CHAPTER 4 :OPTIMIZATION OF EXTRACTION AND ANTIMICROBIAL ACTIVITY OF AJWA DATES

4.1 Introduction

Studies by Nagourne (1998) found that the antimicrobial activity of plants was highly dependent on the preparation used for extraction (Nagourney, 1998), which was the second crucial step in the preparation of plant potential drug candidates in the field of natural product drug discovery. The extraction procedure determined the quantity and quality of the crude extracts obtained from each extraction solvent. Furthermore, the requirement for the selection of the most appropriate extraction methodology was observed from the application of different methods on the same plant material and solvent, including the significant variation of extraction efficiency (Jadhav et al., 2009). Many types of solvents are available for plant material extraction, including water, methanol, ethanol, acetone, chloroform, hexane, and petroleum ether, with water and ethanol as the widely used solvents.

Previous studies recorded the antibacterial activity of date fruits against *S. aureus*, *E. coli*, *P. aeruginosa*, *S. pyogenes*, *B. subtilis*, *S. Typhimurium*, *B. cereus*, and *L. monocytognes* (Al-Daihan & Bhat, 2012; Al-seeni, 2012; Amira et al., 2012; Al-Judaibi et al., 2014; Bouhlali et al., 2016). Although a study was conducted on antimicrobial activities methanol extract of Ajwa dates, there were no published data on antibacterial activity of aqueous extraction of Ajwa dates. Provided that water is a universal solvent for the extraction of compounds from medicinal plants (Ncube et al., 2008), the

antimicrobial activity of hot and cold aqueous extracts was assessed. Some plants retained their antimicrobial activity after exposure at high temperature (121^{o}C), while the antimicrobial activity was lost in some plants after treatment at 60^{o}C (Ginovyan, 2017). Although many studies presented the antimicrobial activity of date fruits, the study on the effects of antimicrobial on the preparation of the date fruits extracts, such as the thermo-stability of date fruits on their antibacterial activity, was lacking.

Many studies were performed on date fruit extract, different result was obtained from each study. The variation of the studies was possibly due to different amounts of dosage used, the sensitivity of test strain, extraction methods, and diffusion capacity of substance (Nimri et al., 1999; Kaneria et al., 2009). Therefore, this chapter aims to 1) determine the thermo-stability of Ajwa dates on their antibacterial activity, 2) determine antibacterial activity of Ajwa dates with different extraction types and different concentration, and 3) determine the optimum concentration of date fruit extracts for anti-adhesion assay.

4.2 Methodology

4.2.1 Extraction of Ajwa Dates at Different Heat Treatment

The optimisations of hot aqueous extraction were performed by preparing 12 groups of samples consisting of 10 g of deseeded Ajwa dates homogenised in 100 ml of distilled water. Every four groups under these groups received different heating treatments, which were at 1) 60ºC for 30 minutes, 2) 60ºC for 60 minutes, 3) 100ºC for 30 minutes, and 4) 100ºC for 60 minutes. Subsequently, all samples were filtered using cotton gauze, followed by Whatman® No. 1 filter paper. The filtrates were then subjected into three groups; 1) unreduced, 2) reduced to dryness at 40ºC oven, and 3) reduced to dryness at 60ºC oven. The employed methods are summarised in **Table 4.1.**

Table 4.1: Temperature and time used for the optimisation of extraction.

Heating Treatment		Further, Reduce	
Temperature (ºC)	Time (Minutes)	Temperature (ºC)	Final volume
60	30	-	Unreduced
		40	Dryness
		60	Dryness
60	60	-	Unreduced
		40	Dryness
		60	Dryness
100	30	-	Unreduced
		40	Dryness
		60	Dryness
100	60	-	Unreduced
		40	Dryness
		60	Dryness

4.2.2 Antibacterial Screening by Well diffusion Assay

As for the thermo-stability experiment of Ajwa dates, dried extracts were dissolved in distilled water at a concentration of 200 mg/ml before the well diffusion assay. For cold aqueous, hot aqueous, and methanol extracts, all samples were dissolved in distilled water at a concentration of 500 mg/ml, 400 mg/ml, 300 mg/ml, 200 mg/ml, and 100 mg/ml, respectively. The entire extracts were then filtered with 0.02 um syringe filter before use. Overall, the extracts were subjected to well diffusion assay after methodology **3.6.1.**

4.2.3 MIC and MBC

MIC and MBC were performed based on the methodology described in **3.6.2** and **3.6.3**.

4.2.4 Bacterial Viability Assay for Bacterial Adhesion Assay

The bacterial inoculums in broths were adjusted to 0.5 McFarland standard (OD at 0.09 - 0.12 at 625 nm). *E. coli* and *V. cholera*e were used in this assay, while 50µL of adjusted inoculum was treated with 50µL hot aqueous, cold aqueous and methanol extract at a concentration of 500 mg/ml, 400 mg/ml, 300 mg/ml, 200 mg/ml, and 100 mg/ml. The bacteria were incubated for two hours, followed by samples were taken and plating on MH agar. The bacteria without any treatment were used as the controls.

4.3 Result

4.3.1 Screening of Antimicrobial Activity by Well diffusion Assay

4.3.1.1 Thermo-stability of Ajwa Date Extracts on Their Antimicrobial Activity.

After the treatment with Ajwa date extracts, the inhibition zone of *S. aureus* was prepared at different heat treatments, as summarised in **Table 4.2.** In the case of the unreduced extract, Ajwa dates heated at 60°C for 30 minutes showed lower antibacterial activity with an inhibition zone of 24.7 ± 0.5 mm compared to those heated at 100 °C for 30 minutes with an inhibition zone of 25.25 ± 0.5mm. However, as for the Ajwa dates heated at 60 °C for 60 minutes, the higher rate of antibacterial activity was observed, with an inhibition zone of 27.75 °C compared to those heated at 100 °C for 60 minutes with the diameter of inhibition zone of 21.75 ± 0.96 mm.

In the case of the extracts dried at 40°C and 60°C in the oven, all extracts showed almost similar antibacterial activity with the inhibition zone ranging from 23.24 mm to 24.5 mm for all heat treatments. Therefore, it was indicated that the filtrated extract did not affect the antibacterial activity in the drying process at a different temperature. Thus, methodology for hot aqueous extraction, the extracts heated at 60 °C for one hour, filtered, and further reduced to dryness at 60°C oven were selected.

Table 4.2: Antibacterial activities of Ajwa dates at different heat treatments. The inhibition zone (mean ± standard deviation) against *S. aureus*, which included 7 mm well hole, is presented.

Heating Treatment		Further, reduce		The diameter of the inhibition zone (mm)
Temperature (^{o}C)	Time (minutes)	Temperature (^{o}C)	Final volume	
60	30	-	Unreduced	24.75 (±0.5)
		40	Dryness	24.5 (±0.58)
		60	Dryness	24.5 (±0.58)
60	60	-	Unreduced	27.75 (±0.5)
		40	Dryness	24.5 (±0.58)
		60	Dryness	23.25 (±0.58)
100	30	-	Unreduced	25.25 (±0.5)
		40	Dryness	24.5 (±1.29)
		60	Dryness	24.75 (±0.5)
100	60	-	Unreduced	21.75 (±0.96)
		40	Dryness	24.75 (±0.5)
		60	Dryness	24.75 (±0.96)

4.3.1.2 Antibacterial Activity of Ajwa Dates Extracts at Different Concentration

Antimicrobial activities of Ajwa date extracts (e.g., cold aqueous, hot aqueous, and methanol) at different concentrations were tested against the selected bacterial gastroenteritis. The Ajwa date extracts were found to be effective for inhibiting the growth of all tested bacteria, as shown in **Table 4.3**. Based on the result, the diameter of the inhibition zone was dose-dependent, where higher doses indicated larger bacteria inhibition zone.

In the case of cold aqueous extracts at a concentration of 300 mg to 500 mg/ml were found to be active against all bacteria, with the inhibition zone diameters ranging from 12.33 mm to 37.67 mm. Furthermore, the extracts at the concentration of 200

mg/ml were recorded as active against all bacteria except for *E. coli*. However, none of the 100 mg/ml of extracts exhibited antibacterial activity against all tested bacteria.

In the case of hot aqueous extracts, extracts at a concentration of 200 mg to 500 mg/ml were found to be active against all bacteria with inhibition zone diameters ranging from 13.30 mm to 35.00 mm. However, none of 100 mg/ml of extracts exhibited antibacterial activity against all tested bacteria.

In the case of the methanol extract, the extracts at a concentration of 200 mg to 500 mg/ml were found to be active against all bacteria with the inhibition zone diameters ranging from 15.33 mm to 45.67 mm. None of 100 mg/ml of extracts exhibited antibacterial activity. Notably, the methanol extract was found to be the most active extract compared to aqueous extract. The inhibition zone diameters had a higher range from 15.33 mm to 45.67 mm compared to hot and cold aqueous extracts with an inhibition zone diameter ranging from 12.33 mm to 37.67 mm and 13.30 mm to 35.00 mm.

Generally, *S. aureus* is more susceptible to all date extracts with higher inhibition zone compared to *S. flexneri, E. coli*, *V. cholerae*, *S. Typhi*, and *S. Typhimurium.* The highest antimicrobial activity was observed from methanol extract with a diameter of inhibition zone at 45.67 mm against *S. aureus.* Meanwhile, *E. coli* was the least susceptible to all date extract with lower inhibition zone compared to other tested bacteria.

Table 4.3: The diameter of the inhibition zone (mean ± standard deviation) of aqueous extract (hot/cold) and methanol extracts against the tested bacteria using well diffusion assay method, which also included 7 mm well hole.

Extraction types	Conc. (mg/ml)	Diameter of Inhibition zone (mm)					
		S. aureus	*S. Typhi*	*S. Typhimurium*	*V. cholerae*	*E. coli*	*S. flexneri*
Cold aqueous extract	500	35.67 (±0.58)	29.33 (±0.58)	25.70 (±0.58)	26.33 (±0.58)	23.67 (±0.58)	30.33 (±0.58)
	400	32.33 (±0.58)	24.67 (±2.31)	22.70(±0.58)	25.33 (±0.58)	18.33 (±0.58)	28.33 (±0.58)
	300	30.33 (±0.58)	21.33 (±1.00)	20.00(±0.58)	21.0 (±0.00)	12.33 (±0.58)	26.67 (±1.00)
	200	22.67 (±0.58)	17.67 (±0.58)	14.70 (±0.58)	15.67 (±0.58)	N.D.	22.00 (±1.15)
	100	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Hot aqueous extract	500	35.00 (±1.00)	28.67 (±0.58)	27.00 (±0.00)	27.33 (±1.15)	27.33 (±0.58)	32.33 (±0.58)
	400	32.0 (±1.00)	25.67 (±0.58)	20.67 (±0.58)	25.00 (±0.00)	24.67 (±0.58)	32.00 (±1)
	300	29.67 (±1.15)	20.00 (±0.58)	20.67 (±0.58)	21.00 (±1.00)	22.67 (±0.58)	29.67 (±0.58)
	200	27.33 (±1.15)	16.33 (±0.00)	17.67 (±0.58)	16.33 (±0.58)	17.33 (±0.58)	13.33 (±0.58)
	100	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Methanol extract	500	45.67 (±0.58)	29.00 (±0.00)	25.67 (±0.58)	28.67 (±0.58)	27.67 (±0.58)	34.00 (±0.00)
	400	36.00 (±1.73)	25.33 (±0.58)	21.33 (±0.58)	24.67 (±0.58)	24.67 (±0.58)	31.67 (±0.58)
	300	32.33 (±0.58)	21.33 (±1.15)	17.67 (±0.58)	22.33 (±0.58)	21.67 (±0.58)	29.33 (±1.15)
	200	26.67 (±1.53)	15.33 (±0.58)	17.33 (±0.58)	17.33 (±1.15)	17.33 (±0.58)	24.33 (±1.15)
	100	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Ampicillin	1	45.00 (±0.00)	31.00 (±0.00)	32.00 (±0.00)	31.00 (±0.00)	33.00 (±0.00)	31.00 (±0.00)

N.D.= Not Determined

4.3.2 Minimum Inhibitory Concentration (MIC) And Minimum Bactericidal Concentration (MBC)

The MIC and MBC values of Ajwa date extracts against all tested bacteria are presented in **Table 4.4**. While these MIC values for hot aqueous extract ranged from 500 mg/ml to 1000 mg/ml, the MIC values for cold aqueous extract ranged from 500 mg/ml to 1000 mg/ml. Furthermore, the MIC values for methanol extract ranged from 250 mg/ml to 500 mg/ml.

In the case of cold aqueous extract, the lowest MIC value was at 500 mg/ml against *V. cholerae* and *E. coli*. MIC values against *S. aureus* and *S. Typhimurium* was at 1000mg/ml. However, the MIC values for *S. Typhi* and *S. flexneri* could not be determined. Overall, all MBC value for cold extract against all tested bacteria were similar to MIC values.

In the case of hot aqueous extract, the lowest MIC value was at 250 mg/ml against *V. cholerae* and *S. aureus*, while the MIC value against *S. Typhimurium*, *S. Typhi*, *S. flexneri*, and *E. coli* were at 500 mg/ml. The MBC value for hot extract against all tested bacteria were similar to the MIC values except for *V. cholerae* with a value of 500 mg/ml.

As for the methanol extract, the lowest MIC value was at 250 mg/ml against *V. cholerae*, *E. coli*, and *S. aureus*, while the MIC values against *S. Typhimurium*, *S. Typhi*, *and S. flexneri* were at 500 mg/ml. Notably, the MBC values for the hot extract against all tested bacteria were equal to the MIC values.

Among all extracts, methanol extract was found to exhibit more potent antibacterial activity, with lowest MIC values at 250 mg/ml against *E. coli.* Notably, the MIC values of hot aqueous extract and methanol extract against *S. aureus*, *V. cholerae* were similar at 250 mg/ml and *S. Typhimurium*, *S. Typhi*, *S. flexneri* at 500 mg/ml. Furthermore, the hot aqueous extract showed more potent antibacterial activity, with lower MIC values ranging

from 250 to 500 mg/ml compared to cold aqueous extract ranging from 250 to 500 mg/ml against *S. aureus*, *S. Typhimurium*, *S. Typhi*, *S. flexneri,* and *V. cholerae.*

Table 4.4: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) for Ajwa date extract against all tested bacteria. The MIC/MBC values are presented as mg/ml of three replicates.

Organism		MIC AND MBC value (mg/ml)		
		Hot aqueous extract	Cold aqueous extract	Methanol extract
S. aureus	MIC	250	1000	250
	MBC	250	1000	250
S. Typhimurium	MIC	500	1000	500
	MBC	500	1000	500
S. Typhi	MIC	500	N.D.	500
	MBC	500	N.D.	500
S. flexneri	MIC	500	N.D.	500
	MBC	500	N.D.	500
V. cholerae	MIC	250	500	250
	MBC	500	500	250
E. coli	MIC	500	500	250
	MBC	500	500	250

N.D.= Not Determined

4.3.3 Bacteria Viability for Adhesion Assay

The bacterial viability, e.g. *E. coli* and *V. cholerae* for bacterial adhesion assay was determined after pre-incubation with Ajwa date extracts at different concentration (100 mg/ml to 500 mg/ml). The results are presented in **Figure 4.1** and **Figure 4.2**. As for *V. cholerae,* the bacteria were not viable after treatment when all the Ajwa date extracts were at the concentration of 500 mg/ml and 400 mg/ml. *V. cholera*e was viable after the treatment with all Ajwa date extracts at the concentration of 100 mg/ml. Meanwhile, the viability of *E. coli* decreased when incubated in Ajwa date extracts at the concentration of 500 mg/ml, 400 mg/ml. and 300 mg/ml. Provided that it was viable after the treatment with all Ajwa date extracts at the concentration of 100 mg/ml, the date fruit extracts at 100 mg/ml concentration will be used in the bacterial adhesion assay.

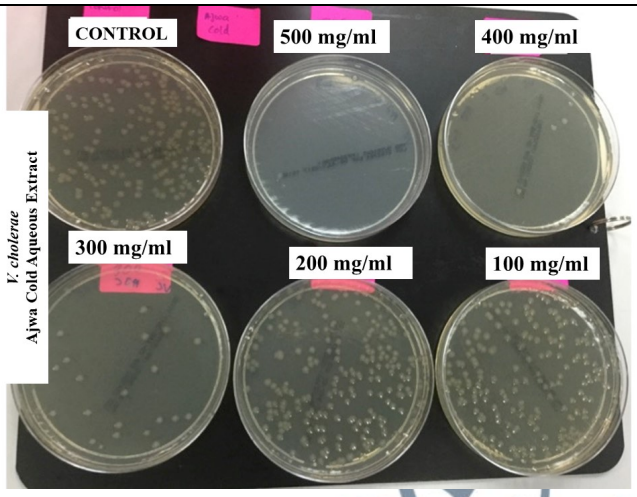
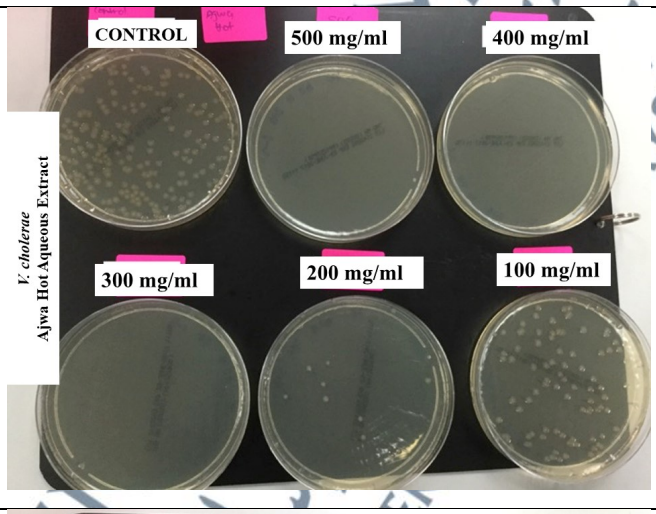
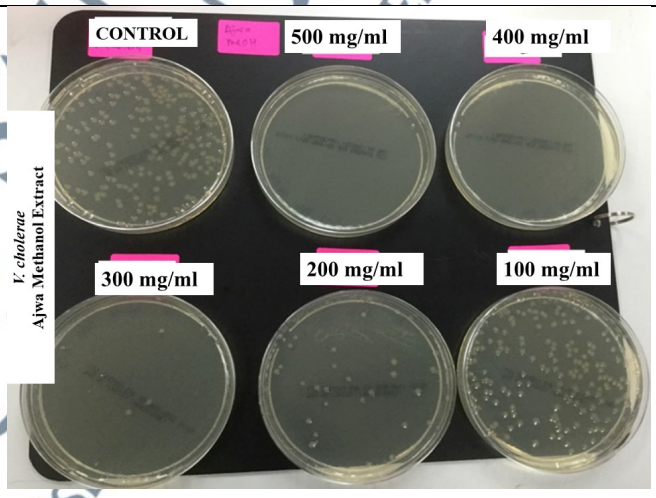
Ajwa cold	
Ajwa methanol	
Ajwa hot	

Figure 4.1: *V. cholerae* colonies after two-hour pre-incubation with Ajwa hot aqueous extracts at the concentration of 100 mg/ml to 500 mg/ml

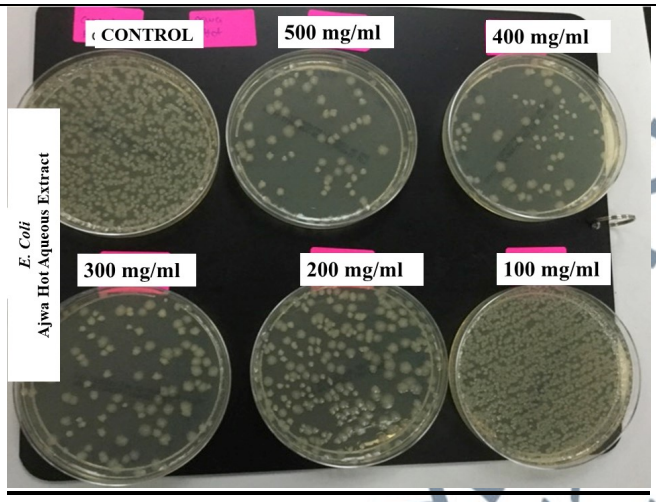
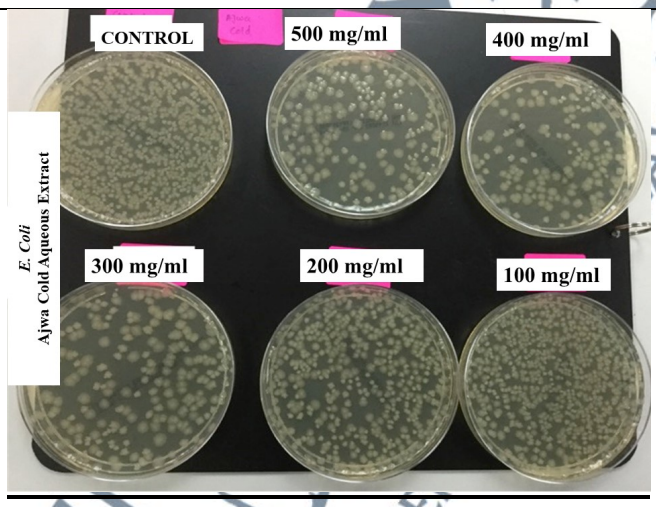
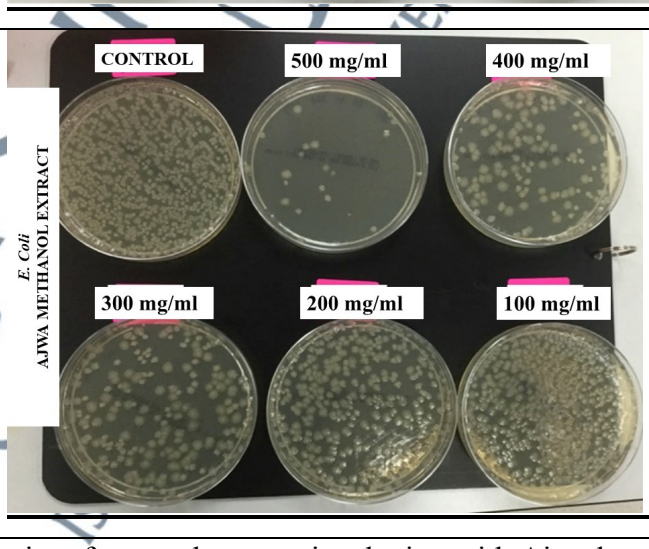
Ajwa hot aqueous extract	
Ajwa cold aqueous extract	
Ajwa methanol extract	

Figure 4.2: *E. coli* colonies after two-hour pre-incubation with Ajwa hot aqueous extract at the concentration of 100 mg/ml to 500 mg/ml.

4.4 Conclusion

The antibacterial activity in Ajwa dates increased with heat-treatment. However, longer heating duration and higher temperature will reduce the antibacterial activity in the Ajwa date. Therefore, an optimum temperature of 60^{o}C for 60 minutes was determined for hot aqueous extraction. The antibacterial activity in Ajwa date extracts was dose-dependent, with higher concentration indicating larger bacterial inhibition zone. Methanol extracts were the most active extracts compared to aqueous extracts, including higher inhibition zone and lower MIC/MBC values against all tested bacteria.