CHAPTER 7 :ANTIMICROBIAL ACTIVITY OF ISOLATED ACTIVE COMPOUNDS AND THE EFFECTS ON BACTERIAL MORPHOLOGICAL CHANGES VIA ELECTRON MICROSCOPY

7.1 Introduction

Based on the initial screening in this study, date extracts comprised significant antibacterial and anti-adhesion activity against all tested bacteria, as verified through a series of data presented in the previous chapters. It was suggested from the findings that Ajwa date hot aqueous extracts possessed the highest potency for antibacterial and anti-adhesion effects. Therefore, Ajwa date hot aqueous extract was selected to elucidate the active compounds that responsible for the antibacterial activity. The reverse phase, specifically preparative high-performance liquid chromatography (RP-HPLC), was employed for the fractionation of the crude extracts. The research finding presented evidence for the contribution of date fruits active compounds towards its antimicrobial effect. Furthermore, it is essential to uncover the fundamental basis of the inhibitory mechanism, by which active compound in Ajwa dates responsible for the antibacterial activity.

Additionally, the evaluation of the mechanisms involved in the Ajwa date extract and isolated active compounds to inhibit or kill the selected tested bacteria is important. The method used in this study was electron microscopy (EM), which is one of the techniques suitable to show definite details of bacteria morphology in a natural environment. This method consisted of two steps, namely scanning electron microscopy

(SEM) and transmission electron microscopy (TEM). Notably, SEM is useful for viewing three-dimensional (3D) image of the specimen, while TEM allows the observation of the internal structure of the cells as the specimens are cut to thin slice before being viewed using TEM.

This chapter aims to isolate the bioactive phytochemical from Ajwa date extract that responsible for the antibacterial activity and to examine the antibacterial mode of action of Ajwa date extracts and isolated active compound by observing the changes to the cell morphology and membranes through scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

7.2 Methodology

7.2.1 Antibacterial activity

As for the good diffusion assay, each dried isolated HPLC fraction was prepared by dissolving the samples with nutrient broth at a concentration of 200 mg/ml. All fractions were filtered using a sterile 0.20 µm membrane filter before use. All samples were subjected to methodology **3.6.2.** Meanwhile, each active fraction of MIC was prepared by dissolving it with nutrient broth at two-fold dilution, which formed a concentration from 100 mg/mL to 6.25 mg/ml.

The MIC for 96-wells plate template is illustrated in **Figure 8.1**. After incubation, Alamar Blue reagent (resazurin) was added to all well (15uL per well), and further incubated for two to four hours to observe the colour change. Upon the completion of incubation, the well with no colour change (blue resazurin remain unchanged) was scored as MIC value (Elshikh et al., 2016). Moreover, MBC was determined using methodology **3.6.3.** For bacteria adhesion assay, all fractions were

prepared based on bacteria dilution below their MIC values. In this case, all active fractions were subjected to methodology **3.7.3.**

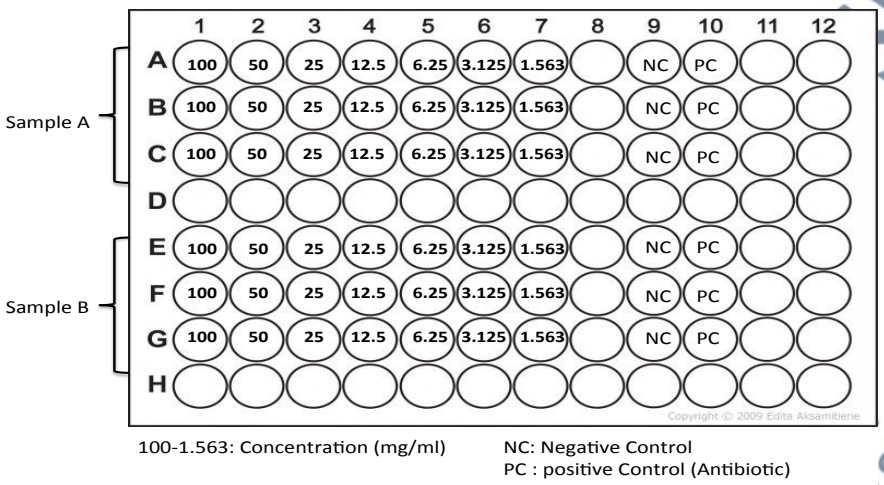

Figure 7.1: Minimum Inhibitory Concentration (MIC) Layout in 96-wells plates for active fractions.

7.2.2 Electron Microscopy

The samples for SEM and TEM were prepared. For Ajwa hot aqueous extract, all bacteria were treated with Ajwa date hot extract at a concentration of 500 mg/m, while the *V. cholerae* for isolated flavonoid fraction was treated with fractions 4, 5, and 6 at a concentration of 3.125 mg/ml. Notably, only *V. cholerae* was selected in this study due to the death or bactericidal of *V. cholerae* after the treatment with all active fractions. The samples were then fixed with 2.5% glutaraldehyde. The detailed process for SEM is described in methodology **3.9.1**, while the detailed process for TEM is described in methodology **3.9.2**.

7.3 Results

7.3.1 Isolation of Active Compound from Ajwa Dates Extract Using Prep-HPLC

Isolation was performed using Ajwa date hot aqueous extracts. The chromatograms of the extracts (see **Figure 8.2**) demonstrated several major peaks within 15 minutes of the separation. Upon HPLC separation, time collection began from zero to 67.3275 minutes, while a total of 45 fractions were collected. The data for fraction collection is illustrated in **Figure 8.3**. Each peak was collected and pooled together manually according to the retention time, while each collected fraction was freeze-dried before the antimicrobial activity was tested.

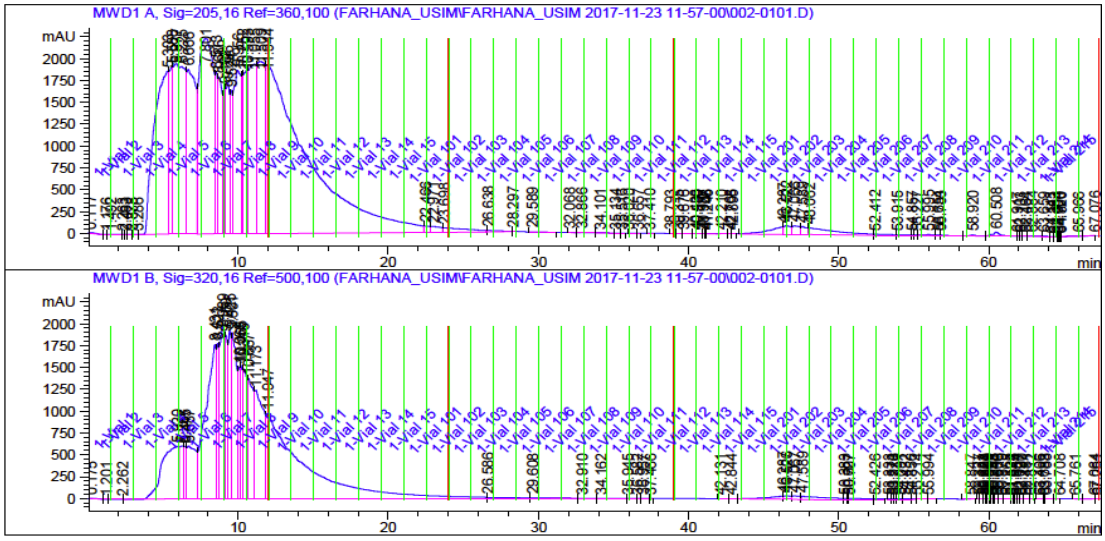

Figure 7.2: Preparative-HPLC chromatograms as monitored by UV absorption at 206nm and 320nm. The Lines in the chromatograph represent the fraction collected for isolation.

Fraction Information

Fraction collection using a timetable

Frac #	Well #	Location	Volume [µl]	BeginTime [min]	EndTime [min]	Reason	Mass
1	1	1-Vial 1	4495.63	0.0015	1.5000	Time	
2	1	1-Vial 2	4464.37	1.5119	3.0000	Time	
3	1	1-Vial 3	4470.63	3.0098	4.5000	Time	
4	1	1-Vial 4	4470.63	4.5098	6.0000	Time	
5	1	1-Vial 5	4469.38	6.0104	7.5003	Time	
6	1	1-Vial 6	4433.12	7.5223	9.0000	Time	
7	1	1-Vial 7	4470.63	9.0098	10.5000	Time	
8	1	1-Vial 8	4469.38	10.5102	12.0000	Time	
9	1	1-Vial 9	4470.63	12.0098	13.5000	Time	
10	1	1-Vial 10	4467.50	13.5111	15.0002	Time	
11	1	1-Vial 11	4424.37	15.0252	16.5000	Time	
12	1	1-Vial 12	4470.63	16.5098	18.0000	Time	
13	1	1-Vial 13	4470.63	18.0098	19.5000	Time	
14	1	1-Vial 14	4470.63	19.5098	21.0000	Time	
15	1	1-Vial 15	4471.25	21.0098	22.5002	Time	
16	1	1-Vial 101	4433.12	22.5223	24.0000	Time	
17	1	1-Vial 102	4470.63	24.0098	25.5000	Time	
18	1	1-Vial 103	4470.63	25.5098	27.0000	Time	
19	1	1-Vial 104	4470.63	27.0098	28.5000	Time	
20	1	1-Vial 105	4471.25	28.5098	30.0003	Time	
21	1	1-Vial 106	4441.25	30.0196	31.5000	Time	
22	1	1-Vial 107	4470.63	31.5098	33.0000	Time	
23	1	1-Vial 108	4469.38	33.0103	34.5000	Time	
24	1	1-Vial 109	4470.63	34.5098	36.0000	Time	
25	1	1-Vial 110	4467.50	36.0111	37.5003	Time	
26	1	1-Vial 111	4433.75	37.5223	39.0003	Time	
27	1	1-Vial 112	4353.13	39.0490	40.5000	Time	
28	1	1-Vial 113	4470.63	40.5098	42.0000	Time	
29	1	1-Vial 114	4470.63	42.0098	43.5000	Time	
30	1	1-Vial 115	4471.25	43.5098	45.0002	Time	
31	1	1-Vial 201	4438.75	45.0205	46.5000	Time	
32	1	1-Vial 202	4470.63	46.5098	48.0000	Time	
33	1	1-Vial 203	4470.63	48.0098	49.5000	Time	
34	1	1-Vial 204	4470.63	49.5098	51.0000	Time	
35	1	1-Vial 205	4471.25	51.0098	52.5002	Time	
36	1	1-Vial 206	4443.75	52.5188	54.0000	Time	
37	1	1-Vial 207	4470.63	54.0098	55.5000	Time	
38	1	1-Vial 208	4470.63	55.5098	57.0000	Time	
39	1	1-Vial 209	4470.63	57.0098	58.5000	Time	
40	1	1-Vial 210	4471.25	58.5098	60.0002	Time	
41	1	1-Vial 211	4430.63	60.0232	61.5000	Time	
42	1	1-Vial 212	4470.63	61.5098	63.0000	Time	
43	1	1-Vial 213	4470.63	63.0098	64.5000	Time	
44	1	1-Vial 214	4469.38	64.5102	66.0000	Time	
45	1	1-Vial 215	3953.13	66.0098	67.3275	Time	

Figure 7.3: Fraction information of all collected 45 fractions collected, including the information of the start/end time of each collection and the total volume collected.

7.3.2 Antibacterial Activity of Isolated Fraction of Ajwa Dates

Antibacterial activity of isolated Ajwa dates fractions was screened using well diffusion assay. Each fraction was tested for antibacterial activity against *Vibrio cholerae, S. flexneri* and *S. Typhimurium* at the concentration of 200 mg/ml. These organisms were selected due to the highest sensitivity of the bacteria to Ajwa date extract and limited quantity/volume of the isolated substance. After the screening of each fraction collected on the selected organism, antimicrobial activity was detected only from fraction 4, 5, and 6 (see **Table 8.1**). The aforementioned fractions were recorded with antibacterial activity with *S. flexneri*, which was the most sensitive organism with an inhibition zone ranging from 21.67 - 29.67mm, followed by 12 - 20.33 mm for *V. cholerae* and 13 - 17.33 mm for *S. Typhimurium.* However, no antibacterial activity against the selected, tested bacteria was recorded from other fractions.

Table 7.1: The diameter of the inhibition zone (mean ± standard deviation) of isolated fraction against tested bacteria using well diffusion assay method. Inhibition zone comprised 7 mm well hole, with N.I. - No inhibition. All fractions were tested at a concentration of 200 mg/ml.

	Inhibition zone (mm)		
Bacteria	*S. Typhimurium*	*S. flexneri*	*V. cholerae*
Fraction 1	N.I.	N.I.	N.I.
Fraction 2	N.I.	N.I.	N.I.
Fraction 3	N.I.	N.I.	N.I.
Fraction 4	17.33(±0.58)	29.67(±0.58)	20.33(±0.58)
Fraction 5	13.00 (±0.00)	26.33(±1.00)	12.00(±0.00)
Fraction 6	13.50(±0.58)	21.67(±0.58)	13.33(±0.58)
Fraction 7	N.I.	N.I.	N.I.
Fraction 8	N.I.	N.I.	N.I.
Fraction 9	N.I.	N.I.	N.I.
Fraction 10	N.I.	N.I.	N.I.
Fraction 11	N.I.	N.I.	N.I.
Fraction 12	N.I.	N.I.	N.I.
Fraction 13	N.I.	N.I.	N.I.
Fraction 14	N.I.	N.I.	N.I.
Fraction 15	N.I.	N.I.	N.I.
Fraction 16	N.I.	N.I.	N.I.
Fraction 1	N.I.	N.I.	N.I.
Fraction 17	N.I.	N.I.	N.I.
Fraction 18	N.I.	N.I.	N.I.
Fraction 19	N.I.	N.I.	N.I.
Fraction 20	N.I.	N.I.	N.I.
Fraction 21	N.I.	N.I.	N.I.
Fraction 22	N.I.	N.I.	N.I.
Fraction 23	N.I.	N.I.	N.I.
Fraction 24	N.I.	N.I.	N.I.
Fraction 25	N.I.	N.I.	N.I.
Fraction 26	N.I.	N.I.	N.I.
Fraction 27	N.I.	N.I.	N.I.
Fraction 28	N.I.	N.I.	N.I.
Fraction 29	N.I.	N.I.	N.I.
Fraction 30	N.I.	N.I.	N.I.
Fraction 31	N.I.	N.I.	N.I.
Fraction 32	N.I.	N.I.	N.I.
Fraction 33	N.I.	N.I.	N.I.
Fraction 34	N.I.	N.I.	N.I.
Fraction 35	N.I.	N.I.	N.I.

Fraction 36	N.I.	N.I.	N.I.
Fraction 37	N.I.	N.I.	N.I.
Fraction 38	N.I.	N.I.	N.I.
Fraction 39	N.I.	N.I.	N.I.
Fraction 40	N.I.	N.I.	N.I.
Fraction 41	N.I.	N.I.	N.I.
Fraction 42	N.I.	N.I.	N.I.
Fraction 43	N.I.	N.I.	N.I.
Fraction 44	N.I.	N.I.	N.I.
Fraction 45	N.I.	N.I.	N.I.

7.3.3 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC of active fractions is presented in **Table 8.2**, in which the MIC values for all fractions range from 3.125 mg/ml - 6.25 mg/ml. Notably, *V. cholerae* was the most sensitive bacteria to the MIC value of 3.125 mg/ml for all the tested active fractions, while fraction 6 had the lowest sensitivity against *S. Typhimurium* and *S. flexneri* with the MIC value of 12.5 mg/ml. The MIC values for fraction 5 and fraction 6 against *S. flexneri* and *S. Typhimurium* amounted at 6.25 mg/ml.

As seen from **Table 8.3**, the MBC values for fractions 4, 5, and 6 against *V. cholerae* amounted to 3.125 mg/ml. However, the MBC value for fraction 4, 5, and 6 against *S, Typhimurium* and *S. flexneri* could not be determined.

Table 7.2: Minimum Inhibitory concentration (MIC) for active fractions. The MIC values are given in mg/ml of three replicates.

	Minimum inhibitory concentration (MIC) (mg/ml)		
	Vibrio cholerae	*S. Typhimurium*	*S. flexneri*
Fraction 4	3.125	6.25	6.25
Fraction 5	3.125	6.25	6.25
Fraction 6	3.125	12.5	12.5

Table 7.3: Minimum bactericidal concentration (MBC) for active fractions. The MBC values are presented in mg/ml of three replicates.

	Minimum bactericidal concentration (MBC) (mg/ml)		
	Vibrio cholerae	*S. Typhimurium*	*S. flexneri*
Fraction 4	3.125	ND.	ND.
Fraction 5	3.125	ND.	ND.
Fraction 6	3.125	ND.	ND.
N.D. - Not determined.			

7.4 Bacterial adhesion assay

The anti-adhesion activity of active isolated fraction was determined using bacterial adhesion assay, which demonstrated the inhibition of bacterial adhesion from all fractions.

The bacterial ability of *V. cholerae* (see **Figure 8.4** and **Table 8.4**) to adhere to caco-2 cells amounted to 83.52%, 63.12%, and 73.85% after the treatment with fractions 4, 5, and 6, respectively. Specifically, fraction 5 showed a significantly lower percentage of bacterial adhesion compared to control (non-treated cells) ($p = 0.0171$).

Meanwhile, the bacterial ability of *S. Typhimurium* (see **Figure 8.4** and **Table 8.5**) to adhere to caco-2 cells amounted to 75%, 53%, and 58.45% after the treatment with fraction 4, 5, and 6, respectively. All fractions demonstrated a significantly lower percentage of bacterial adhesion compared to control (non-treated cells) ($p < 0.05$).

Furthermore, the bacterial ability of *S. flexneri* (see **Figure 8.4** and **Table 8.6**) to adhere to caco-2 cells amounted to 88.85%, 79.22%, and 73.06% after the treatment with fractions 4, 5, and 6, respectively. Specifically, fraction 6 was recorded with a significantly lower percentage of bacterial adhesion compared to control (non-treated cells) ($p = 0.0205$).

Table 7.4: Relative percentage of *V. cholerae* adhesion to Caco-2 cell monolayer after the treatment with an isolated active fraction. The data are presented as the mean percentage ± SD of three samples.

Relative percentage of bacteria adhesion (%)	
Fraction 4 (1.563mg/ml)	83.52 (±6.11)
Fraction 5 (1.563mg/ml)	63.12 (±12.36)
Fraction 6 (1.563mg/ml)	73.85 (±4.25)

Table 7.5: Relative percentage of *S. Typhimurium* adhesion to caco-2 cell monolayer after treatment with an isolated active fraction below MIC values. The data are presented as the mean percentage ± SD of three samples.

Relative percentage of bacteria adhesion (%)	
Fraction 4 (12.5 mg/ml)	75.00 (±2.50)
Fraction 5 (12.5mg/ml)	53.00 (±10.15)
Fraction 6 (25mg/ml)	58.45 (±5.80)

Table 7.6: Relative percentage of *S. flexneri* adhesion to caco-2 cell monolayer after the treatment with an isolated active fraction below MIC values. The data are presented as the mean percentage ± SD of three samples.

Relative percentage of bacteria adhesion (%)	
Fraction 4 (12.5mg/ml)	88.85 (±5.55)
Fraction 5 (12.5mg/ml)	79.22 (±9.60)
Fraction 6 (25mg/ml)	73.06 (±0.84)

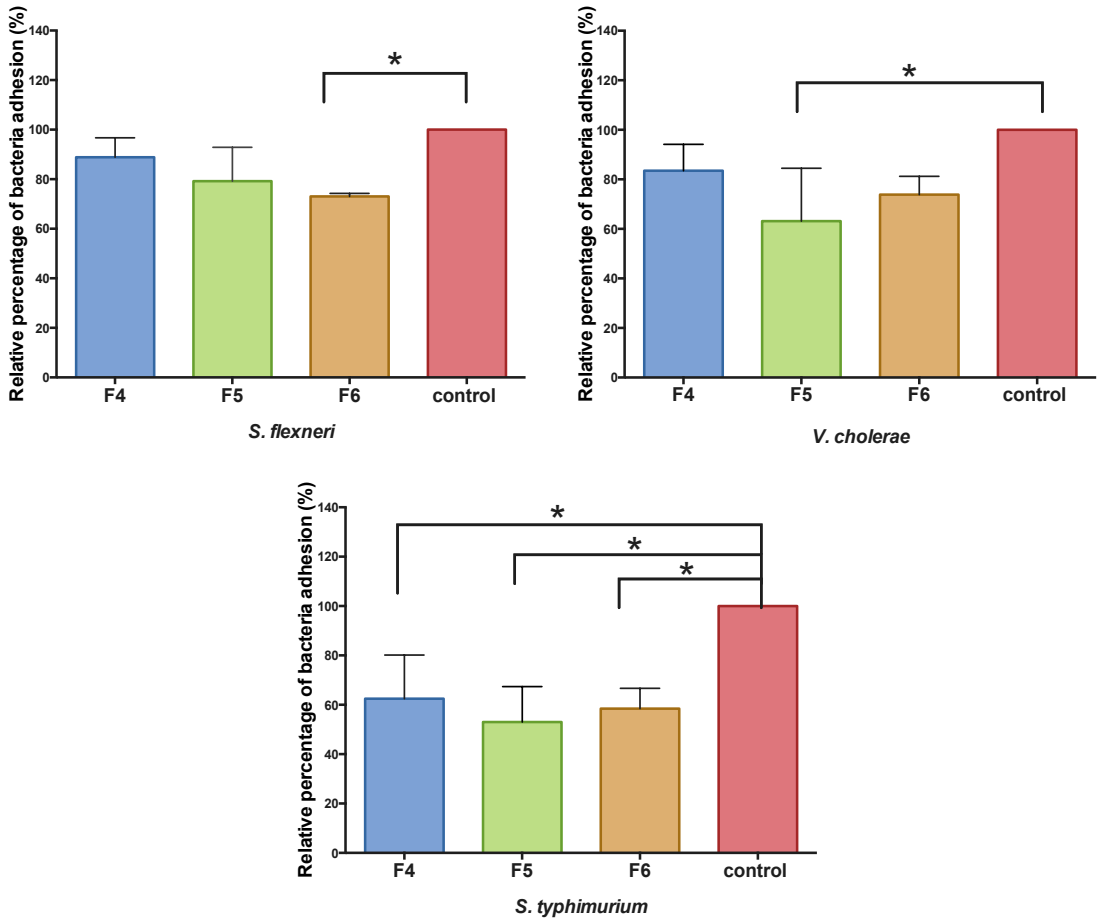

Figure 7.4: Relative percentage of bacterial adhesion to caco-2 cell monolayer after the treatment with an isolated active fraction. Asterisk* indicates the significant difference (*$p < 0.05$) compared to the control (100%).

7.5 Identification of active compound using UHPLC-ESI-QTOF-MS/MS

After screening the antibacterial activity of isolated fraction on the tested organisms, the active fractions were identified using UHPLC-ESI-QTOF-MS/MS system with Sciex 3200QTRAP hybrid mass spectrometer, which was coupled with Perkin Elmer FX-15 UHPLC. Fraction 4 (see **Figure 8.5 and Table 8.7**) consisted of flavonoid fractions, that including Catechin 7,4'-dimethyl ether, Hexa-acetyl-pyracanthoside, and Luteolin 7, 3',4'-trigluconide. Although fraction 5 (see **Figure 8.6** and **Table 8.8**) consisted of few compounds, the most prominent peak was identified as Kaempferol 3-(2",3"-diacetyl-4"-p-coumarylrhamnoside, which was a flavonoid compound. Moreover, fraction 6 (see **Figure 8.7** and **Table 8.9**) consisted of few compounds, with the most prominent peak identified as Cyanidin 3-rutinoside-5-glucoside, which was a Flavone Glucoside. Therefore, flavonoids and their derivatives had a role in the antimicrobial and anti-adhesion activities of Ajwa dates.

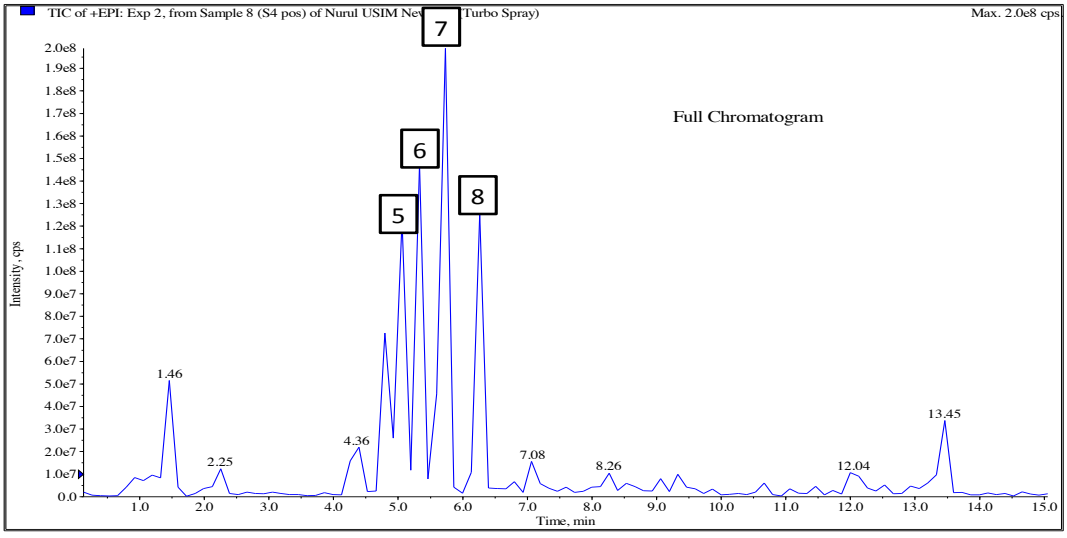

Figure 7.5: Full chromatogram of phytochemical compounds detected in fraction 4 using UHPLC-ESI-QTOF-MS/MS

Table 7.7: Phytochemical compound detection of fraction 4 using UHPLC-ESI-QTOF- MS/MS

Peak	RT	ms	Ms/ms	Compound	Reference
1	1.26	355	144.04, 161.10, 209.15, 191.11, 265.14, 337.16, 355.14	Unknown	-
2	2.25	268	146, 163.07, 150.02, 162.10, 174.08, 176.11, 178.09, 198.11, 232.18, 250.11,268.16	Unknown	-
3	4.36	436	68.81, 95.97, 113.99, 209.19, 226.21,	Unknown	-
4	4.794	590	571.47, 589.45	Unknown	-
5	5.061	703	684.74, 702.62	Hexaacetylpyranthoside (flavanone glucoside) $[M+H]^+$ Exact mass = 702.1796	NCBI&(Bilia et al., 1991)
6	5.328	816	797.99, 815.79	Luteolin 7, 3',4'-trigluconide (flavonoid glucoside) $[M-H\}^+$	NCBI
7	5.720	1020	548.65, 664.79, 775.02, 889.12, 907.15, 1002.23, 1020.04	Fatty acids	NCBI
8	6.26	319	69.85, 101.99, 212.29, 256.30, 300.32, 318.31	m7,4'-dimethyl ether (flavonoid) $[M+H]^+$	NCBI

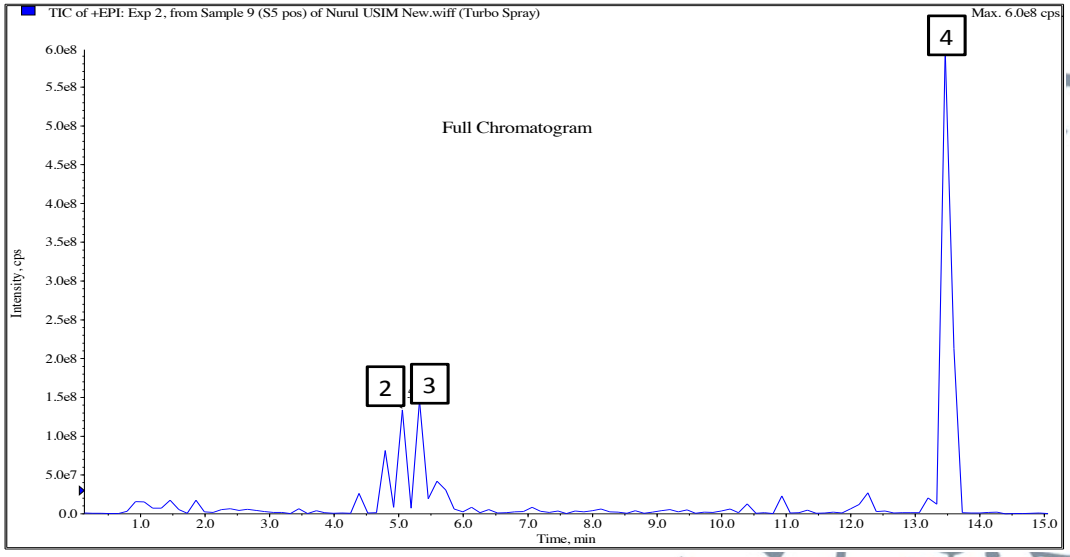

Figure 7.6: Full chromatogram of phytochemical compounds detected in fraction 5 using UHPLC-QTOF-MS/MS

Table 7.8: Phytochemical compound detected Fraction 5 using UHPLC-ESI-QTOF-MS/MS

Peak	RT	m/z	Ms/ms	Compound	References
1	4.787	590	571.48, 589.46	Unknown	-
2	5.055	703	684.78, 702.63	Hexaacetylpyracanthoside (flavanone glucoside) $[M+H]^+$ Exact mass = 702.1796	NCBI and (Bilia et al., 1991)
3	5.323	816	797.94, 815.79	Luteolin 7, 3',4'-trigluconide (flavonoid glucoside) $[M-H\}^+$ Exact mass = 814.611	NCBI
4	13.462	663	143.23, 251.41, 307.43, 327.42, 383.48, 439.45, 495.32, 551.42, 607.47, 663.52	Kaempferol 3-(2",3"-diacetyl-4"-p-coumarylrhamnoside Exact mass = 662.1636	NCBI

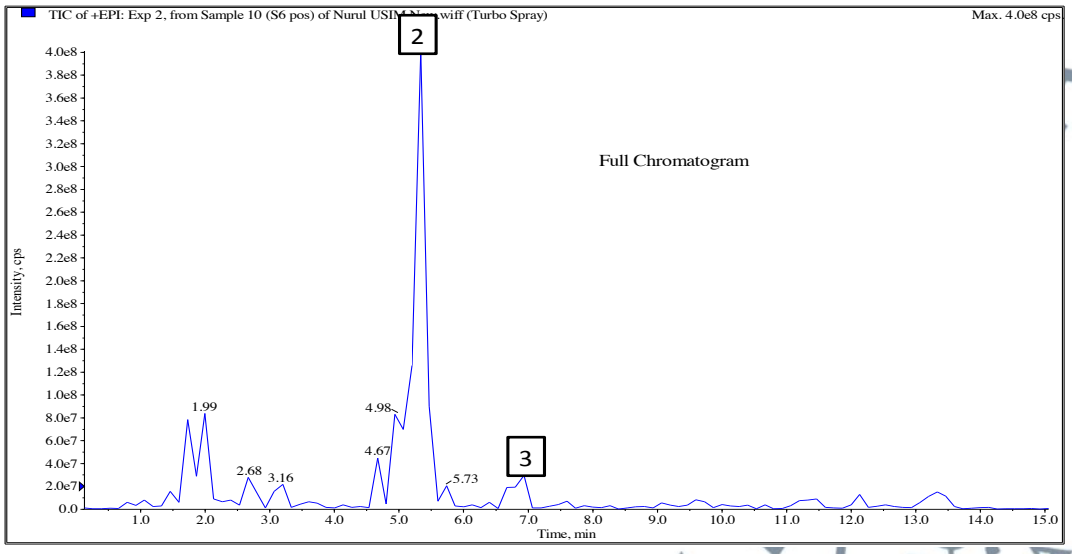

Figure 7.7: Full chromatogram of phytochemical compounds detected in Fraction 6 by using UHPLC-QTOF-MS/MS

Table 7.9: Phytochemical compound detected Fraction 6 using UHPLC-ESI-QTOF-MS/MS

Peak	RT	m/z	Ms/ms	Compound	Reference
1	1.996	425	168.11, 211.15, 229.14, 247.16, 291.15, 407.17, 425.13	Unknown	-
2	5.336	793	113.96, 209.30, 228.30, 322.39, 341.37, 435.39, 435.39, 454.38, 548.67, 565.73, 661.84, 679.87, 757.09, 774.96, 792.77	Cyanidin 3-rutinoside-5-glucoside (Flavone Glucoside)	NCBI
3	6.933	906	209.17, 322.25, 435.25, 531.37, 548.41, 566.45, 661.57, 679.75, 792.75, 887.81, 906.35, 1018.96, 1133.09, 1246.18	4-Chlorobenzoyl-CoA	NCBI

7.5.1 Electron Microscopy

7.5.1.1 Observation of Changes and Structural Damage of Bacterial Incubated in Ajwa Dates Hot Aqueous Extract By SEM

All bacteria treated with Ajwa date hot aqueous extracts were then observed by SEM for any morphological changes in the appearance. Subsequently, physical damages were observed in the bacterial cell wall, while morphological alteration to all tested bacteria after threated with Ajwa dates extract. Meanwhile, no sign of structural degeneration was observed from the bacterial in non-treated cells (control). Moreover, they retained their natural shape as *S. aureus* (see **Figure 7.8; A**) was found to have an intact smooth round shapes surface. After the treatment with Ajwa dates extract, *S. aureus* (see **Figure 7.8; A-2**), several cells were found to shrunken/flatten (arrow A), while other cells were completely lysed (arrow B).

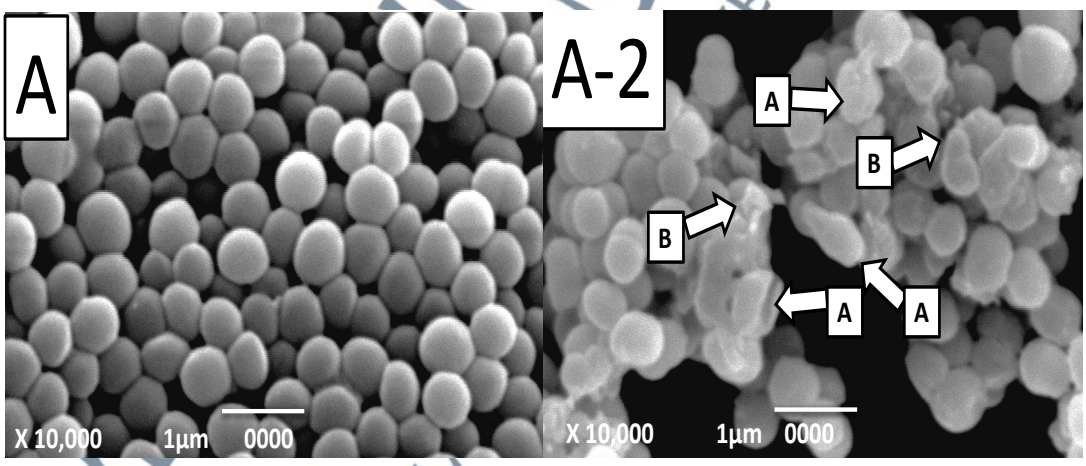

Figure 7.8: SEM micrograph observation of *S. aureus* for untreated (control) and treated Ajwa date hot aqueous extract.

Untreated *S. Typhi* (see **Figure 7.9; B**) and S. *Typhimurium* (see **Figure 7.9; C**) were found to have intact smooth rod shapes. After the treatment with Ajwa dates extract, almost the same cell damage was recorded from both bacteria. While the deformity of the cell wall (arrow A) was currently present, formations of dimples at the edge and surface of the cells (arrow B), a blister on the cell surface after treatment, and some cell lysis (arrow C) were also identified. Cell length was found to be shorter compared to control (arrow D).

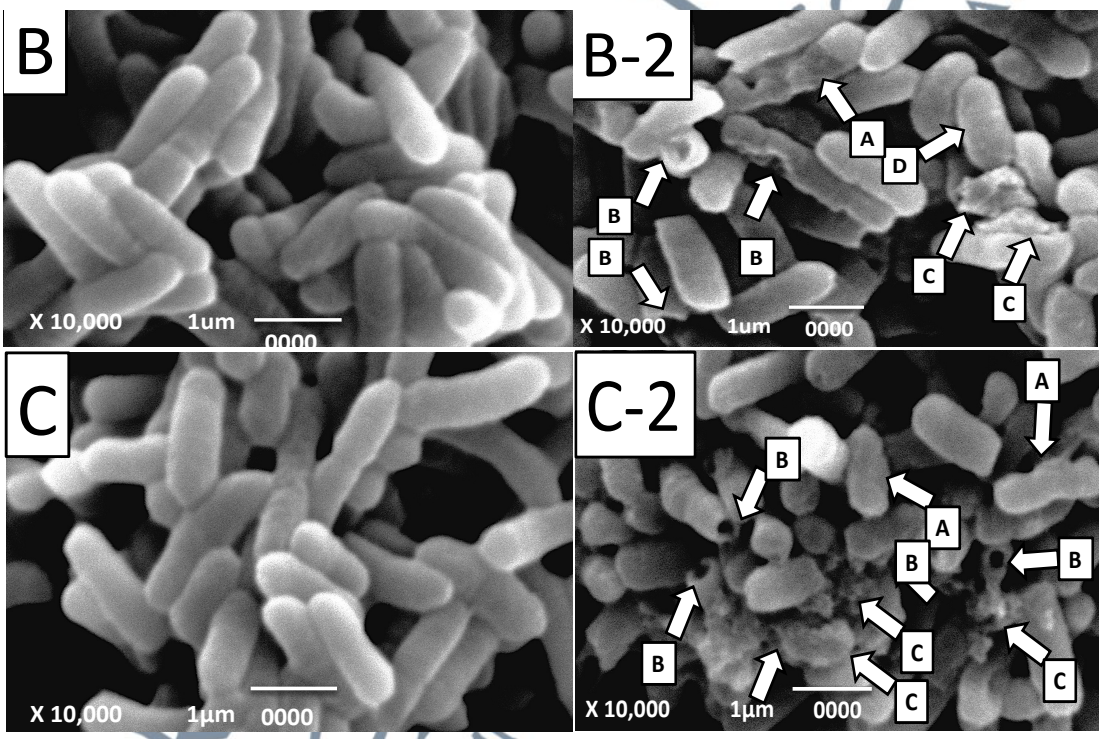

Figure 7.9: SEM micrograph observation of *S. Typhi* and S. *Typhimurium* for untreated (control) and treated Ajwa date hot aqueous extract

Untreated *E. coli* (see **Figure 7.10; D**) was found to have intact smooth rod shapes surface. After the treatment with Ajwa date extract *E. coli* (see **Figure 7.10; D-2**), surface irregularities were observed, in which the bacterial surfaces appeared to be rougher and shrinking compared to the control (arrow A) and cell lysis (arrow B).

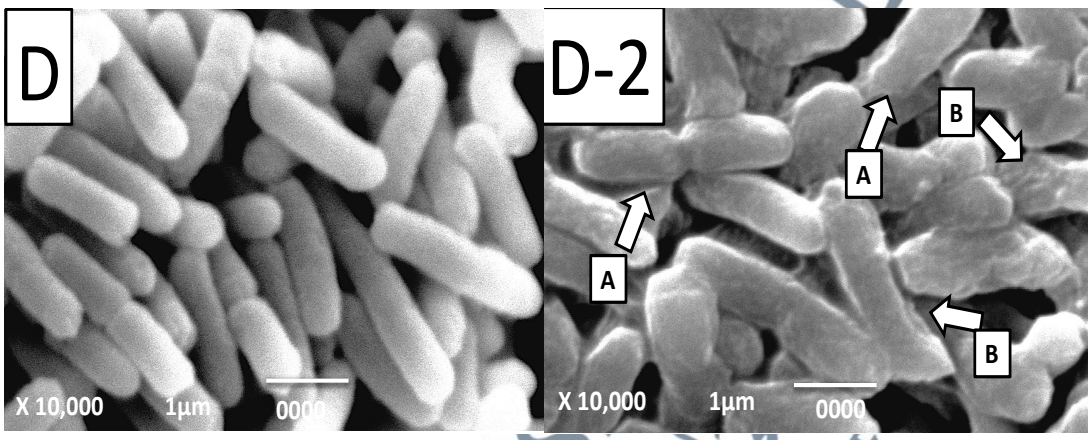

Figure 7.10: SEM micrograph observation of *E. coli* for untreated (control) and treated Ajwa date hot aqueous extract

Untreated *S. flexneri* (see **Figure 7.11; E**) as found to have intact smooth rod shapes surface. After the treatment with Ajwa dates extract *S. flexneri* (see **Figure 7.11; E-2**), several cells appeared with deformity (arrow A) and complete lysis (arrow B), followed by the formations of debris-likes substance, which were considered cellular debris appearing from cell lysis (arrow C).

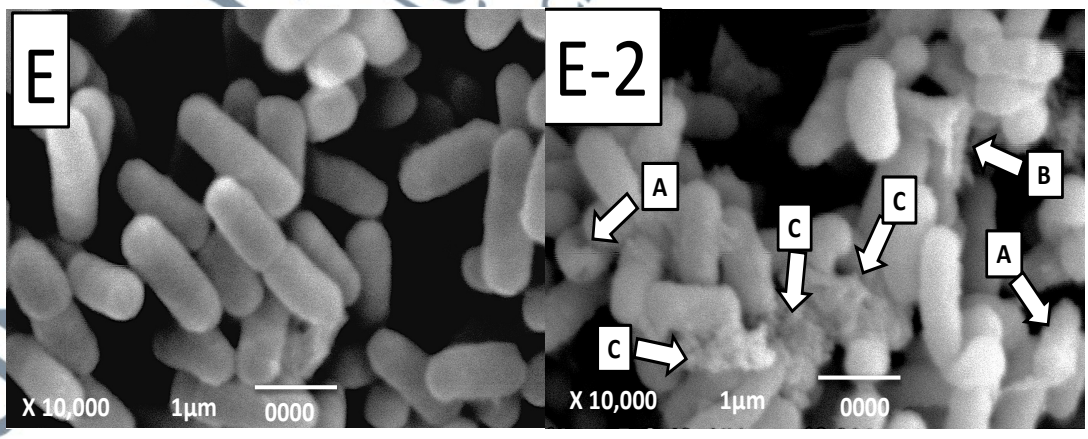

Figure 7.11: SEM micrograph observation of *S. flexneri* for untreated (control) and treated Ajwa date hot aqueous extract

Untreated *V. cholerae* (see **Figure 7.12; F**) was observed with a slightly curved rod (comma shape) with a smooth surface. After the treatment with Ajwa dates extract *V. cholerae* (see **Figure 7.12; F-2**), some cells were recorded with deformity (arrow A) and complete lysis (arrow B), followed by the formations of debris-like substance, which was considered the cellular debris appearing from cell lysis (arrow C).

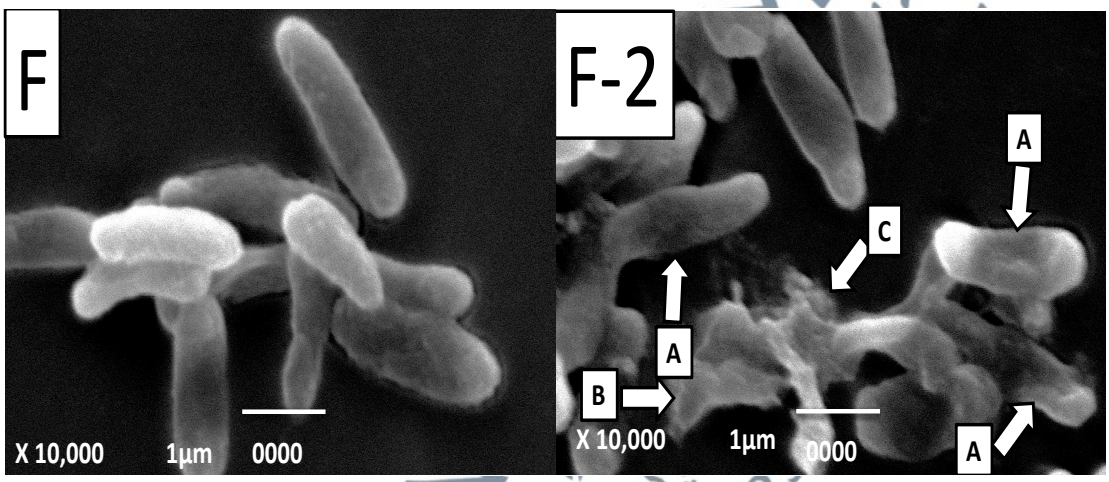

Figure 7.12: SEM micrograph observation of *V. cholerae* for untreated (control) and treated Ajwa dates hot aqueous extract

7.5.2 Observation of changes and structural damage of bacterial incubated in isolated active fraction of Ajwa dates by Electron Microscopy

7.5.2.1 Observation Using Scanning Electron Microscopy (SEM)

The observation of bacterial treated with active fraction was performed on *V. cholerae* as the *S. Typhimurium* and *S. flexneri* ere not killed after the treatment with the active fraction as shown previously. In this case, MBC values could not be determined. The untreated *V. cholerae* (see **Figure 7.13**; A) cells have the shape of slightly curve rod (comma shape) with an intact smooth surface. In comparison, there were ultra-structural changes in the cellular structure of *V. cholerae* after treatment with an active fraction (see **Figure 7.13**; B, C, D). SEM observation showed most of the cells treated with fraction 4 (see **Figure 7.13**; B) were damaged and shrunken with cell wall deformity (arrow A). Besides, most cells were clustered, stick to each other and melted (arrow B).

Bacterial cells treated with fraction 5 (see **Figure 7.13**; C) were also observed to be deformed and shrunk (arrow A), clustered, and sticking to each other (arrow B). Meanwhile, the cells treated with fraction 6 (see Figure **Figure 7.13**; D) were recorded with surface irregularities, in which swelling on the surface of the bacteria and bigger cell diameter were observed (arrow A), the cell surface seem to rougher, shrunken (arrow B) and there was a formation of dimple (arrow C). Some cells were observed to be completely lysis.

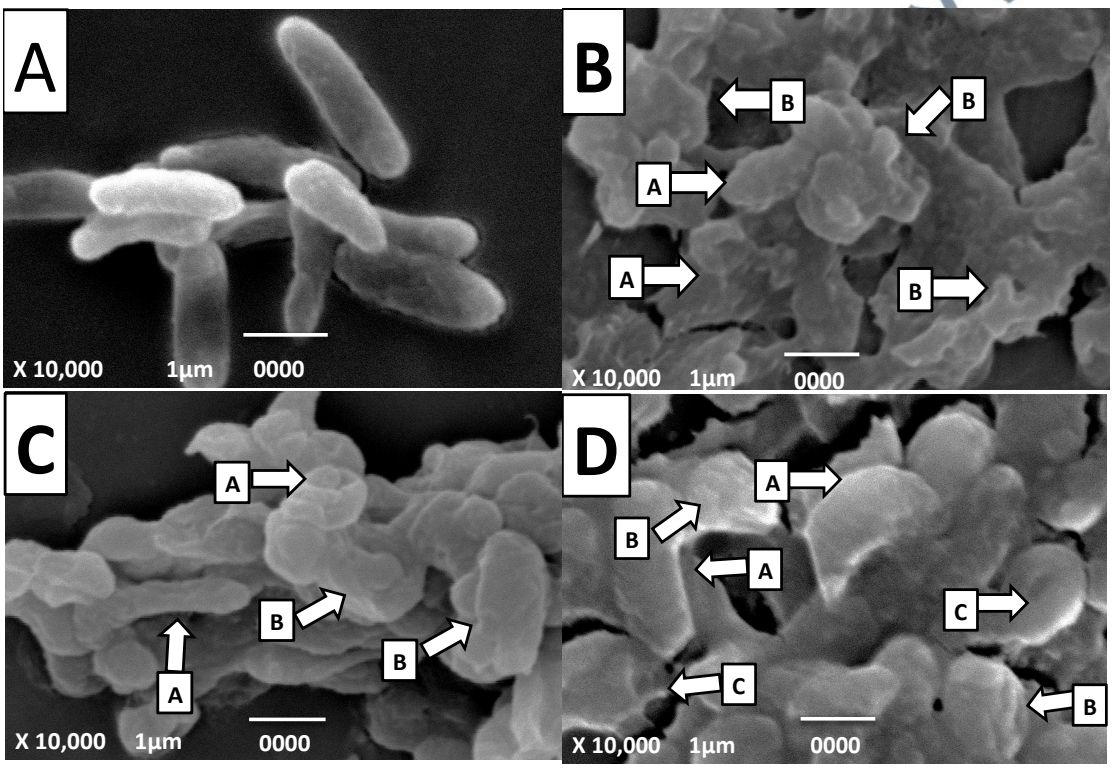

Figure 7.13 :SEM micrograph observation of *V. cholerae* for untreated (control) and after treated with the active fractions. **A:** untreated (control); **B:** Fraction 4; **C:** Fraction 5; **D**: Fraction 6

7.5.2.2 Observation Using Transmission Electron Microscopy (TEM)

The cells were observed under TEM to determine the degeneration effects of treatment on the inside and cross-sectional of the cells. In this case, *V. cholerae* was selected for this parameter due to good antibacterial activity on Ajwa dates extract and active fraction. The treatment of the cells was performed in the same method to SEM observation.

Figure 7.14(A) presents the *V. cholerae* cells without any treatment (control), in which all cells were intact with no sign of structural degeneration. The cell wall and organelle were completely intact with the slightly waved outer membrane, while the periplasmic was thin and had a uniformed appearance. However, after treated with Ajwa dates hot aqueous extract and flavonoids fraction of Ajwa dates, *V. cholerae* cells were deformed completely.

As seen in **Figure 7.14 (B)**, after the treatment with Ajwa date hot aqueous extract, TEM appeared with the rippling appearance and disorganised surface of cell membrane (arrow A), cellular cytoplasmic constituent/organelle were release in some cells (arrow B), and plasmolysis occur where detachment or shrinking of cellular cytoplasm from the cell membrane (arrow C).

After the treatment with fraction 4 (see **Figure 7.14 (C)**), plasmolysis was seen in the TEM images with detachment of cellular cytoplasm from cell membrane (arrow C). Cytoplasmic constituent/organelle which was fully released from the cell membrane also observed in some cells (arrow B).

After the treatment with fraction 5 (**Figure 7.14 (D)**), cells rippling appearance and disorganised cell membrane (arrow A) were observed, which included disintegration of the cells component (arrow D).

After the treatment with fraction 6 (**Figure 7.14 (E)**), rippling appearance and disorganised cell membrane (arrow A) was observed, including the appearance of cytoplasmic released from the cell membrane(arrow B) and the cellular disintegration (arrow D). The detachment of cellular cytoplasm from cell membrane (arrow C) also seen from the TEM images.

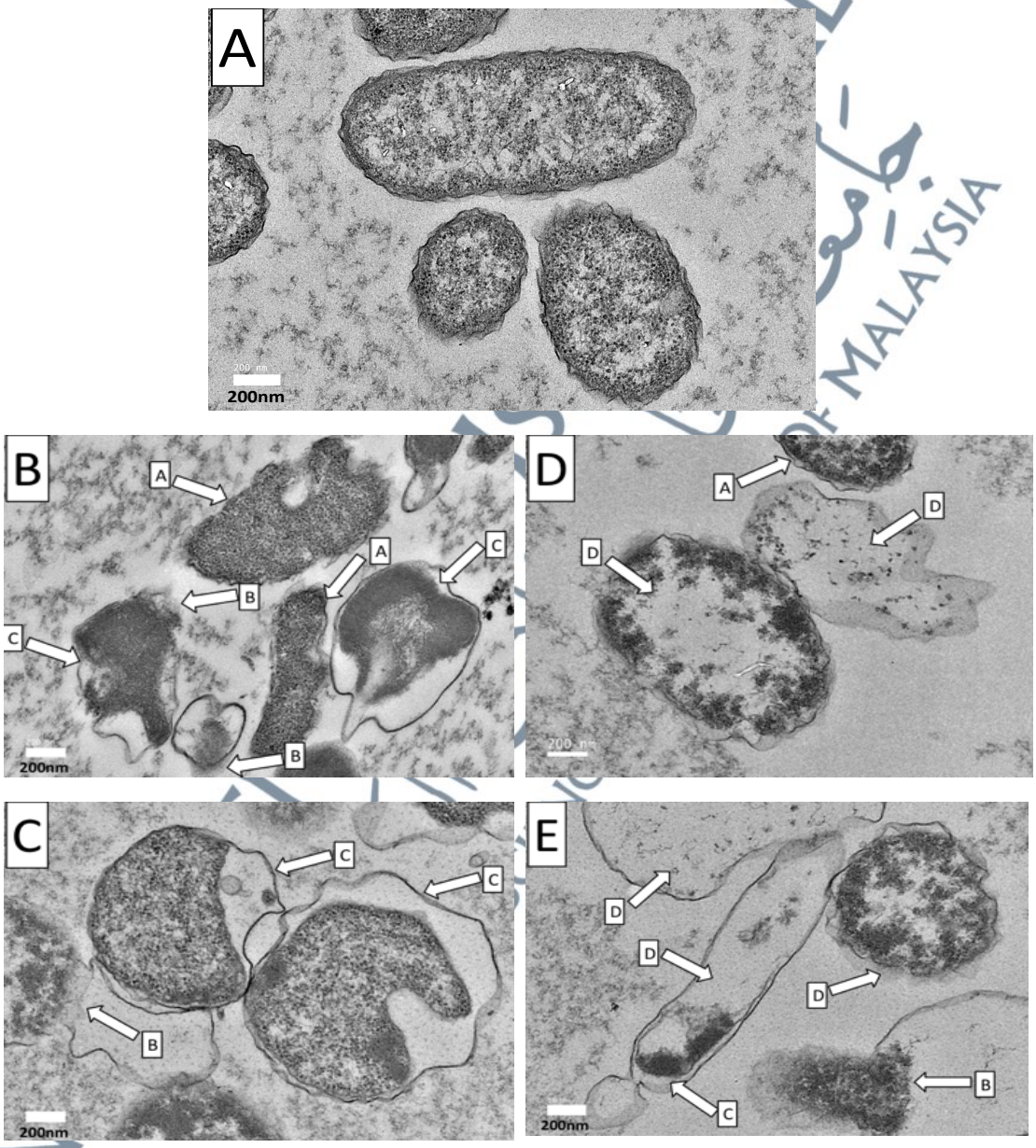

Figure 7.14: TEM micrograph observation of *V. cholerae* for untreated (control) and after treated with an active fraction. **A:** untreated (control); **B:** Ajwa dates hot aqueous extract; **C:** Fraction 4; **D:** Fraction 5; **E**: Fraction 6.

7.6 Conclusion

Flavonoids were found to be the responsible compounds for the antibacterial and anti-adhesion activity of Ajwa date. These compounds were Catechin 7,4'-dimethyl ether, Hexa-acetyl-pyracanthoside and Luteolin 7, 3',4'-trigluconide, Kaempferol 3-(2",3"-diacetyl-4"-p-coumarylrhamnoside and Cyanidin 3-rutinoside-5-glucoside. These results indicated that flavonoid compounds in Ajwa dates were responsible for inhibiting/killing and prevent the adhesion of bacteria that led to gastroenteritis.

The mode of action of isolated flavonoids and Ajwa date extracts antibacterial activity, is through disruption of the cell wall and intracellular alteration of the cells, which are vital for the survival of the bacteria. Therefore, morphological damages and alteration lead to bacterial cells death.