly higher TPC and TFC compared to Medjool and Mariami dates.

- 4) Flavonoids compounds in Ajwa dates consisted of Catechin 7,4'-dimethyl ether, Hexa-acetyl-pyracanthoside, Luteolin 7, 3',4'-trigluconide, Kaempferol 3-(2",3"-diacetyl-4"-p-coumarylrhamnoside, and Cyanidin 3-rutinoside-5-glucoside. These compounds were responsible for the antibacterial and anti-adhesion activities of Ajwa date. This result indicated that the flavonoid compounds in Ajwa dates had a role in inhibiting/killing and preventing the adhesion of bacterial gastroenteritis. SEM and TEM found that the mechanism of Ajwa dates extracts and flavonoids antibacterial activity involved in the disruption of the cell wall and intracellular alteration of the cells were vital for the survival of bacteria. Therefore, the morphological damages and alteration led to bacterial cells death.

8.1 Optimisation of Extraction and Antibacterial Activity of Ajwa Dates

8.1.1 Optimisation of Extraction of Ajwa Dates

The initial stage of studying medicinal plants in the preparation of plant samples to preserve their biomolecules in the biomass before the extraction. In most cases, the dried sample is preferred as fresh samples are fragile and tend to deteriorate faster compared to dried samples. Ajwa dates at the dried stage (Tamar) were used in this study due to the aforementioned reason and their easy accessibility in Malaysia.

Extraction is the second crucial step in the preparation of potential plant candidates for natural product drug discovery. This process determines the quantity and quality of the crude extracts (Azwanida, 2015; Altemimi et al., 2017). Notably, when different methods are applied on the same plant material with the same solvent, extraction efficiency can vary significantly, which indicates the importance of the selection of the most appropriate extraction methodology (Jadhav et al., 2009). Many types of solvents are available for extracting plant materials, including water, methanol, ethanol, acetone, chloroform, hexane, and petroleum ether, with water and ethanol, and methanol being the most widely used solvents.

A previous study recorded that methanol was more effective in extracting the higher amount of phenolic, flavonoids, and alkaloids constituent (Koffi et al., 2010; Altemimi et al., 2017; Truong et al., 2019). Furthermore, a systematic review study by Farhana et al. (2017) demonstrated that dates fruit methanol extract involved the highest antibacterial activity compared to aqueous and other solvents (Farhana et al., 2017). Despite the previous report regarding antimicrobial activities of Ajwa date methanol extract (Al-Judaibi et al., 2014; Samad et al., 2016), the published data on aqueous extracts of Ajwa date were either scarce or absent. Therefore, hot aqueous and cold

aqueous extraction was conducted to assess the antibacterial activity of Ajwa dates. For solvent extraction, methanol was used to assess date fruit antibacterial activity. Additionally, aqueous extracts were selected to follow the practice of the Prophet Muhammad (PBUH) in the consumption of dates.

In some extraction processes, heat treatment is required for the extraction of active constituent in plants. The thermal processing may release more bound phenolic acids from the breakdown of cellular constituents (Chism & Haard, 1996). However, provided that some plants are labile to heat, the suitable temperature to extract the active compounds should be determined. For this reason, Ajwa dates were extracts subjected to different heat temperatures and heating times to observe the thermostability of Ajwa dates on its antimicrobial activities.

It was observed in this study that the extract heated at 100 °C for 30 minutes was recorded with higher inhibition zone diameter compared to the extract heated at 60 °C. However, the extracts heated at 100 °C for 60 minutes showed lower inhibition zone diameter compared to the extracts heated at 60°C. According to Shi et al. (2003), the heating process may soften the plant tissue and weaken the phenol-protein and phenol polysaccharide interactions in the plant materials. Therefore, more phenols would transfer to the solvent portion (Shi et al., 2003), leading to an increase in antimicrobial activity of the plant extracts. However, excessive heating may lead to compound degradation (Muhamad et al., 2015). The decrease in antibacterial property caused by heating was possibly due to volatilisation and/or the physical and chemical changes occurring during heating (Durairaj et al., 2009). Provided that plants are labile to heat, the suitable temperature to extract the compounds should be determined. The results of this study that heat treatment increased the antibacterial activity were supported although heating at a higher temperature and longer durations would reduce the

antimicrobial activity. Therefore, heating at 60°C for 60 minutes was the optimum temperature and time for extraction, which was also selected for hot aqueous extraction.

8.1.2 Ajwa Date Extract Has Antimicrobial and Anti-adhesive Properties Against Selected Bacteria Causing Gastroenteritis

After extraction, the antimicrobial properties of Ajwa date extracts were assessed. Subsequently, antibacterial and anti-adhesion activities against the selected bacterial gastroenteritis were identified. Notably, this study finding matched the findings observed in an earlier study by Samad et al. (2016), which recorded that antibacterial activity against *S. aureus*, *B. cereus*, *E. coli* and *S. marcescens* (Samad et al., 2016) took place in Ajwa date methanol extracts. However, a study by Al-Judaibi et al. (2014) reported that Ajwa date methanol extract did not show antibacterial activity against MRSA ATCC335910 and *S. aureus* which contradict with this study (Al-Judaibi et al., 2014). Furthermore, it was observed that antimicrobial activity results of the same tested plant part varied from researcher to researcher as plant constituents could vary due to geographical location, age of the plant, topographical factors, and the nutrient concentrations of the soil (Kaneria et al., 2009; Doughari, 2012). Accordingly, the number of doses used, extraction methods, diffusion capacity of a substance, the method used for the antimicrobial study, and different sensitivity of test strain led to antimicrobial activity differences (Nimri et al., 1999; Kaneria et al., 2009; Aziman et al., 2014).

Based on the antimicrobial activity assessment using well diffusion assay and MIC/MBC on the degree of the diameters of inhibition zone and assorted MIC/MBC values, the varieties were based on the type of extraction and tested bacteria used. The

methanol extract showed higher antibacterial activity with a higher diameter of inhibition zone and lower MIC value against all tested bacteria compared to aqueous extract. Meanwhile, methanol is a polar solvent, and antimicrobial compounds, such as phenolic, flavonoids and tannin, have been identified to be soluble in polar solvents (Cowan, 1999; Bimakr et al., 2011; Altemimi et al., 2017).

Among aqueous extracts, the hot extract exhibited higher antibacterial activity compared to cold extract. The heating process may soften the plant tissue and weaken the phenol-protein and phenol polysaccharide interactions in the plant materials. Therefore, a mass transfer would be promoted, while more phenols would transfer to the solvent portion (Shi et al., 2003; Spigno & Dante Marco, 2007; Dorta et al., 2012).

The antimicrobial activity of Ajwa date extracts, which was dose-dependent, was determined at different concentrations. Different extract concentrations would yield different results related to the inhibitory effect on microorganism growth. Specifically, higher concentrations exhibited a higher diameter of inhibition zone against all tested bacteria as the date extracts at higher concentrations contain more antimicrobial compounds. Studies related to antimicrobial activity were also observed to produce similar results (Joshi & John, 2002; Ali et al., 2005; Prasad et al., 2008; Chua & Aminah, 2017). All extracts at the concentrations ranging from 200 mg/ml to 500 mg/ml exhibited antimicrobial activity although the cold aqueous extract at the concentration 200mg/ml did not exhibit antibacterial activity against *E. coli*. Moreover, all extracts tested at the concentration of 100 mg/ml did not exhibit any antimicrobial activity. While the extract at this concentration may contain antimicrobial constituent, the amount was not sufficient to be effective. Overall, this result matched the results observed in an earlier study by Samad et al. (2016), which found that Ajwa methanol

extracts at a concentration of 100 mg/ml did not exhibit any antibacterial activity against *S. aureus*, *B. cereus*, *E. coli*, and *S. marcescens* (Samad et al., 2016).

In this present study, the gram-positive *S. aureus* was found to be more sensitive to Ajwa date extracts due to the relatively higher inhibition zone compared to other gram-negative bacteria. Gram-negative bacteria were more resistant due to the presence of lipopolysaccharides in their cell membrane, which was impermeable to lipophilic compounds (Stefanello et al., 2008; Aziman et al., 2014). Provided that active phytochemicals, such as phenolic and flavonoids present in Ajwa dates are lipophilic compounds, the compounds were more permeable and absorbed to phospholipid bilayer of the gram-positive bacterial cell membrane (Liu et al., 2014; Xie et al., 2015; Khalid et al., 2017).

Besides determining the inhibition/killing effects of date fruit extract, the anti-adhesive activity of Ajwa dates extracts against tested bacteria was evaluated using caco-2 cells models. The prevention of infection by anti-adhesion is directed to the initial attachment of the bacteria to human cells, which is the first step of microbial colonization and pathogenesis (Toivanen et al., 2017). Therefore, anti-adhesion is one of the approaches using an agent, which will inhibit the ability of bacteria to adhere to the host and prevent infection (Brück et al., 2006; Cozens & Read, 2012). Provided that anti-adhesive agents are not bactericidal, the spread of bacterial resistance is less likely to occur. Prior the bacterial adhesion assay, Ajwa date extracts at different concentrations were evaluated to ensure that the concentration did not eliminate the bacteria. Subsequently, it was confirmed that anti-adhesion activity was not caused by the inhibition/killing of the bacteria. The findings indicated that the extract at a concentration of 100 mg/ml did not affect the bacteria viability compared to control.

Therefore, this concentration was selected for bacterial adhesion assay for crude extracts of dates.

8.2 Antimicrobial Activity of Ajwa Dates Prepared Following the Practice of Prophet (PBUH) Toward Bacterial Causing Gastroenteritis

Antimicrobial activity against all tested bacteria was present in the consumption of Ajwa dates-infused water according to the practice of the Prophet Muhammad (PBUH). The antibacterial activity had a significant positive correlation with the level of phenolic, tannin, and flavonoids content in Ajwa date infused water.

The antimicrobial activity of Ajwa date infused water was time-dependent and dose-dependent in terms of the number of date fruits soaked in the water. Meanwhile, the findings corresponded with the TPC, TFC, and TTC of Ajwa dates-infused water. The initial increase in the antimicrobial activity and phytochemical content of Ajwa dates-infused water could be explained using Fick's second law of diffusion, in which the solute concentration in the plant would dissolve after the contact with the solvent until a final equilibrium state was achieved at a certain point (Paul et al., 2014; Pang et al., 2017). Therefore, longer infusing time and the higher number of date fruits would increase the antibacterial activity until the equilibrium was achieved.

The final equilibrium of solute in solvent led to a static or plateau in antimicrobial activity in Ajwa dates-infused water against tested bacteria. This could be demonstrated by the antibacterial activity of Ajwa dates-infused water against *S. aureus*. Furthermore, the bacterial inhibition zone remained constant at a particular point of time, indicating that the antimicrobial compounds in Ajwa dates infused water reached the maximal efficacy to inhibit *S. aureus*. While extraction efficiency increased with the increase in extraction duration within a certain time range, the increase in time would not affect the

extraction after the equilibrium of the solute was achieved inside and outside the solid material (Zhang et al., 2018). Meanwhile, a decrease in TFC values of Ajwa dates-infused water was observed after 10 hours, while this decrease at a longer extraction duration was possibly due to degradation polyphenols (Muhamad et al., 2015; P. Y. Lim et al., 2018).

The phytochemical quantifications of the Ajwa dates-infused water showed the contents of phenolic, tannin, and flavonoids in it. Previous studies found that Ajwa date fruit was enriched with polyphenols and flavonoids, which included rutin, tannin, catechins, iso-flavonoids, and sterols (Hamad et al., 2015; Samad et al., 2016 & Yaacob, 2016). Furthermore, the phytochemical content in Ajwa dates infused waters was dose and time-dependent. The increase in the number of used dates and extraction time lead to a significant increase in the TPC, TTC, and TFC. This finding was supported by studies by Tan et al. (2013), where the TPC of henna stem extracts increased gradually the increase in extraction time (Mallhi et al., 2014).

Spearman correlation coefficient analysis was conducted to observe the relationship between the bacteria inhibition zone and the concentration of the phytochemical in Ajwa dates-infused water. It was recorded from the analysis that a significant positive correlation was present between the concentration of TPC, TTC, and TFC and bacterial inhibition zone ($p < 0.05$). Previous studies proved that phytochemicals in the plant, such as phenolic, tannin, and flavonoids had a role in plant's antibacterial activity (Cowan, 1999; Cushnie & Lamb, 2005; Daglia, 2012). It was suggested that antibacterial activity of Ajwa dates-infused water was caused by the phytochemicals (e.g., phenolic, tannin, and flavonoids) individually or in a combination against all tested bacteria. This finding was also supported by the study by Zhao and

Shah (2015), which recorded strong correlations between the total phenolic content in all pure plant extracts and the size of the inhibition zone (Zhao & Shah, 2015).

8.3 Comparison of Antimicrobial Activity and Phytochemical of Three Date Fruits Variety

8.3.1 Antimicrobial Activity

Besides the Ajwa dates, various date varieties are available in the market. To assess the antimicrobial activity in the dates, three different date varieties were selected in this study, namely Ajwa, Medjool, and Mariami due to different reasons. Specifically, Ajwa was selected due to its exceptional nutritional value. It was also one of date fruits variety consumed by the Prophet Muhammad (PBUH), as mentioned in various Quranic verses and hadith. Meanwhile, Medjool date was selected as this variety was the most well-known variety in the world besides Deglet-Nour. Being widely grown and available in the market worldwide, these varieties are genetically distinguished (Elhoumaizi et al., 2006). Mariami dates were selected due to the easy accessibility of this variety in Malaysia market and affordable price compared to Medjool and Ajwa varieties. As a result, all three varieties of dates showed antimicrobial activity and different degrees of antimicrobial susceptibility. The difference between the activities of the varieties was possibly due to the high diversity in chemical composition depending on the genotype, environment, season, and cultural practices of these palm dates (Guido et al., 2011; Farag et al., 2014; Khalid et al., 2017).

Ajwa dates extracts were mostly recorded with significantly higher antibacterial activity compared to Medjool and Mariami dates extracts possibly due to the phytochemical content in Ajwa dates. From the phytochemical qualitative screening, it

was found that Ajwa dates contain higher phytochemical compounds compared to Medjool and Mariami, which amounted to 78 compounds. Furthermore, these dates consisted of phenolic acids, flavonoids, and terpenoids, which are known for antibacterial properties (Kurekci et al., 2013; Laouini et al., 2013; Üstündağ et al., 2016; Ng et al., 2019). It was found from the phytochemical quantification that Ajwa date contained significantly higher TPC and TFC values compared to Medjool and Mariami dates.

In the recent studies by Saleh et al. (2011), Eid et al. (2013), and Hamad et al. (2015) comparing the phytochemicals (e.g., phenolic and flavonoid contents) of various date varieties, it was found that Ajwa dates had a higher level of phenolic (e.g., caffeic acid, ferulic acids, gallic acids, and syringic acid) and flavonoid (catechin, quercetin, and rutin) compared to other date varieties, such as Khalas, Sukkari, Nabtit Ali, Mabroom, Khla Al Qassim, Khlas Al Ahsa, Khals Elshiokh, Nabot Saif, Saffawy, Barni, and Rashodia (Saleh & Otaibi, 2013; Eid et al., 2014; Hamad et al., 2015). These phytochemicals were the active compounds with a role in the antibacterial activity, which justified the higher antimicrobial activity in Ajwa dates.

8.3.2 Anti-adhesive Activity

One strategy to avoid bacterial infections includes the anti-adhesive agents, which might prevent the attachment of bacteria to human cells. Recently, many researchers focused on the potential anti-adhesion agent from the plant base. It due to higher resistance of the bacteria to being cleared using normal cleansing mechanisms (e.g.; such as normal immune factor and bacteriolytic enzyme) or antibiotic when they are adhering to the epithelial surface. The acquirement of nutrient by these bacteria enhances the bacteria ability to survive and infect the host (Ofek et al., 2003). Moreover,

an adherent condition, which is the initial step in pathogenesis, is advantageous for bacterial survival (Ofek et al., 2003). Therefore, control of bacteria adhesion to the host is important for disease prevention.

In the anti-adhesion assay, all dates varieties were recorded with anti-adhesion activity against all tested bacteria. Specifically, Ajwa date extracts showed good anti-adhesion activity compared to Mejdool and Mariami dates. However, no conclusion was made regarding the difference between anti-adhesion activities in each date variety due to the lack of available published data on this matter. It was suggested that polyphenol and saccharide contents in date fruits might be responsible for this activity (Ferrazzano et al., 2009; Wang et al., 2015). Provided that date fruits are rich in carbohydrates, such as glucose, fructose, maltose and galactose, and polyphenols, these compounds are possibly among the responsible compounds for anti-adhesion activity. Provided that phytochemical content in Ajwa, Medjool and Mariami dates are varied, it suggests the difference in term of anti-adhesion activity.

Although the exact mechanism remained undefined in terms of the adhesion-inhibition properties of phenolic extracts, the previous study suggested that phenolic compounds, such as proanthocyanidins, ellagitannins, and isoflavones had a role in blocking or inactivating the adhesins located on a bacterial outer cell wall or fimbriae (Puupponen-Pimiä et al., 2001; Ferrazzano et al., 2009). A study by Wells et al. (1999) on isoflavone, namely genistein derived from soybean, found that genistein anti-adhesion activity was associated with the integrity of enterocyte tight junction and genistein function as a barrier sustaining agent, which inhibited the adhesion and invasion of enteric bacteria (Wells et al., 1999).

Besides polyphenols, the previous study by Wang and co-worker (2015) found that carbohydrate and oligosaccharide from natural sources, such as orange peel, algae

colloid, guar gum, and konjak gum consisted of different degree of anti-adhesion activity against *V. cholerae*, *Escherichia coli*, *S. Typhimurium*, and *C. jejuni* (Wang et al., 2015). Polysaccharide from *Panax ginseng* also demonstrated strong anti-adhesion activities against *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Propionibacterium acnes*, and *S. aureus* (Lee et al., 2009). Oligosaccharide had a role in mimicking the receptor-structure to bacterial adhesins (Shoaf-Sweeney & Hutkins, 2008). Instead of binding to the host cells, the pathogen could adhere to specific oligosaccharide and expelled from the host, leading to the prevention of bacterial pathogenesis (Shoaf et al., 2006).

Among all the tested bacteria, *V. cholerae* was the most affected bacteria after the treatment with date extracts. The adherence of *V. cholerae* to caco-2 cells received the most impact with up to 95% adhesion-inhibition after the treatment with Ajwa hot aqueous extract. A similar finding was recorded by Coppa et al. (2006) on the inhibition of adhesion of pathogenic bacteria by human milk oligosaccharide, where *V. cholerae* was the most affected bacteria after treated with high molecular-weight fraction of human breastmilk compared to *E. coli* and *S. fryis*.

Another study by Sahnshan et al. (2015) showed that *V. cholerae* was the most affected organism after the treatment with *D*-mannose, *D*-fructose, *D*-fucose, *D*-glucose, pectin oligosaccharide, and chitooligosaccharides compared to *E. coli*, *C. jejuni*, and *S. Typhimurium*. This situation was possibly due to the difference between pathogenic mechanisms and adhesion abilities of the organism (S. Wang et al., 2015). *V. cholerae* which exposed to higher osmolality, an antimicrobial compound, and intermittent nutrient deprivation led to challenges in bacterial growth and disruption of the gene encoding the general stress response regulator RpoS (σ S) or the RNA polymerase σ E subunit (RpoE). These genes mediated the envelope stress response,

leading to a significant weakening of *V. cholerae* virulence and its capacity to colonise the small intestine (Merrell et al., 2000; Kovacicova & Skorupski, 2002; Louis & O'Byrne, 2010; Silva & Benitez, 2016).

8.3.3 Phytochemical Analysis of Date Fruits Extracts.

The acquirement of plant extracts led to crucial challenges in the identification and characterisation of bioactive compounds as most plant extracts occurred as a combination of various types of bioactive compounds or phytochemicals with different polarities. Notably, phytochemical screening assay is a simple, swift, and inexpensive procedure, which provides a quick answer about various types of phytochemicals or secondary metabolites found in plants (Sasidharan et al., 2011). In this study, a qualitative method was performed on the bioactive composition from date fruits, namely Ajwa, Medjool, and Mariami with different extraction types using high-performance liquid chromatography-electrospray time of flight mass spectroscopy (UHPLC-ESI-QTOF-MS/MS) in negative ionisation mode.

It could be summarised that all date fruit varieties consisted of a phenolic compound, flavonoids, organic acid, and fatty acid. This study data regarding Ajwa dates were in line with the previous studies by Ahmed et al. (2016), Eid et al. (2013), and Hamad et al. (2015) on the phenolic and flavonoid content in Ajwa dates. Although organic acids, including oxalic acids, succinic acid, malic acids, citric acids, and formic acid were previously reported in Ajwa dates (Hamad et al., 2015), this study identified gluconic acid, penylvaleric acids, and tartaric acid in the dates. Meanwhile, the previous study observed terpenoids, such as lupeol and lup-20(29)-en-3-one in Ajwa date (C.-R. Zhang et al., 2013), although new terpenoid compounds were found in this study, namely Dihydroxydimethyl19[(Dglucopyranosyl)oxy]-19-oxo-ent-labda-8(17),13-

dien-16,15-olidederivative and Oleanolic acid. This difference was possibly due to the solvent for extraction and methodology for identification of the compound. A study by Hamad et al. (2015) used methanol and chloroform for extraction, while identification of compound was carried out using Proton nuclear magnetic resonance ($^1\text{H NMR}$). This difference was also in terms of plant origin, topographical factors, and nutrient concentration of soil (Kaneria et al., 2009; Doughari, 2012).

Between all date varieties, Ajwa date contains more flavonoid compounds compared to Medjool and Mariami dates. Similarly, a previous study found that polyphenolic flavonoids were the most abundant phenolic compound found in Ajwa dates (Eid et al., 2013). The flavonoid compounds, such as Chrysoeriol hexadecyl sulfate, Isoquercetrin acetate, Hexosyl luteolin sulphate, and Myricetin-3-galactoside were found in Ajwa dates, however, were not present in Medjool and Mariami dates. Flavonoid is well known as an antibacterial agent, which contributes to the higher antibacterial activity of Ajwa dates. Moreover, Ajwa dates contains fatty acid and terpenoids, which were not present in Mariami and Medjool date. Terpenoids is known to have antimicrobial properties (Guimarães et al., 2019). A high degree of variability in terms of the phytochemical content in date variety might be present in terms of genetic variation, geographic location, soils, and environment status (Kim et al., 2006; D'Archivio et al., 2010; Khalid et al., 2017).

The quantification of an active compound was determined, while three colorimetric assays were selected to calculate the total phenolic, tannin, and flavonoid in date extracts. These assays were selected as phenolic, tannin, and flavonoids were the plant active compounds, as discussed in many previous works and literature regarding the antimicrobial activity of date fruits (Cowan, 1999; Baliga et al., 2011; Vayalil, 2012).

From our present study, the total phytochemical content of date fruits varied based on date variety and extraction type. Ajwa dates significantly contain higher TPC values compared to Medjool and Mariami dates. The previous study also demonstrated that Ajwa date contained higher phenolic compared to Mariami, Sukari, Safawi, Mariami Khalas, Barni, Nabot Saif, Rashodia, Khlas Al Ahsa, Sokary, Mabroom, and Khla Al Qassim varieties (Saleh et al., 2011; Eid et al., 2013; Hamad et al., 2015; Samad et al., 2016; Khairuddin et al., 2017). Moreover, previous data demonstrated the TPC values of Ajwa dates were varied in each study (Hamad et al., 2015; Khalid et al., 2017). The difference between TPC values was possibly due to extraction type and stage of plant collection (Eid et al., 2013; Hamad et al., 2015), where Ajwa date contained higher phenolic content at the Kimri stage, followed by Khalal, Rutab, and Tamar stages (Eid et al., 2013). Hot aqueous extracts TPC values higher compared to methanol extract. It was found that aqueous extract constantly increased phenol compared to alcoholic extracts (Saleh & Otaibi, 2013; El Sohaimy et al., 2015; Hamad et al., 2015), which was in line with previous study results.

Medjool date hot aqueous extracts and methanol extracts contained higher tannin compared to Mariami and Ajwa date. In line with this result, the previous study recorded that Medjool date contained tannin up to 1146 mg GAE/100 g at different ripening stages (Martín-Sánchez et al., 2014) compared to Ajwa date contained 500 mg/100 g of tannin (Bhatti & Bhatti, 2019). Hot aqueous extract for all date varieties showed higher TTC value compared to cold aqueous and methanol extracts. Provided that the heating process might soften the plant's tissue and weaken the phenol-protein and phenol polysaccharide interactions in the plant materials, more compounds would transfer to the solvent portion (Shi et al., 2003; Spigno & Dante Marco, 2007; Dorta et al., 2012).

Ajwa date cold and methanol extracts contained higher flavonoids content compared to Medjool and Mariami dates. This result was in line with previous studies, which recorded that Ajwa date contained higher flavonoids compared to Nabot Saif, Rashodia, Khlas Al Ahsa, Sokary, Mabroom, Khla Al Qassim, and Khalas (Saleh et al., 2011; Hamad et al., 2015). Hamad et al. (2015) demonstrated that Ajwa date total flavonoids amounted to 2.78 mg/100 g, while this amount ranged from 77 to 216 mg/ml in this study according to the extraction types. The difference between the total flavonoids was attributed to the methodology and extraction solvent. Flavonoid content was quantified by Hamad et al. (2015) using acetone-water and HPLC approaches, which were more precise compared to the colorimetric assay method used in this study. Hot aqueous extracts of Mariami dates were found to contain significantly higher flavonoid content compared to Ajwa and Medjool date. A study by Sani et al. (2015) demonstrated that Mariami date extracted using methanol produced higher flavonoid content compared to water-ethanolic and aqueous extracts. On the contrary, this study recorded that hot aqueous extracts contained higher flavonoids compared to methanol extracts. The difference between the current and previous study findings was due to the use of cold extraction and the different compound used for the standard curve in the previous study.

8.4 Isolation and antibacterial activity of Active Compound from Ajwa Dates That Responsible for Their Antibacterial Activity

8.4.1 Isolation of Active Phytochemicals from Ajwa dates extracts

An excellent antibacterial and anti-adhesion activity led to the selection of Ajwa date hot aqueous extracts in this study. However, little information was obtained about the actual mechanism of action of the antibacterial compounds derived from these plants. Several methods were employed to extract potential antibacterial compounds from natural products. The conventional way of testing an antibacterial compound from natural products is the preparation of crude extract, followed by purification, identification of compound, and testing against bacterial strains (Kang et al., 2013; Ejele et al., 2014; Khurram et al., 2015). During the last few decades, several compounds were isolated from different plants, where the bioassay-guided isolation was used and reported to have significant biological activities. Notably, some compounds have received interest for potential drug development (Kang et al., 2013; Ejele et al., 2014; Khurram et al., 2015).

Bioassay-guided isolation was used in this study, which began with a testing of a crude extract to confirm its activity, followed by separation of the compounds in the extracts and *in-vitro* biological testing of purified compounds.

8.4.2 Antibacterial and Anti-Adhesion Activity of Isolated Active Phytochemicals

Only three organisms, namely *S. Typhimurium*, *S. flexneri*, and *V. cholerae* were selected in this parameter, where the exhibited good antibacterial and anti-adhesion activity was due to a limited amount of fractions collected. Three fractions, namely fractions 4, 5, and 6 were found to be active and exhibited antimicrobial activity against *S. Typhimurium*, *S. flexneri*, and *V. cholerae*. Notably, *S. flexneri* was the most sensitive organism to the treatment with active fraction, with higher inhibition zone compared to *V. cholerae* and *S. Typhimurium*. Same observation was found that *S. flexneri* had low efficacy in the antimicrobial bioactive compound, with less potential for developing resistance (J. H. Doughari et al., 2007). The sensitivity of bacteria toward antimicrobial agent is affected by the genotypic and phenotypic variation of the organism, thus justify the sensitivity differences (Othman et. al., 2019).

Although the MBC value for *V. cholerae* was 3.125 mg/ml for three active fractions, the MBC value against *S. Typhimurium* and *S. flexneri* cannot be determined. It was found that the active fraction was only bacteriostatic against *S. Typhimurium* and *S. flexneri*, while the crude extract of Ajwa at concentration 500mg/ml could kill those organism. It was demonstrated that the single active compounds in Ajwa dates were only able to inhibit the bacterial growth, while the combination of active compounds had a role in killing *S. Typhimurium* and *S. flexneri*. These active fractions also showed moderate anti-adhesion activity after the treatment with an active fraction. The anti-adhesion activity of active fractions was lower compared to Ajwa date crude extracts. As proposed in other studies, it was proven that multiple anti-adhesive agents should be employed to perform actions on different types of adhesins, while a multi-component system could be more effective at blocking pathogenic bacterial adhesion to enterocytes

(Ofek et al., 2003 2003).

The identification of fractions 4, 5, and 6 was performed using UHPLC-ESI-QTOF-MS/MS and active fractions, which consisted of various flavonoid substances, such as Catechin, Kaempferol, Luteolin, and Cyanidin 3-rutinoside-5-glucoside. Polyphenolic flavonoids were the most abundant phenolic compound found in Ajwa date (Eid et al., 2013). Using the LC/MS technique, the previous study also found that Ajwa dates had high quantities of flavonoids composition, such as naringenin, luteolin, apigenin, myricetin, quercetin, and kaempferol (Eid et al., 2013).

Well known as antibacterial agents against a wide range of pathogenic microorganism, flavonoids fall under the primary class of polyphenols with C6-C3-C6 skeleton. To date, a total of 8000 known flavonoid molecules are present (Bhattacharya, 2011). Flavonoids could be categorised into various subclasses, including flavanone, flavones, flavanols, flavanonols, isoflavones, bioflavonoids, chalcones and hydrochalcones, aurones, and anthocyanidins, which are also noted for antimicrobial activities (Falcone et al., 2012). The antibacterial activity of flavonoids could be employed in many ways, such as directly killing the bacteria (membrane disruption, inhibition of cell envelope synthesis, inhibition of nucleic acid synthesis), weakening bacterial pathogenicity (biofilm formation, inhibition of bacterial toxins), and synergistically activating the antibiotics (antibacterial action of flavonoids-metal complex) (Xie et al., 2014; Górnjak et al., 2019). The antibacterial mode of action of flavonoids varies depending on the structures, specifically the substitutions on the aromatic rings.

Fraction 4 consists of Hexa-acetyl-pyracanthoside, Luteolin, and Catechin in a subclass flavone, flavanone, and bioflavonoid. Each flavonoid subclass consists of different actions on its antibacterial mechanism. Luteolin is in a flavanone subclass,

while the antibacterial activity in flavones has been widely investigated. Flavone acts as a prenylation (addition of hydrophobic molecules to a protein or chemical compound), while the substitutive phenyl group reacts to the adjacent hydroxyl group to form a heterocycle of six members, which might reduce the bacterial activity (Xie et al., 2015). Besides prenylation, a study by Wang and Xie (2010) found that Luteolin comprised antibacterial activity against *Staphylococcus aureus*, while the antibacterial mechanism of luteolin occurred through the inhibition of the activities of DNA topoisomerase I and II, which reduced the nucleic acid and protein synthesis. Meanwhile, Hexa-acetyl-pyracanthoside is in flavanone subclass, where flavanone has the saturated C3-C4 bond of C ring, which creates the nuclear skeleton of flavanone non-planar. Notably, many of the active antibacterial flavanones are also prenylation. Tsuchiya et al. (1996) found that flavanones were active as anti-MRSA agents. Furthermore, Celiz et al. (2011) recorded that the attachment of a saturated aliphatic chain with 10 - 12 carbon atoms to the sugar attached to the A ring of naringenin significantly increased the anti-listerial and anti-staphylococcal activity. Also, Catechin is in flavonol subclass, which is characterised by an additional hydroxyl group at the C-3 position. It has been widely studied in terms of its antimicrobial properties against both gram-positive and gram-negative bacteria. Catechin kills the bacterial through the rupture of the bacterial membrane by binding the lipid bilayer and inactivating/inhibiting the synthesis of an intracellular and extracellular enzyme (Reygaert, 2014). Overall, this result was in line with the current study result presented in electron microscopy analysis, with damage in *V. cholerae* cells was present as a deformity or shrinking of the cell wall after the treatment with this flavonoids fraction.

Fraction 5 majorly consists of kaempferol, which is present in a flavonol subclass. Activity against *S. aureus* and *E. coli* in Kaempferol was recorded in a

previous study (Tatsimo et al., 2012). It was also found keampferol mediate the displacement of AHL molecules from LuxR-type transcriptional activator protein (Roy et al., 2018). Keampferol also reported inhibit FAS-1, where FAS-1 is the fatty acids synthesis of mycobacteria, and the biosynthesis disruption directly killed the bacteria (Li & Tian, 2004). Kaempferol was also recorded with significantly inhibited DNA gyrase from *E. coli*.

Fraction 6 majorly consists of Cyanidin 3-rutinoside-5-glucoside in anthocyanin subclasses. Anthocyanin is a naturally occurring pigment, which is normally associated with the blue, purple, and red colours of different plant parts (flowers, fruits, and other plant tissues). Despite the intensive studies performed on the antimicrobial activity of crude extracts of plant phenolic compounds against human pathogens, the available information about the antimicrobial activity of the pure anthocyanins is scarce. Inhibitory effects were observed from the cranberry concentrate rich in anthocyanin on the growth of *Listeria monocytogenes*, *S. aureus*, *B. subtilis*, *Enterococcus faecalis*, *Citrobacter freundii*, *E. coli*, *P. aeruginosa*, and *S. Typhimurium* (Wu et al., 2008; Lacombe et al., 2010). It was also found that the exposure to cranberry concentration of 5 $\mu\text{L}/\text{mL}$ resulted in morphological damage of bacterial cells, such as the loss of structural integrity of the wall, membrane, and intracellular matrix on *E. coli*, *L. monocytogenes*, *S. Typhimurium*, and *S. aureus*. It was also found from TEM that anthocyanin fraction from cranberry concentrate led to localised disintegration and irregularity in the outer member and leaking of cytoplasm on *E. coli* (Lacombe et al., 2010).

All flavonoids fraction showed moderate anti-adhesion activity against all tested bacteria. This result was in line with a previous study, which suggested that phenolic compound might be responsible for the anti-adhesion properties. Anti-adhesion activity

was observed from the phenolic compounds, such as proanthocyanidins, ellagitannins, and isoflavones. These compounds may have a role in blocking or inactivating the adhesins located on a bacterial outer cell wall or fimbriae (Puupponen-Pimiä et al., 2001; Ferrazzano et al., 2009). A study by Wells et al. (1999) recorded that isoflavone had a function in enhancing the tight-junction integrity of caco-2 enterocyte, which reduced bacterial internalisation on caco-2 enterocyte (Wells et al., 1999).

Citrus flavonoids, such as apigenin, kaempferol, quercetin and naringenin, are proven to be an effective antagonist of cell-cell signalling (Vikram et al., 2010). Cell-cell signalling is a significant regulatory factor for the biofilm production in *Escherichia coli*, *Vibrio* spp., and *Salmonella Typhimurium* (Vikram et al., 2010). This fact may justify that the active flavonoids fraction of Ajwa dates could reduce the adhesion possibly through different mechanisms, including blocking/inactivating bacteria adhesins, enhancing enterocytes integrity, or reducing the ability of bacterial adhesion through the antagonists of cell-cell signalling to reduce bacterial adhesion. Comprehension study in molecular level is crucial to develop understanding about the mechanism of Ajwa dates responsible for the anti-adhesion activity against bacterial gastroenteritis.

8.4.3 Effects of Ajwa Date Extracts and Isolated Active Flavonoids on Bacterial Morphological Change Via Electron Microscopy

In examining the antibacterial mechanism of Ajwa date extracts and the active compounds, the morphology of the bacteria was observed using electron microscopy. While SEM could view the 3-dimensional surfaces of an object, TEM enables the observation of the internal structure of the cells as the specimens are cut using a microtome instrument into an extremely thin slice.

In this study, *S. aureus*, *S. Typhi*, *S. Typhimurium*, *E. coli*, *S. flexneri*, and *V. cholerae* were treated with Ajwa hot aqueous extract and observed using a scanning electron microscope. This observation identified the physical damages and considerable morphological alteration to all tested bacteria after the treatment with Ajwa date extract. Given the important function of the bacterial cell wall, including the presence of strength and integrity, the damage to the cell wall would disrupt this function and lead to cell death (Talaro & Chess, 2018). Provided that Ajwa dates are rich in phenolic compounds, the structural changes observed in this study were possibly due to the effect of phenolic compound.

Antimicrobial mechanism of phenol was attributed to the presence of hydroxyl group, where the accumulation of phenolic group in lipids bilayer would disrupt the lipid-protein interaction. This situation led to alteration of membrane structure, increase the leakage of the intracellular constituent, and destroy the membrane integrity to facilitate the entry of antimicrobial agent (Char et al., 2010). Other literature also suggested that the phenol might be bound to the bacterial cell surface and penetrate through the target sites, such as the phospholipid bilayer of the cytoplasmic membrane and membrane-bound enzymes (Shan et al., 2007). The easy entry of the antimicrobial

compounds into the cells through these lesions also accelerates the leakage of the cell contents. These antimicrobials may also react with the bacterial DNA, eventually leading to cell death. Although the consequences after the cell wall are weakened, low osmotic pressure leads to cells burst (Black, 2008).

Active flavonoids fraction antibacterial mode of action was also observed using SEM and TEM against *V. cholerae*, which was selected due to the highest antibacterial activity after the treatment with active fractions. For SEM, the damage was present in the cells treated with active flavonoids fraction, which included the shrunk cell wall and the clustered and melted cells. Overall, these results were in line with previous studies, which showed that flavonoid from *Melastoma malabathricum* Linn leaves and phenolic from Oregano and Fennel could cause morphological changes in pathogenic bacteria (Soylu et al., 2007 & Kurt, 2007; Alwash et al., 2013 2013). Many cells were found to be clustered, while the alteration of the physical cell structure would cause the expansion and destabilisation of the membrane and increase membrane fluidity, which in turn would increase passive permeability. Leakages of various vital intracellular constituents were also present, such as ions, ATP, nucleic acids, and amino acids (J. Kim et al., 1995; Cox et al., 1998; Helander et al., 1998).

TEM revealed that *V. cholerae* treated with flavonoids fraction in Fraction 4 and fraction 5 showed damage as rippling and disorganize appearance of cell membrane, detachments of cytoplasm from cellular membrane, and outflow of intracellular constituent in cells were observed. The same observation also reported by Feng Xu et.al. (2019), where flavonoid treated *Aeromonas* were totally deformed, with loss of physical integrity of cell membrane and led to the loss of cell constituents (Xu et. al, 2019) . Moreover, He et. al (2014) also observed from the TEM that *E. coli* treated

with kaempferol indicated membrane disrupted, and plasmolysis where outflow of intracellular constituents in cells (He et. al., 2014). The damage from flavonoid treated bacteria cell, may due to flavonoids that interfering the protein synthesis resulting in leakage of protein in damaged cell membrane (Zhao et. al., 2015)

V. cholerae treated with Fraction 6 which primarily consisted of anthocyanidin, were deformed with rippling appearance, disorganised cell surface, cytoplasmic released from the cell membrane, and cellular disintegration. This finding was in line with the research by Lacombe et al. (2010), which found that anthocyanin fraction from cranberry concentrate led to localised disintegration and irregularity in the outer member, including the leaking of cytoplasm on *E. coli* (Lacombe et al., 2010). Furthermore, *V. cholerae* is specifically a gram-negative bacterium, which regulates permeability through hydrophilic channels known as porins. These channels allow the nutrients of a relatively low molecular weight (< 600 Da) to enter the cell cytoplasmic membrane (Nohynek et al., 2006). The irregularity in the cell shape supports the theory of localised chemical interactions at the cell surface. When the membrane is penetrated, smaller phenolic compounds could enter the cell and disrupt metabolism (Kwon et al., 2007). These SEM and TEM images conform that Ajwa date extracts and Ajwa date's flavonoids fractions exhibited strong antibacterial effect that cause severe damage to membrane integrity, which is followed by cell death. Additionally, antimicrobial compounds enter the cells easily through these lesions, which also facilitate leakage of the cell contents, eventually resulting in cell death