CHAPTER 4

RESULTS AND DISCUSSION

4.1 Chemical Compounds in Ajwa Dates Flesh

Nutritional profiling of Ajwa dates fruits have been based on GC-MS and LC-QToF-MS. Meanwhile, the fingerprint analysis of Ajwa dates fruit has been conducted using FTIR.

4.1.1 GC-MS Analysis of Ajwa Dates

Other studies reported that dates fruits contain flavonoids, sterols, anthocyanins, carotenoids (Baliga et al., 2011) and other nutritional composition such as fiber and minerals content (El-Sohaimy and Hafez, 2010). According to Kchaou et al (2013), most of previous researches used the mixture design concept for extraction, by combining few polar solvents to investigate antioxidants activity in dates fruits (Saeed et al., 2012) and antibacterial in Manjakani (Nur Syukriah et al., 2014).

Thus, we studied the effects of selective extracting solvents and their combination on extraction of chemical compounds in Ajwa dates using three mixture designs. By combining different polarities of extracting solvents, we believed that the combination will give new finding or extra information on the nutritional value of Ajwa dates fruits compared to single solvent extraction. In GC-MS analysis, all chemical compounds were selected based on similarity index (SI) is 80 % and above. The basis of selection also depends on the area of the peak. If the peak area is less than 0.5 %, the SI from 60 % to 80 % will also be considered if, the compound is detected in other design. This is because of the noises that decrease the SI of the peak.

4.1.1.1 Methanol Extract (D1)

Methanol extract is labeled as Design 1 (D1). Methanol, a polar organic solvent with polarity index 5.1 is commonly used in extraction of chemical compounds. 12 chemical compounds were identified; 3, 5-dihydroxy-2-methyl-4H-pyran-4-one; 2, 3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one; 5-hydroxymethylfurfural; octanoic acid; n-decanoic acid; dodecanoic acid; tetradecanoic acid; n-hexadecanoic acid; 9, 12-octadecadienoic acid; 9-octadecenoic acid; cis-13-octadecenoic acid and octadecanoic acid. Chemical compounds that identified in D1 are tabulated in Table 4.1 and the chemical structures are shown in Appendix 10.

Table 4.1: Chemical Compounds Extracted using D1

Retention Time	Compounds	Area (%)
8.37	2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	6.41
8.77	octanoic acid	1.11
9.01	3,5-dihydroxy-2-methyl-4H-pyran-4-one	0.22
10.08	5-hydroxymethylfurfural	3.97
12.36	n-decanoic acid	1.18
16.02	dodecanoic acid	6.85
19.04	tetradecanoic acid	2.99
21.96	n-hexadecanoic acid	1.41
24.26	9,12-octadecadienoic acid	1.41
24.35	9-octadecenoic acid	1.42
24.39	cis-13-octadecenoic acid	0.31
24.62	octadecanoic acid	0.39

The highest concentration of compound in methanol extract with percentage area 6.85 is dodecanoic acid (lauric acid). Meanwhile the lowest concentration is 3, 5-dihydroxy-2-methyl-4H-pyran-4-one with percentage area 0.22.

Most of the identified compounds are acids and the longer the compound remains in the column, indicated that the compound is more nonpolar than others. This is because of the column used is nonpolar column, thus, "like to like" concept has applied. Two direct ways of measuring polarity are dipole moments and dielectric constant. The dipole moment and dielectric constant increases concomitant with the increasing the polarity of the solvent (Khan et al., 2016). Chromatogram of D1 is shown in Appendix 11.

4.1.1.2 Chloroform Extract (D2)

As a semi polar organic solvent; chloroform with polarity index 4.1 is used as the extracting solvent which labelled as D2. The greatest numbers of compounds were identified in D2 compared to other designs. 13 compounds were identified including octanoic acid trimethylsilyl ester, decanoic acid trimethylsilyl ester, dodecanoic acid trimethylsilyl ester, D-psicofuranose pentakis(trimethylsilyl) ether, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-D-fructose, tetradecanoic acid trimethylsilyl ester, hexadecanoic acid ethyl ester, hexadecanoic acid trimethylsilyl ester, heptafluorobutyric acid n-tetradecyl ester, octadecanoic acid ethyl ester, 9,12-octadecadienoic acid (Z,Z)-trimethylsilyl ester, 11-trans-octadecenoic acid trimethylsilyl ester and trans-9-octadecenoic acid, trimethylsilyl ester. Table 4.2 showed the compounds that identified in D2.

Table 4.2: Chemical Compounds Extracted using D2

Retention Time	Compounds	Area (%)
10.18	octanoic acid, trimethylsilyl ester	2.39
13.87	decanoic acid, trimethylsilyl ester	2.31
17.24	dodecanoic acid, trimethylsilyl ester	37.54

19.45	d-Psicofuranose, pentakis(trimethylsilyl) ether (isomer1)	0.22
19.57	1,3,4,5,6-pentakis-O-(trimethylsilyl)- D-Fructose	0.63
20.26	tetradecanoic acid, trimethylsilyl ester	10.16
22.36	hexadecanoic acid, ethyl ester	1.21
23.04	hexadecanoic acid, trimethylsilyl ester	7.18
23.61	heptafluorobutyric acid, n-tetradecyl ester	0.35
25.01	octadecanoic acid, ethyl ester	0.67
25.18	9,12-octadecadienoic acid, trimethylsilyl ester	0.24
25.26	11-trans-octadecenoic acid, trimethylsilyl ester	4.32
25.35	trans-9-octadecenoic acid, trimethylsilyl ester	1.01

Generally, more ester compounds were extracted. The trimethylsyilyl ester is expected from Trimethylsilyl (TMS) during the derivatization process. Chloroform is a strong organic solvent because this solvent reacts vigorously with chemically active metal, strong oxidants, rubber, coatings and plastic. Chromatogram of D2 is shown in Appendix 12.

Compared to D1, dodecanoic acid that identified in D2 is more concentrated with percentage area 37.54. Other similar compounds also have the percentage area more than in D1. There are few compounds that identified in D2 but not identified in D1 such as, D-psicofuranose, pentakis(trimethylsilyl) ether, 1,3,4,5,6-pentakis-O-(trimethylsilyl)- D-fructose, heptafluorobutyric acid, n-tetradecyl ester, 11-trans-octadecenoic acid, trimethylsilyl ester and trans-9-octadecenoic acid, trimethylsilyl ester. This means that these compounds are dissolved more in chloroform than methanol. Ethyl ester or methyl ester may occur naturally in plant (Herrera-Valencia et al., 2012). Not all compounds can be detected in GC-MS, so derivatization is needed. Typically, derivatization is done to convert the analyte properties to improve the separation and enhancing method sensitivity (Moldoveanu and David, 2019; Zheng et al., 2016).

4.1.1.3 Hexane Extract (D3)

Hexane is a nonpolar organic extracting solvent with polarity index 0.0. In this design, 5 chemical compounds were identified; dodecanoic acid; trimethylsilyl ester; hexadecanoic acid, trimethylsilyl ester; octadecanoic acid; trimethylsilyl ester; vitamin E and β-sitosterol. All compounds that identified in this design are presented in Table 4.3 and the chromatogram is in Appendix 13.

Table 4.3: Chemical Compounds Extracted using D3

Retention Time	Compounds	Area (%)
17.21	Dodecanoic acid, trimethylsilyl ester	1.08
23.03	Hexadecanoic acid, trimethylsilyl ester	5.43
25.59	Octadecanoic acid, trimethylsilyl ester	2.98
30.62	Vitamin E	7.70
42.15	β-Sitosterol	0.23

Vitamin E and β-sitosterol are only identified in D3, which hexane is the solvent. β-sitosterol is a polar (hydrophilic) molecule in which its dipole moment is -2.5 D. However, according to Kurban et al. (2010), extra ethyl group in β-sitosterol makes this compound differ from cholesterol, which providing more bulk. Consequently, β-sitosterol is more hydrophobic than cholesterol, which poorly soluble in oil and water phases. This is the reason why β-sitosterol is extracted in nonpolar solvent instead of polar solvent.

4.1.1.4 Methanol: Chlorofom Extract (D4)

A total of 8 chemical compounds were identified in Design 4 (D4), which was a combination of methanol: chloroform with ratio 1:1. Those chemical compounds detected in D4 including 2, 3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, hexadecanoic acid, methyl ester, n-hexadecanoic acid, hexadecanoic acid, ethyl ester,

9,12-octadecadienoic acid, methyl ester, 9,12-octadecadienoic acid, octadecanoic acid and octadecanoic acid, ethyl ester. These compounds were found in both methanol (D1) and chloroform (D2). No new compounds were identified in this design. These compounds are tabulated in Table 4.4 and the chromatogram of this extract is illustrated in Appendix 14.

Table 4.4: Chemical Compounds Extracted using D4

Retention Time	Compounds	Area (%)
7.92	2, 3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	0.33
21.40	hexadecanoic acid, methyl ester	0.39
21.89	n-hexadecanoic acid	2.17
22.35	hexadecanoic acid, ethyl ester	0.67
23.70	9,12-octadecadienoic acid, methyl ester	0.31
24.19	9,12-octadecadienoic acid	1.47
24.58	octadecanoic acid	0.90
25.00	octadecanoic acid, ethyl ester	0.35

4.1.1.5 Methanol: Hexane Extract (D5)

Combination of strong polar organic solvent (methanol) and strong non polar organic solvent (hexane) formed two layers as both organic solvents are immiscible. This is due to the attraction between methanol and hexane molecules are different from attraction of individual solvents. The mixing causes the disruption of the structure of the liquid, so very little mixing take places. The layers are labeled as D5M for methanol extract layer and D5H for hexane extract layer. Chemical compounds that were identified in D5M are presented in Table 4.5.

Table 4.5: Chemical compounds Extracted using D5M

Retention Time	Compounds	Area (%)
8.06	2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	1.45
9.83	5-hydroxymethylfurfural	31.18

21.90	n-hexadecanoic acid	1.73
24.21	9,12-octadecadienoic acid (Z,Z)-	1.65
24.29	cis-13-octadecenoic acid	1.85
24.58	octadecanoic acid	0.36

Compounds identified in D5M are octadecanoic acid, 5-hydroxymethylfurfural, n-hexadecanoic acid, cis-13-octadecenoic acid, 9, 12-octadecadienoic acid and 2, 3-dihydro-3, 5-dihydroxy-6-methyl-4H-pyran-4-one. New compound was extracted in D5 in methanol layer, which is 5-hydroxymethylfurfural (HMF) with high percentage area 31.18. This compound was found in methanol layer (D5M) and methanol alone (D1), but the yield of this compound is higher in D5M. Based on other research, to extract high yield of HMF is by adjusting the mixture properties; polarity and viscosity (Yu et al., 2017). The chromatogram of D5M extract is illustrated in Appendix 15.

Meanwhile in D5H, compounds identified are hexadecanoic acid trimethylsilyl ester, nonadecyl pentafluoropropionate, 9, 12-octadecadienoic acid trimethylsilyl ester, cis-13-octadecenoic acid trimethylsilyl ester, octadecanoic acid trimethylsilyl ester and longifolenaldehyde. Among these two compounds were identified in hexane layer (D5H); nonadecyl pentafluoropropionate and longifenaldehyde but were not identified in hexane alone (D3). This outcome revealed that by combining extracting solvents, other compounds could be extracted. Chemical compounds that were identified in D5H are presented in Table 4.6 and the chromatogram is shown in Appendix 16.

Table 4.6: Chemical compounds Extracted using D5H

Retention Time	**Compounds**	**Area (%)**
23.03	hexadecanoic acid, trimethylsilyl ester	32.43
25.17	9,12-octadecadienoic acid, trimethylsilyl ester	22.61

25.25	cis-13-octadecenoic acid, trimethylsilyl ester	25.75
25.59	octadecanoic acid, trimethylsilyl ester	12.07
26.28	nonadecyl pentafluoropropionate	1.55
27.39	longifolenaldehyde	1.88

4.1.1.6 Chloroform: Hexane Extract (D6)

Combination of semi polar organic solvent and strong non polar solvent is labelled as D6. Compounds identified in this design are hexadecanoic acid ethyl ester, hexadecanoic acid trimethylsilyl ester and octadecanoic acid trimethylsilyl ester with the percentage area 3.52 %, 14.31 % and 6.67 %, respectively. No new compounds were identified in D6. Table 4.7 showed the list of compounds identified in D6. The Chromatogram of this design is shown in Appendix 17.

Table 4.7: Chemical compounds Extracted using D6

Retention Time	**Compounds**	**Area (%)**
22.35	hexadecanoic acid, ethyl ester	3.52
23.03	hexadecanoic acid, trimethylsilyl ester	14.31
25.59	octadecanoic acid, trimethylsilyl ester	6.67

4.1.1.7 Methanol: Chloroform: Hexane Extract (D7)

Combination of three extracting solvents with different polarities (methanol, chloroform and hexane) is labeled as D7. Chemical compounds identified in this design are 5-hydroxymethylfurfural, n-hexadecanoic acid, hexadecanoic acid, ethyl ester, 9, 12-octadecadienoic acid, octadecanoic acid and octadecanoic acid, ethyl ester. All compounds are identified in other designs as well. These compounds are presented in Table 4.8. The Chromatogram of this design is illustrated in Appendix 18.

Table 4.8: Chemical compounds Extracted using D7

Retention Time (min)	Compounds	Area (%)
9.61	5-Hydroxymethylfurfural	32.17
21.88	n-Hexadecanoic acid	3.25
22.35	Hexadecanoic acid, ethyl ester	0.78
24.19	9,12-Octadecadienoic acid (Z,Z)-	1.20
24.57	Octadecanoic acid	1.46
24.99	Octadecanoic acid, ethyl ester	0.60

4.1.1.8 Summary of Compounds Extracted in All Designs

Some studies reported that 2, 3-dihydro-3, 5-dihydroxy-6-methyl-4H-pyran-4-one and 3, 5-dihydroxy-2-methyl-4H-pyran-4-one have antioxidant activity (Yu et al., 2013). The former compound was identified in D1, D4 and D5M which is polar design. Other antioxidants identified in D3 and D5H are β-sitosterol (Mahaddalkar et al., 2015) and nonadecyl pentafluoropropionate (Renukadevi et al., 2011), respectively. Both designs are nonpolar design. Therefore, these results are in agreement with those studies who reported that dates fruits as a good source of natural antioxidant compounds (Harthi et al., 2015).

Furthermore, three fatty acids; capric (n-decanoic acid) (Huang et al., 2014), lauric (dodecanoic acid) (Nitbani et al., 2016) and caprylic acid (octanoic acid) (Kim and Rhee, 2016) have been reported as potential antimicrobial compounds where in the case of caprylic acid, the antimicrobial or antibacterial activity is due to the ability of these medium chain saturated fatty acids to act against several Gram negative and Gram positive bacteria. Besides, Huang et al. (2014) reported that lauric and capric acids have anti-inflammatory activities while other functions of caprylic and capric acids include anticonvulsant properties in seizures (Wlaz et al., 2015), by decreasing blood glucose and alternatively increasing β-hydroxybutyrate concentrations.

Palmitic acid (hexadecanoic acid) and stearic acid (octadecanoic acid) are reported as anticholesterol (Andrea & Scott, 1988). Additionally, Nai-Sheng et al (2014) stated that the benefit of fatty acids on human health such as myristic (tetradecanoic acid) and myristoleic acids is accumulation in muscle which might have beneficial effects for human health. Summary of compounds which extracted in all designs are tabulated in Table 4.9. The information arranged in this table is the name of compounds, the extraction design and the percentage area (the value in the center of the table).

Table 4.9: Compounds in Ajwa Dates

Compounds	**D1**	**D2**	**D3**	**D4**	**D5M**	**D5H**	**D6**	**D7**
2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	6.41	-	-	0.33	1.45	-	-	-
octanoic acid	1.11	-	-	-	-	-	-	-
3,5-dihydroxy-2-methyl-4H-pyran-4-one	0.22	-	-	-	-	-	-	-
5-hydroxymethylfurfural	3.97	-	-	-	31.18	-	-	32.17
n-decanoic acid	1.18	-	-	-	-	-	-	-
dodecanoic acid	6.85	-	-	-	-	-	-	-
tetradecanoic acid	2.99	-	-	-	-	-	-	-
n-hexadecanoic acid	1.41	-	-	2.17	1.73	-	-	3.25
9,12-octadecadienoic acid	1.41	0.24	-	1.47	1.65	-	-	1.20
9-octadecenoic acid	1.42	-	-	-	-	-	-	-
cis-13-octadecenoic acid	0.31	-	-	-	1.85	-	-	-
octadecanoic acid	0.39	-	-	0.90	0.36	-	-	1.46
octanoic acid, trimethylsilyl ester	-	2.39	-	-	-	-	6.67	-
decanoic acid, trimethylsilyl ester	-	2.31	-	-	-	-	-	-
dodecanoic acid, trimethylsilyl ester	-	37.54	1.08	-	-	-	-	-
D-psicofuranose, pentakis(trimethylsilyl) ether	-	0.22	-	-	-	-	-	-
1,3,4,5,6-pentakis-O-(trimethylsilyl)- D-fructose	-	0.63	-	-	-	-	-	-
tetradecanoic acid,	-	10.16	-	-	-	-	-	-

trimethylsilyl ester								
hexadecanoic acid, ethyl ester	-	1.21	-	0.67	-	-	3.52	0.78
hexadecanoic acid, trimethylsilyl ester	-	7.18	5.43	-	-	32.43	14.31	-
heptafluorobutyric acid, n-tetradecyl ester	-	0.35	-	-	-	-	-	-
octadecanoic acid, ethyl ester	-	0.67	-	0.35	-	-	-	0.60
octadecanoic acid, trimethylsilyl ester	-	-	2.98	-	-	12.07	-	-
11-trans-octadecenoic acid, trimethylsilyl ester	-	4.32	-	-	-	-	-	-
trans-9-octadecenoic acid, trimethylsilyl ester	-	1.01	-	-	-	-	-	-
vitamin E	-	-	7.70	-	-	-	-	-
β-sitosterol	-	-	0.23	-	-	-	-	-
hexadecanoic acid, methyl ester	-	-	0.39	-	-	-	-	-
9,12-octadecadienoic acid, methyl ester	-	-	0.31	-	-	-	-	-
9,12-octadecadienoic acid, trimethylsilyl ester	-	-	-	-	-	22.61	-	-
cis-13-octadecenoic acid, trimethylsilyl ester	-	-	-	-	-	25.75	-	-
nonadecyl pentafluoropropionate	-	-	-	-	-	1.55	-	-
longifolenaldehyde	-	-	-	-	-	1.88	-	-

According to previous studies, β-sitosterol known to has antioxidant (Mahaddalkar et al., 2015), anti-inflammatory and anticancer (Lomenick et al., 2015), anti-cholesterol (Robert, 2010), antimicrobial (Bildziukevich et al., 2015) and anti-asthmatic (Mahaddalkar et al., 2015) activity. The presence of longifolenaldehyde and nonadecyl pentafluoropropionate in D5H extract suggests the capacity of dates to act as antifungals (Zhang et al., 2014) and antimicrobial (Renukadevi et al., 2011), respectively. Other chemical compounds identified in Ajwa flesh is 5-hydroxymethylfurfural (5-HMF). 5-HMF content known to vary in various foods such as honey, jam, biscuits and apple since this compound is a result of sugar degradation via the Maillard reaction when carbohydrate-rich foods are heated (Gurkan &

Altunay, 2015). Shapla et al. (2018) stated that HMF is known to have antioxidant and anti-inflammatory activities. Table 4.10 is the summary of the compounds identified in Ajwa dates in this study in which might have potential beneficial effects on human health (based on the review from other studies on the particular compounds). Chemical structures of chemical compounds that extracted using three mixture designs are shown in Appendix 10.

Table 4.10: Summary of Compounds Identified in This Study with Potential Beneficial Effects on Human Health (Based on Review)

	Compound Identified in This Study	**References**
Antioxidant	2, 3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	(Yu et al., 2013)
	3,5-dihydroxy-2-methyl-4H-pyran-4-one	(Yu et al., 2013)
	β-sitosterol	(Mahaddalkar et al., 2015)
	nonadecyl pentafluoropropionate	(Renukadevi et al., 2011)
	5-hydroxymethylfurfural	(Shapla et al., 2018)
Antimicrobial	lauric acid	(Nitbani et al., 2016)
	capric acid	(Huang et al., 2014)
	caprylic acid	(Kim and Rhee, 2016)
	β-sitosterol	(Bildziukevich et al., 2015)
	nonadecyl pentafluoropropionate	(Renukadevi et al., 2011)
Anti-inflammatory	lauric acid	(Huang et al., 2014)
	capric acid	(Huang et al., 2014)
	5-hydroxymethylfurfural	(Shapla et al., 2018)
	β-sitosterol	(Lomenick et al., 2015)
Anticonvulsant	capric acid	(Wlaz et al., 2015)
	caprylic acid	(Wlaz et al., 2015)
Anticholesterol	palmitic acid	(Andrea & Scott, 1988)
	stearic acid	(Andrea & Scott, 1988)
	β-sitosterol	(Robert, 2010)
Muscle	myristic acid	(Nai-Sheng et al., 2014)
Anticancer	β-sitosterol	(Lomenick et al., 2015)

Anti-asthmatic	β-sitosterol	(Mahaddalkar et al., 2015)
Antifungal	longifolenaldehyde	(Zhang et al., 2014)

4.1.1.9 Principal Components Analysis (PCA)

Multivariate statistical analysis often used for examining relationships among multiple variables at a time. Principal Component Analysis (PCA) is one of unsupervised data analysis approaches which used to obtain an overview of the data sets. It provides a visual representation of the relationships between samples and variables (CAMO Software India). Typically, multivariate data in term of table (samples against variables) were analyzed simultaneously to obtain an overview pattern of the data sets using scores and loadings plot. The data matrix from Table 4.9 is subjected to pre-processing (mean normalization) before analysis by PCA.

PCA overview of chemical compounds (scores plot) and mixture design (loadings plot) was shown in Figure 4.1 (a) and Figure 4.1 (b), respectively. Apparently, the data set was clustered into four groups. The relationships between Scores plot and Loadings plot were labelled as group I, II, II and IV on both plots. Scores plot represent the chemical compounds while Loadings plot represent the mixture design. Group I in Scores plot is belongs to group I in Loadings plot. In which, β-sitosterol and vitamin E is detected in D3 (hexane). The same goes to group II, III and IV in Scores plot are belong to group II, III and IV in Loadings plot, respectively. This is due to the position of these groups are identical in both plots.

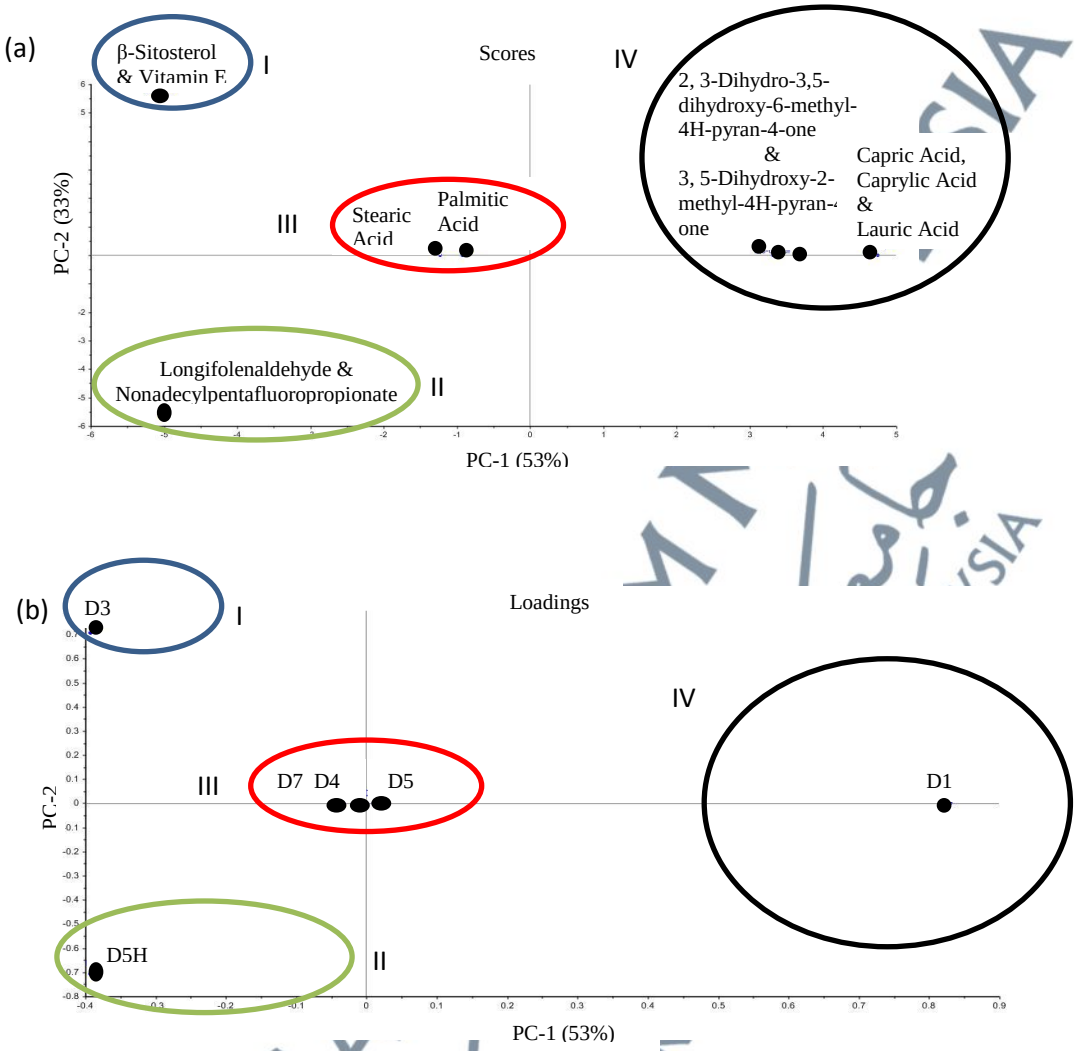

Figure 4.1: Scores (a) and Loadings (b) Plots of Chemical Compounds in Ajwa Dates Flesh

The pattern of PCA plot could be seen clearly as the samples and variables were distributed far from each other. Group IV located far from group I and II because of the difference polarity of the solvents (Loadings plot) and the chemical compounds (Scores plot) that merely detected in non-polar design D3 and D5H, which are hexane and hexane layer, respectively. Nonetheless, group III located near group I since chemical compounds in group III similarly identified in group I.

4.1.2 LC-QToF-MS Analysis of Ajwa Dates

Both positive and negative ionization were performed in LC-QToF-MS analysis on Ajwa dates extracts to increase the variety of metabolites (Naz et al., 2014) and provide a reasonable breadth of coverage (Lu et al., 2008). Acquiring data in both polarities (positive and negative ionization mode) is in order to maximize the information (Jarmusch et al., 2016). Chemical compounds that identified in Ajwa dates flesh are tabulated in Table 4.11 (positive ionization), Table 4.12 (negative ionization) and Table 4.13 (both ionization). Therefore, compounds that had been identified are quercetin, apigenin, caffeic acid, rhoifolin, crocetin, procyanidin, cyanidin, ferulic acid, trimethylgallic acid, quercimetrin, proanthocyanidin, pyrogallic acid, luteolin, β-sitosterol, elaeocyanidin and glucosyringic acid. All these compounds were selected based on confirmation criterias of UNIFI Scientific Information System Software. The criterias are mass error (mDa) between -2>x<2, isotope match intensity RMS (percent) is less than 10 %, isotope match m/z RMS (ppm) is less than 5 ppm and for theoretical fragments found, which is the greater the number of fragment found the high potential of compound to be true.

Table 4.11: Chemical Compounds in Ajwa Dates in Positive Ionization

Group	Compound name
Flavonoids	3-O-β-D-galactopyranosyl quercetin
	6-C-arabinose-8-C-glucose-apigenin
	7-O-methyl luteolin-6-C-β-D-glucoside
	apigenin-7-O-acetyl-β-D-glucoside
	apigenin-7-O-β-D-glucopyranoside
	dihydroquercetin
	luteolin-7-O-[β-D-apiofuranosyl(1→6)]β-D-glucopyranoside
	luteolin-7-O-glucuronide ethyl ester
	quercetin-3,3'-dimethyl-ether
	quercetin-3,7-O-β-D-diglucopyranoside
	quercetin-3-O-(6''-O-acetyl)-β-D-

	glucopyranoside quercetin-3-O-(6-O-feruloyl-β-D-glucopyranosyl)-(1→2)-β-D-galactopyranosyl-(1→2)-β-D-glucopyranoside quercetin-3-O-β-D-glucopyranosyl=(1→4)-α-L-rhamnopyranoside quercetin-3-rhamnogentiobioside quercetin-6-O-glucoside
Anthocyanidins	cyanidin 3-glucoside
Phenolics	pyrogallic acid rhoifolin trimethylgallic acid
Sterols	β-sitosterol-3-O-gentiobioside

In other studies, dates fruits were reported to have flavonoids (Farag et al., 2014), phenolics (Benmeddour et al., 2013), sterols, anthocyanins, carotenoids and procyanidins (Baliga et al., 2011) compounds which identified using liquid chromatography technique. The ratio and concentrations of these constituents depend on the type of the fruit, stage of fruitpicking, location and soil conditions (Baliga et al., 2011). According to Benmeddour et al (2013) who studied Algerian dates stated that the results obtained for phenolic content were much higher than Omanian dates. Farag et al. (2014) indicated that mono and di-glycosides identified in dates fruits were mostly flavonol derivatives (quercetin) and flavone derivatives (luteolin and apigenin).

Table 4.12: Chemical Compounds in Ajwa Dates in Negative Ionization

Group	**Compounds Name**
Flavonoids	luteolin luteolin 7-β-neohesperidoside 3,8-di-C-glucosylapigenin 3-O-acetyl-caffeic acid apigenin-6-C-galactosyl-8-C-arabinoside apigenin-7-O-β-D-rutinoside

	quercetin-3-gentiobioside
	quercetin-3-O-xyloside
	quercetin-3-O-β-D-glucopyranosyl (1→2) β-D-galactopyranosyl-(1→2)-β-D-glucopyranoside
	quercetin-3-sulphate
	quercetin-7-O-[β-D-glucopyranosyl(1→6)-β-D-glucopyranoside]
	quercimeritrin
Anthocyanidins	cyanidin 3-(2G-xylosylrutinoside)
	cyanidin 3-(4''-acetylglucoside)
	cyanidin 3-(6''-ferulyl-2'''-sinapylsambubioside)-5-(6-malonylglucoside)
	cyanidin 3-(6-malonylglucoside)-7-(6-caffeoylglucoside)-3'-glucoside
	cyanidin 3-(6''-malylglucoside)
	cyanidin 3-(6''-p-coumaryl-2'''-sinapylsambubioside)-5-(6-malonylglucoside)
	cyanidin 3-(6''-p-coumaryl-2''-sinapylsophoroside)-5-glucoside
	cyanidin 3-(6''-p-coumarylglucoside)-5-4''',6'''-dimalonylglucoside)
	cyanidin 3-(6''-p-coumarylsambubioside)
	cyanidin 3-(6''-sinapylsophoroside)-5-glucoside
	cyanidin 3,5-di-(6-malonylglucoside)
	cyanidin 3-[6-(6-p-hydroxybenzoylglucosyl)-2-xylosylgalactoside]
	cyanidin 3-lathyroside
	cyanidin 3-O-(6''-O-succinyl-β-glucopyranoside)
	cyanidin 3-sophoroside-5-glucoside
	proanthocyanidin
	procyanidin C-1
	elaeocyanidin
Phenolics	methyl-5-O-caffeoylquinate
	decaffeoylacteoside
	sinapic acid
	1-O-caffeoyl-β-D-glucopyranoside
	1-O-methyl-3,5-O-dicaffeoylquinic acid methyl ester
	caffeoylxanthiside
	chlorogenic acid
	crocetin

From Table 4.11 and Table 4.12, we can see that more flavonoids were identified in positive and negative mode ionization. Nevertheless, cyanidins group were

identified more in negative mode ionization instead. No similar cyanidin compounds were identified in both ionizations (Table 4.13). Meanwhile, sterols compound was identified in positive ionization only. These findings showed that it is important to perform both positive and negative ionization to obtain more information as both identified different compounds.

Table 4.13: Chemical Compounds in Ajwa Dates in Both Ionizations

Group	Compounds Name
Flavonoids	luteolin-7-O-α-D-glucoside quercetin-3-rhamnogentiobioside quercetin-6-O-glucoside apigenin-6-C-glucosylglucoside apigenin-7-O-α-L-rhamnose(1→4)-6"-O-acetyl-β-D-glucoside
Phenolics	ferulic acid glucosyringic acid trimethylgallic acid 2-methoxy cinnamic acid 3,4-dihydroxycinnamic acid 3,4-dimethoxy-cinnamic acid 4-O-β-D glucopyranosyl-cis-cinnamic acid

Typically researchers maily focused on the positive ionization due to the higher efficiency compared to negative mode (Zheng et al., 2016). However, in this study, 21 compounds were identified in positive mode while 39 compounds were reported in negative mode and 12 compounds identified in both ionization.

4.1.3 FTIR Fingerprint of Ajwa Dates

The purpose of using this method is to investigate the specific fingerprint in order to differentiate between ten selected dates fruits with different varieties. FTIR spectrum can be divided into two regions which are functional group region and

fingerprint region (Alex et al., 2018). The wavelengths for infrared spectra are ranging from 4000 cm^{-1} to 450 cm^{-1} the functional group region lies from 4000 cm^{-1} to 1450 cm^{-1} (Alex et al., 2018) while the other study stated that vibrational fingerprint region lies from 1000 cm^{-1} to 2000 cm^{-1} (Rehault et al., 2017).

In this study, ten dates fruits were analyzed using ATR-FTIR from 4000 cm^{-1} to 600 cm^{-1}. Figure 4.2 shows the overlaid spectrum of ten different varieties of dates fruits. The spectrum can be considered as identical because the lines of the spectra are aligned. Thus, from this information, we can assume that dates fruits with different varieties have equal beneficial effects on human health as Ajwa dates (dates fruits that being used in this study). However, the wavelength ranging from 600 cm^{-1} to 2000 cm^{-1} has slightly different in term of the value of Transmittance (%T).

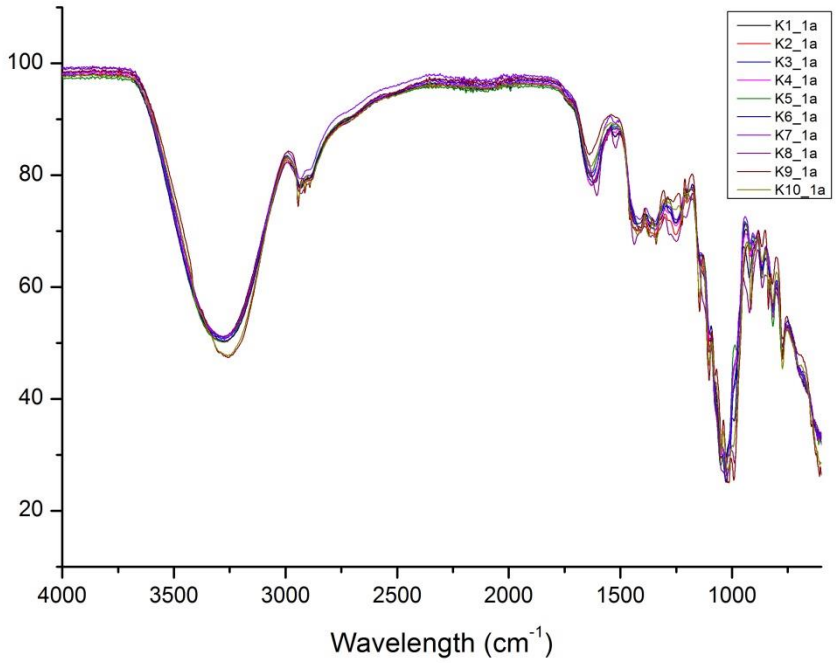

Figure 4.2: Overlaid Spectrum of Ten Different Varieties of Ajwa Dates Fruits

Three peaks were observed in the functional group region for all samples except for samples labeled as K8 and K9 which have four peaks. A strong and broad band in all spectra K1 to K10 with peak ranging from 3258.19 cm^{-1} to 3284.95 cm^{-1} corresponding to alcohols and phenols (O-H stretch, H-bonded) were remarked. Meanwhile, peak ranging from 2928.38 cm^{-1} to 2942.97 cm^{-1} is a characteristic of aliphatic C-H stretch present in alkanes were observed in all spectra except for K9, which is has two peaks, 2943.60 cm^{-1} and 2913.31 cm^{-1}. A medium intensity band at 1605.59 cm^{-1} to 1638.52 cm^{-1} were observed representing –C=C- stretch in alkenes in all spectra. A new peak was observed in K8 at a peak 1520.27 cm^{-1} usually represents C=C in benzene ring. All spectra are illustrated in Appendix 19.

The peak appearing in fingerprint region in all spectra except K2 and K6 indicates the presence of CH_2 asymmetric bending; K1 at 1411.94 cm^{-1}, K3 at 1408.66 cm^{-1}, K4 at 1408.06 cm^{-1}, K5 at 1407.91 cm^{-1}, K7 at 1412.16 cm^{-1}, K8 at 1437.72 cm^{-1}, K9 at 1425.84 cm^{-1}, and K10 at 1423.92 cm^{-1}. The next peak which represents CH_2 bending in the sample analyzed were observed in all spectra; K1 at 1343.65 cm^{-1}, K2 at 1344.01 cm^{-1}, K3 at 1344.33 cm^{-1}, K4 at 1341.10 cm^{-1}, K5 at 1341.33 cm^{-1}, K6 at 1340.56 cm^{-1}, K7 at 1343.81 cm^{-1}, K8 at 1341.72 cm^{-1}, K9 at 1339.71 cm^{-1}, and K10 at 1339.16 cm^{-1}. The presence of C-O stretching was revealed due to the presence of peak at 1250.54 cm^{-1} in the sample K1, peak at 1250.36 cm^{-1} in the sample K2, peak at 1250.90 cm^{-1} in the sample K3, peak at 1250.59 cm^{-1} in the sample K4, peak at 1248.77 cm^{-1} in the sample K5, peak at 1250.71 cm^{-1} in the sample K6, peak at 1247.61 cm^{-1} in the sample K7, peak at 1249.28 cm^{-1} in the sample K8, two peaks presence at 1223.75 cm^{-1} and 1202.40 cm^{-1} in the sample K9, and two peaks presence at 1224.26 cm^{-1} and 1202.78 cm^{-1} in the sample K10. A high intensity peak ranging 1000 cm^{-1} to 1100 cm^{-1} was observed in spectrum K1 to K7 denoting the

presence of C-O stretch present in alcohol, carboxylic acid, ester and ether. However, a small unique peak (1102.31 cm^{-1}) was attached to the high intensity peak ranging 1000 cm^{-1} to 1100 cm^{-1} was observed in K8 denoting the presence of C-O stretch present in alcohol, carboxylic acid, ester and ether. Meanwhile, three sharp peaks in K9; 1145.64 cm^{-1}, 1102.61 cm^{-1} and 1047.63 cm^{-1} and in K10; 1145.43 cm^{-1}, 1102.99 cm^{-1} and 1047.78 cm^{-1} were attached to the high intensity peak ranging 1000 cm^{-1} to 1100 cm^{-1} was observed denoting the presence of C-O stretch present in alcohol, carboxylic acid, ester and ether, C-N or C-C stretching.

Several peaks appearing below than 1000 cm^{-1} indicates the presences of C-H bending. The peaks spotted are at 917.10 cm^{-1}, 864.07 cm^{-1}, 816.49 cm^{-1} and 775.16 cm^{-1} in K1, peaks at 917.63 cm^{-1}, 864.24 cm^{-1}, 816.89 cm^{-1} and 774.99 cm^{-1} in K2, peaks at 917.99 cm^{-1}, 864.09 cm^{-1}, 816.56 cm^{-1} and 775.03 cm^{-1} in K3, peaks at 916.53 cm^{-1}, 865.72 cm^{-1}, 816.40 cm^{-1} and 775.74 cm^{-1} in K4, peaks at 918.43 cm^{-1}, 865.48 cm^{-1}, 816.51 cm^{-1} and 776.73 cm^{-1} in K5, peaks at peak 921.99 cm^{-1}, 863.76 cm^{-1} and 817.90 cm^{-1} in K6, and peaks at 917.60 cm^{-1}, 864.24 cm^{-1}, 816.33 cm^{-1} and 775.07 cm^{-1} in K7 and peaks at 988.83 cm^{-1}, 921.57 cm^{-1}, 864.00 cm^{-1} and 818.96 cm^{-1} in K8, peaks at 991.91 cm^{-1}, 915.29 cm^{-1}, 865.55cm^{-1}, 837.29 cm^{-1} and 774.46 cm^{-1} in K9 and peaks at 915.60 cm^{-1}, 864.56 cm^{-1}, 837.27 cm^{-1}, 817.20 cm^{-1} and 7743.86 cm^{-1} in K10. Three samples; K8, K9 and K10 have more peaks than other samples might be due to the present of other added nutrient such as honey.

Figure 4.3 illustrates the overlaid spectrum of two different Ajwa dates. K1 is Ajwa dates from Egypt Bazaar and K4 is Ajwa dates which was used as main sample in this study. The overlaid spectrum indicates that both Ajwa are identical as the spectrum is overlapping each other. Therefore, we assume that both Ajwa samples have equal beneficial effects on human health.

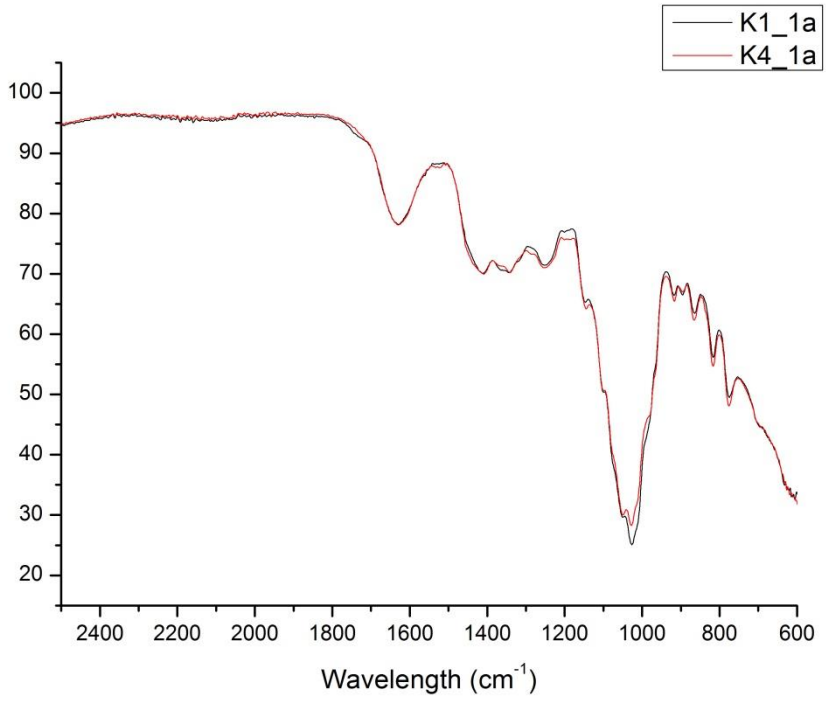

Figure 4.3: The Overlaid Spectrum of Two Different Ajwa Dates

4.2 Urine Metabolites

4.2.1 ^{1}H-NMR Analysis of Urine Sample

^{1}H-NMR spectra of urine samples were labeled according to the urine collection as 0 hour (0h), 4 hours (4h), 8 hours (8h), 12 hours (12h) and 24 hours (24h). Figure 4.4 showed the ^{1}H-NMR spectra from blank urine (0h) to 24h of urine collection. Individual spectrum of this analysis is shown in Appendix 20. These ^{1}H-NMR spectra were analyzed using PCA because the data is too complex.

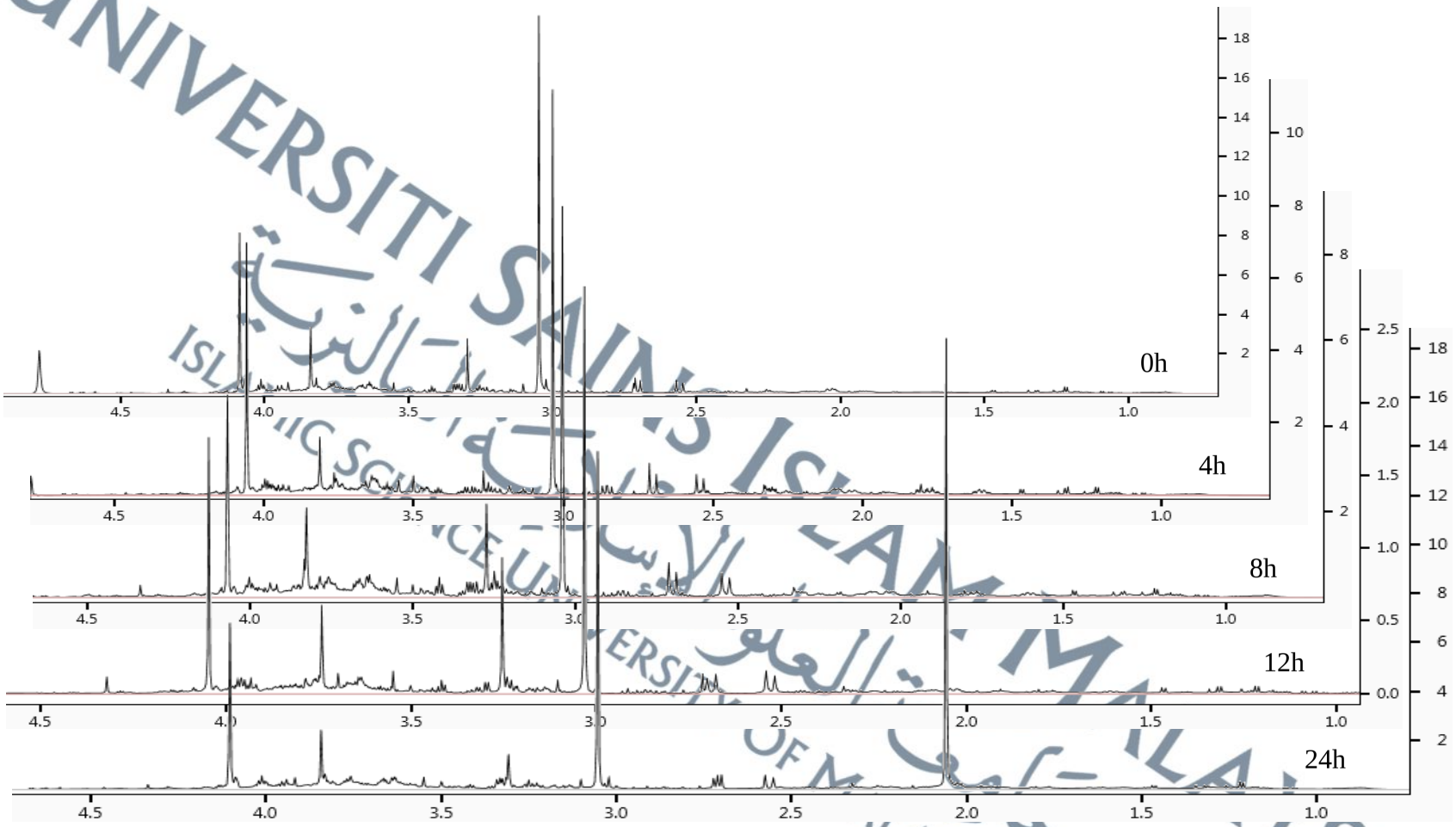

Figure 4.4: ^{1}H-NMR Spectra from Blank (0h) to 24h of Urine Collection

4.2.1.1 Principal Components Analysis (PCA)

PCA was performed using Unscrambler 10.3 (CAMO Software, Norway). PCA model were used to assist in comprehending the clustering patterns of observation. PCA was performed on ^{1}H-NMR spectra (Day 1 to Day 4). Prior to that, preprocessing technique was performed on raw data using max-normalization. Initially, urine samples were collected twice on Day 1, four times on Day 2 which labeled as 0h, 4h, 8h and 12h, twice on Day 2 (24h and 36h) and Day 4 (48h and 60h). Scores plot in Figure 4.5 illustrates the urine metabolites have been divided into two sides which is urine samples collected at 0 hour has been clustered together with urine samples after 24 hours (24 – 60 hours), meanwhile urine samples collected at 4 hours, 8 hours and 12 hours are clustered together in the opposite side.

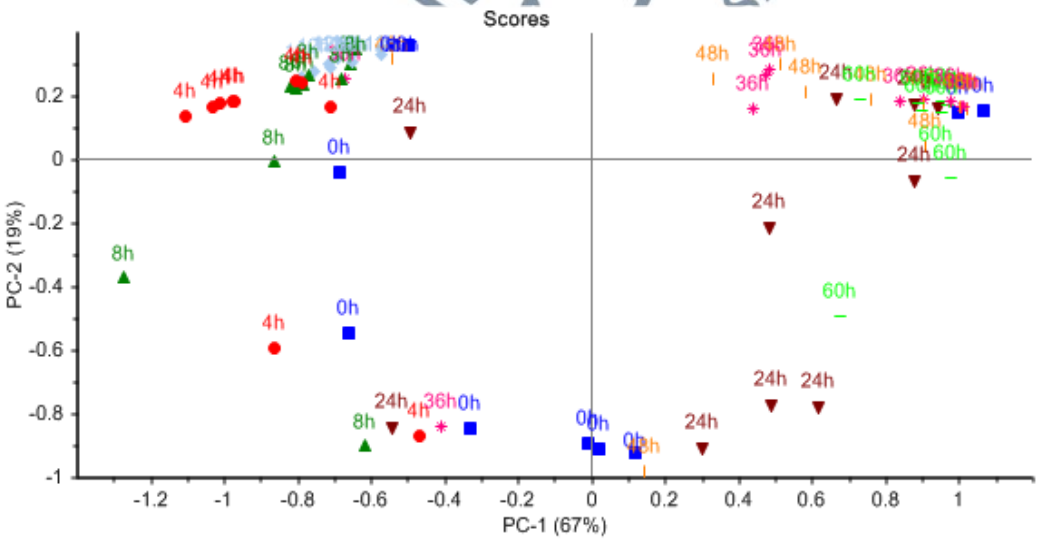

Figure 4.5: Scores Plot of ^{1}H-NMR Spectra from Day 1 to Day 4

Therefore, another PCA plot has been performed on the urine samples labeled 0h, 4h, 8h, 12h and 24h. The Scores plot is illustrated in Figure 4.6. From the plot, the

samples labeled 0h (blank urine before consumption of Ajwa dates) were found located near to the 24h (urine samples collected at 24 hours after consuming the Ajwa dates). This may indicates a complete 24 hours cycle of the effect of Ajwa dates intake. Therefore, it indicates that the effect of Ajwa in human body is within 24 hours. Furthermore, the slightly scattered spots of 4h group, was assumed due to different metabolism rate for each individual. On the contrary, spots in 12h group were highly aggregated compared to 8h, indicated that all subjects have reached the similar metabolism rate.

The first principal component (PC1) and second principal component (PC2) accounted 58 % and 24 % of variance, respectively, was obtained from PCA to produce scores and loadings plot. Scores plot (Figure 4.6) explains the distribution among the time of urine sampling (0 to 24 hours) whereas loadings plot (Figure 4.7) elucidates the chemical shifts that contribute to respective time of sampling in scores plot.

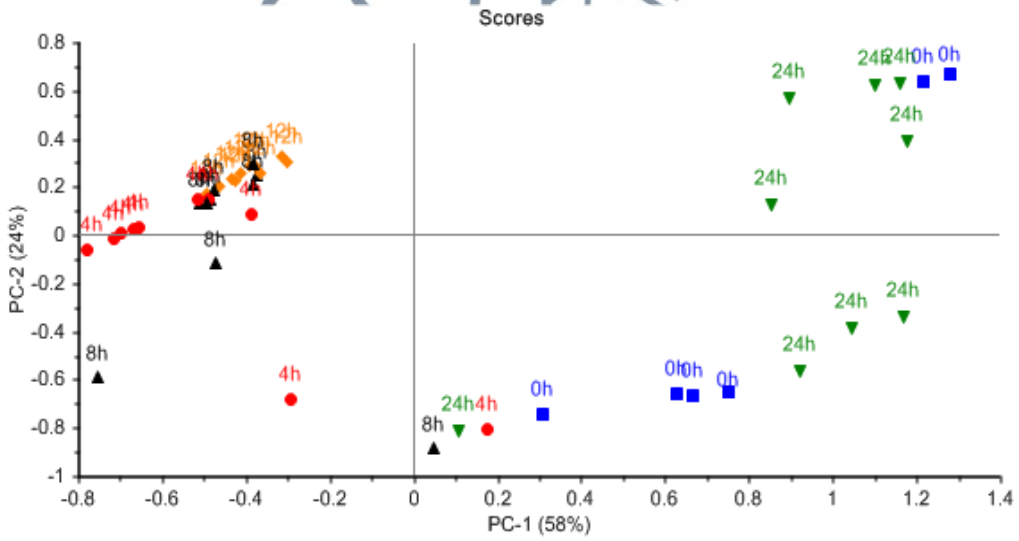

Figure 4.6: Scores Plot of ^{1}H-NMR Spectra of Urine Samples Collected from 0h to 24h

In loadings plot (Figure 4.7), the chemical shifts 3.06 ppm for creatinine contributes to 0 hour while 2.06 ppm for N- acetylcysteine (NAC) contributes to 24 hours in scores plot. This could be explained that those metabolites were more excreted to urine at respective sampling time. Other chemical shifts are insignificant when lies close to origin. ^{1}H-NMR spectra from 0h to 24h samples have been selected for further study because the complete 24 hours cycle.

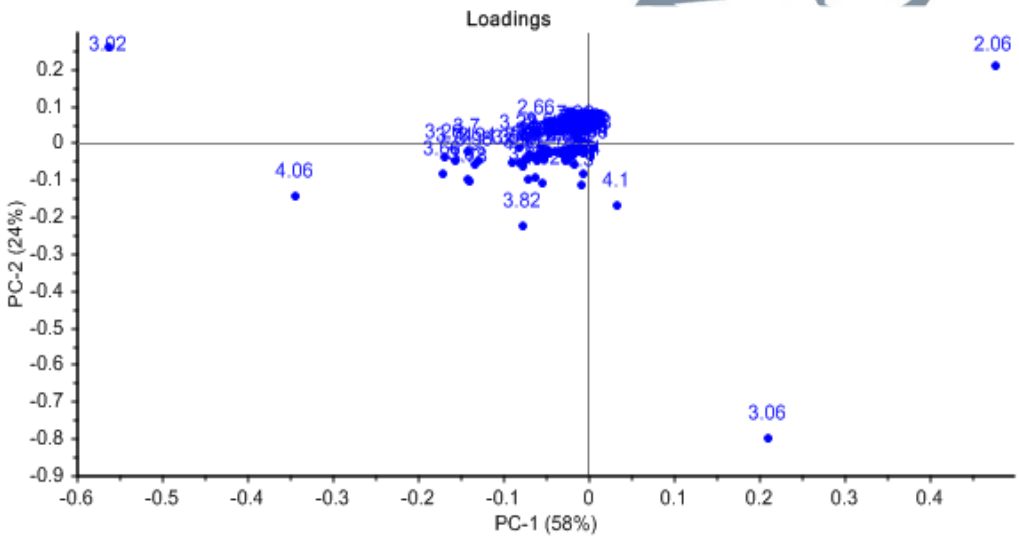

Figure 4.7: Loadings Plot of ^{1}H-NMR Spectra of Urine Samples Collected from 0h to 24h

4.2.1.2 Identification of Urine Metabolites (Metabolic Pathway)

PCA plots showed the metabolites change within 24 hours after consumption of Ajwa dates. Therefore, the focus is on the ^{1}H-NMR spectra from 0h to 24h. Chemical shifts in ^{1}H-NMR spectrum were compared with reference spectra in the Human Metabolome Database (HMDB) (Wishart et al., 2013). From ^{1}H-NMR spectra, the changes are mostly observed for peaks located in the region of 0.0 ppm – 4.0 ppm.

In the aliphatic region, citrate (2.57 and 2.72 ppm), trimethylamine-N-Oxide (3.27 ppm), taurine (3.42 and 3.25 ppm), alanine (1.48 ppm), lactate (1.32 ppm), malonate (3.10 ppm), N- acetylcysteine (2.06 ppm), creatinine (3.05 and 4.05 ppm) and methylhistidine (3.33, 3.84, 4.02) showed significant different from blank to 24 hours. There is also significantly different in aromatic region (6.5 ppm – 9.0 ppm) where there are relatively small peaks from hippurate (7.53, 7.62 and 7.82 ppm) and methylhistidine (7.38 and 8.60 ppm) which had been identified in all the spectra.

The peak of alanine was compared with reference spectra in HMDB and was supported by Marques et al. (2016) which stated that the alanine-CH_3 doublet signal resonating at 1.47 ppm is prominent in the ^{1}H-NMR spectra. Both alanine and taurine are classified as amino acids and also very similar in chemical structure (Nishigawa et al., 2018). Taurine is the end product of metabolic degradation of cysteine. In various stress levels, disrupted taurine metabolism was observed. Increased excretion of taurine was noticed in rats after being exposed to chronic radiation stress, immobilization stress and continuously fasting stress (Feng et al., 2016). Besides, Holmes et al. (1998) reported that the elevation of taurine in urine has been concomitant with hepatoxicity. In our study, the taurine peaks showed no changes in intensity from blank to 24 hours in all samples. Table 4.14 showed the summary of the urine metabolites change at different sampling time.

The level of alanine, hippurate, malonate and citrate were significantly decreased after the consumption of Ajwa dates, but were elevated after 24 hours. These endogenous metabolites were reported as biomarkers of depressive disorder where the concentration of these metabolites increased with depression (Tian et al., 2016). Thus, this could be suggested that Ajwa dates have the ability to reduce the

level of depression. Figure 4.8 shows the overview of metabolic pathway related to metabolites change after Ajwa dates intake

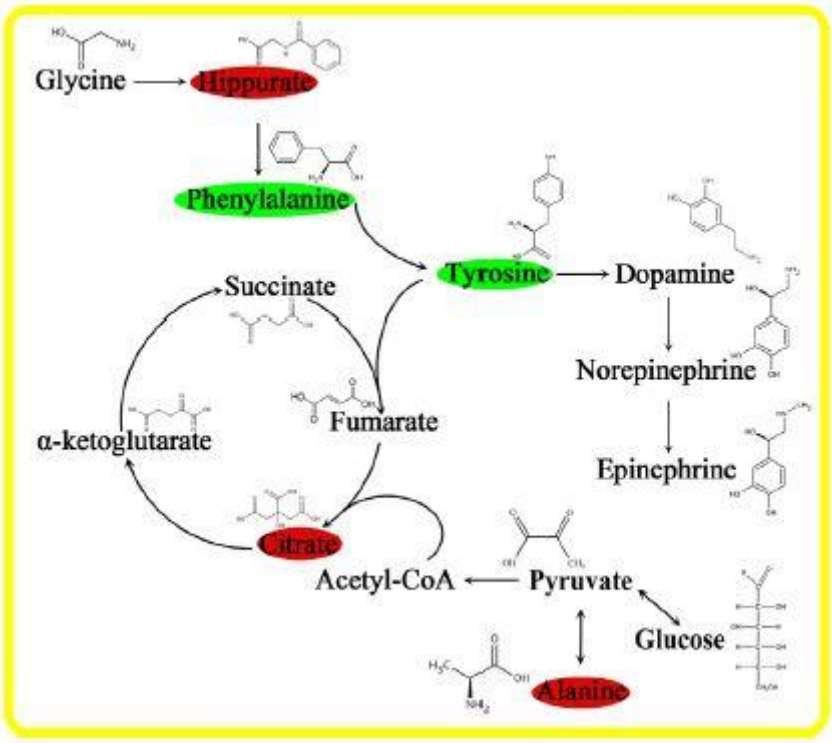

Figure 4.8: An Overview of Metabolic Pathway Related to Metabolites Change after Ajwa Dates Intake (Tian et al., 2015)

Generally, alanine is produced from pyruvate by reversible transmination reactions and highly produced in muscle, which functioning as a major energy source. According to Tian et al (2015), alanine is a regulator in glucose metabolism. Therefore, if the alanine is fully utilized, the level of alanine would decreases as well as the depression level. Most organisms will go through cellular respiration to produce ATP in the presence of oxygen. However, generally human body will develop a way called fermentation to create ATP even the absent of oxygen. This method could only works in anaerobic conditions, which is there is no air. Glucose still goes through glycolysis which creates the pyruvic acid and 2

ATP, but in order to regenerate more NAD the pyruvic acid is then broken down into lactic acid. Although the lactic acid fermentation takes places in muscle, however, as the lactic acid starts to build up in your muscle, your muscle starts to cramp up. Figure 4.9 shows the metabolic pathway of lactate and pyruvate.

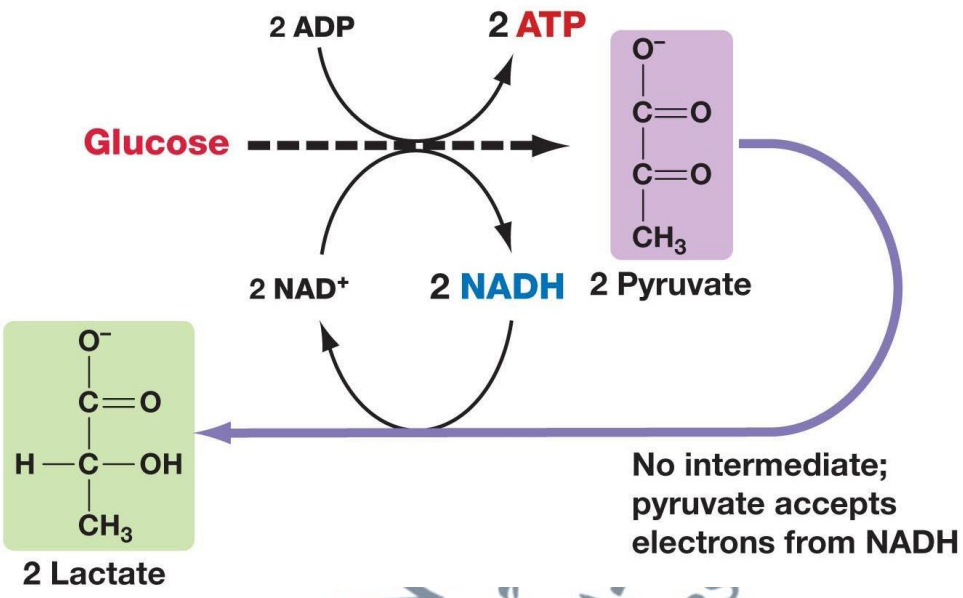

Figure 4.9: Relation of Lactate and Pyruvate in the Presence of Glucose

In addition, several studies reported that the enrichment of lactate, which is an organic molecule produced by most tissues in human body, was an indication of the increased in anaerobic glycolytic activity (Heather et al., 2013), related to diabetes and microalbuminuria (Chou et al., 2015), and major depressive disorder (Chen et al., 2017). Meanwhile, McNiven et al. (2011) stated that colon and stomach tumour tissues have high level of lactate compared to the healthy tissues. In this study, lactate peak was decreased after 4 hours of Ajwa dates consumption. This can be acknowledged as a good effect of Ajwa dates due to the decreasing level of lactate as shown in ^{1}H-NMR spectra.

Table 4.14: Summary of the Urine Metabolites Change at Different Sampling Time

Metabolites	Chemical Shifts, δ (ppm)	Changes of the urine metabolites at different sampling time				
		0H	4H	8H	12H	24H
lactate	1.32	+	-	-	-	-
alanine	1.48	+	-	-	-	+
N- acetylcysteine	2.06	nd	nd	nd	nd	+
citrate	2.57, 2.72	+	-	-	-	+
malonate	3.10	+	-	-	-	+
trimethylamine-N-Oxide	3.27	+	-	-	-	+
taurine	3.42, 3.25	nc	nc	nc	nc	nc
creatinine	3.05, 4.05	+	-	-	-	+
methylhistidine	3.33, 3.84, 4.02, 7.38, 8.60	nc	nc	nc	nc	nc
hippurate	7.53, 7.62, 7.82	+	-	-	-	+

(nc)= no changes, (+)= appear/identified, (-)= decreased, (nd)= not detected

A high intensity peak of N- acetylcysteine (NAC) with chemical shift 2.06 ppm was observed in spectra of all samples at 24 hours. NAC is a source of thiol groups which has been reported to enhance muscle force production and mitigate fatigue (Jannig et al., 2017), acts as an antioxidant (Ommati et al., 2017) and as a treatment alternative for substance use disorders (Squeglia et al., 2018). In all samples, NAC was merely detected in blank and 24 hours spectra. This can be assumed that NAC did not correlate to Ajwa dates intake. Instead, result showed that trimethylamine-N-Oxide (TMAO), a gut microbiota-generated metabolite, was diminished after the consumption of Ajwa dates.

Meanwhile in 24 hours spectrum, TMAO was elevated, showed the significant correlation between TMAO and Ajwa dates intake. Carta et al. (2017) stated that TMAO levels increased due to adverse cardiovascular outcomes and cardio-renal indexes meanwhile Sun et al. (2017) mentioned that TMAO directly contributes to kidney interstitial fibrosis and dysfunction. However, several metabolomics studies reported that meat or fish intake (Dragsted, 2010) could be the reason to the increased

level of TMAO in urine samples since TMAO is a metabolite of L-carnitine and phosphatidylcholine, which is abundant in red meat (Ufnal et al., 2015). Therefore, further work is required to investigate its specificity. TMAO may possibly be more useful as a dietary fingerprint of protein intake (Manach et al., 2014). In other research, Ottiger et al. (2016) reported that TMAO plasma levels showed significantly higher in patients with diabetes mellitus, colorectal cancer, congestive heart failure and chronic kidney disease, compared with controls.

Methylhistidine has been proposed as fingerprint of dietary intake (O'Gorman et al., 2013). On degradation of actin and myosin, the released 3-methylhistidine is neither reutilized for protein synthesis nor metabolized, but is excreted in the urine (Aranibar et al., 2011; Dragsted, 2010). Both 3-methylhistidine and 1-methylhistidine excretion proportionally to red meat intake, meanwhile 1-methylhistidine may be more useful as its excretion is independent of muscle mass and catabolism (Dragsted, 2010; O'Gorman et al., 2013). The peak intensity of methylhistidine found in all samples in this study showed the insignificant fluctuation.

Creatinine is known as an indicator of renal function (Debus et al., 2015). Elevated level of creatinine indicate renal malfunction (Debus et al., 2015), effect of correlation with oxidative stress (Lehtonen et al., 2013) or meat intake (Dragsted, 2010). On the other hand, lower creatinine values can be resulted through the administration of antioxidant (Lehtonen et al., 2013). The intensity of creatinine with chemical shift 3.05 ppm and 4.05 ppm from ^{1}H-NMR spectra showed that the peak was significantly diminishing in spectra of 4, 8 and 12 hours after consuming Ajwa dates and significantly increased at 24 hours. It was speculated that the antioxidant activity of Ajwa dates could be a possible explanation to the changes of the creatinine level. The intensity of creatinine increased 24 hours after Ajwa dates consumption

indicate the antioxidant activity of the dates which gives effect for less than 24 hours. Appendix 21 showed the chemical structures of urine metabolites.

4.2.2 GC-MS Analysis of Urine Sample

GC-MS was used to detect volatile compounds. Extraction method conducted to extract urine metabolites is acid-base extraction. Table 4.15 presenting the metabolites identified in urine samples from 0h to 24h (as mentioned in previous subtopic). The information in the table representing the area under the peaks Metabolites labeled accordingly and followed by abbreviation; acidic condition (A); Alkaline condition (B) and neutral (N). All metabolites were filtered based on retention time and similarity index (SI) more than 70 %.

Most of the compounds are detected in acidic condition including p-cresol (1A), m-cresol (2A), benzoic acid (3A), metylsuccinic acid (4A), 3-methyladipic acid (5A), pimelic acid (6A), 3-hydroxyphenylacetic acid (7A), azelaic acid (8A), 3-hydrobenzoic acid (9A), 4-hydrobenzoic acid (10A), homovanilic acid (11A), suberic acid (12A), hippuric acid (13A), azelaic acid (14A), myristic acid (15A), indoleacetic acid (16A), n-hexadecanoic acid (17A), palmitic acid (18A), triclosan (19A), and stearic acid (20A). In Alkaline condition, few compounds were detected including cadaverine (22B), urea (23B), pimelic acid (6B), suberic acid (12B), hippuric acid (13B), azelaic acid (14B), myristic acid (15B), palmitic acid (18B), (Z)-oleic acid (24B), and stearic acid (20B). Meanwhile, only two compounds were identified in neutral condition which is palmitic acid (18N) and stearic acid (20N). Mostly, compounds that were identified are fatty acids and phenolic acids. p-Cresol is a phenolic compound and major metabolites of aromatic amino acids tyrosine (Verbeke et al., 2015), and also as end-product of protein breakdown (Human Metabolome

Database (HMDB)). All value in table representing the value of area under compound peaks.

Table 4.15: Urine Metabolites Identified in Acid, Alkaline and Neutral Condition

Code	Blank	4 Hours	8 Hours	12 hours	24 Hours
1A	2931146	1749460	0	0	0
2A	922260	1219720	1701821	825205	1891746
3A	1137796	891158	0	0	933112
4A	0	0	0	0	937302
5A	3152763	2718832	820353	0	4478892
6A	0	0	0	0	4211280
7A	0	0	0	0	6215593
8A	0	1229523	0	0	7214706
9A	3283023	3033673	0	0	3884740
10A	0	0	2567035	0	0
11A	6704255	0	0	0	6637033
12A	0	0	2066104	0	2513772
13A	4714201	3189921	0	0	21005056
14A	0	0	730796	0	0
15A	728357	0	728438	0	0
16A	2657509	2476682	0	0	3569077
17A	9547980	9237042	6180389	4022958	5853689
18A	2476026	5965420	9473611	11384934	2447334
19A	6173710	2866010	0	0	0
20A	3613611	4647584	3082570	1925355	4136421
22B	0	0	0	0	17034128
23B	0	0	79129493	58124945	82692830
6B	1440382	0	0	0	0
12B	2234740	0	0	0	0
13B	5070057	0	0	0	0
14B	4072237	0	0	0	0
15B	0	0	2177515	0	1173741
18B	22049641	26413185	31667937	21853946	25205888
24B	0	1019088	1156221	869483	1658964
21B	27728433	31494292	35529267	26931563	29546782
18N	17752263	18006343	18012213	19819401	19742858
21N	21151589	21874510	21487728	24887285	24280779

4.2.3 LC-QToF-MS Analysis of Urine Sample

4.2.3.1 Urine Metabolites

In positive ionization, there are a number of metabolites identified in 0h (blank) such as flavonoids; cnidimol F (Yan et al., 2019); kushenol I (Chen et al., 2018); kushenol M (Chen et al., 2018); kushenol T (Chen et al., 2018); liquiritin (Zhao et al., 2008); pachypodol (Rezazadeh et al., 2017) and sanggenon J (Liu et al., 2018), alkaloids; ephedradine B (Zhang et al., 2018a); isomaistemonine (Arianna, 2018); melicopidine (Isabel, 2017); piperyline (Olalere et al., 2018) and pellitorin (Ngo et al., 2017), phenolics; feruperine (Gomez-Gomez et al., 2018) and glabrol (Lin et al., 2017). Other known compounds that identified in blank urine are isoanhyoicaritin and riboflavin (Bergwik and Akestrom, 2018).

Meanwhile in negative ionization, metabolites that identified in 0h are flavonoids; apigenin-7-O-rhamnoside and chrysosplenetin B, and other compounds such as morusin hydroperoxide, 6,7-dihydroxy-2-(2-phenylethyl) chromone, clerodendrin and hibiscetin-3-O-glucoside. Metabolites identified in 0h (blank) are presented in Table 4.16.

Table 4.16: Metabolites in Human Urine that Identified in Blank (0h)

	Compounds (+H)	**Compounds (-H)**
Flavonoids	cnidimol F	
	kushenol I	
	kushenol M	apigenin-7-O-rhamnoside
	kushenol T	chrysosplenetin B
	liquiritin	
	pachypodol	
	sanggenon J	
Alkaloids	ephedradine B	
	isomaistemonine	
	melicopidine	

	pellitorine piperyline	
Phenolics	feruperine glabrol	
Unknown	riboflavin (Vitamin B2) isoanhyoicaritin	morusin hydroperoxide 6,7-dihydroxy-2-(2-phenylethyl) chromone clerodendrin hibiscetin-3-O-glucoside

Urine metabolite that appeared after 4h consuming Ajwa dates flesh are flavonoids; 2-methoxykurarinone; bavachin (corylifolin) (Song et al., 2017); kaempferol-3-O-α-L-arabinoside (Xue et al., 2008); leachianone G (Bilal et al., 2014) and licoflavone A (Kuang et al., 2018), alkaloids; codonopsine (Wang et al., 2017c) and nigeglanine (Gaikwad e al., 2015). Other compounds that identified after 4h Ajwa dates intake are ophiopogonanone B (Jiang et al., 2016) and retusine (Gupta et al., 2014). All these metabolites were identified in positive ionization. Metabolites that identified in negative ionization for the same sampling time (4h) are flavonoids; astragaline C and sophoranodichromane D and other compounds such as saulatine. All metabolites identified in 4h are tabulated in Table 4.17.

Table 4.17: Metabolites in Human Urine that Identified in 4h

	Compounds (+H)	**Compounds (-H)**
Flavonoids	2-methoxykurarinone bavachin (Corylifolin) kaempferol-3-O-α-L-arabinoside leachianone G licoflavone A	astragaline C sophoranodichromane D
Alkaloids	codonopsine nigeglanine	
Others	ophiopogonanone B retusine	saulatine

Meanwhile, 2 alkaloids; polycanthine, 1 phenolic compound; eugenyl glucoside and 1 sesquiterpene; pseudosantonin were identified in 8h after consumption of Ajwa dates flesh. Geniposidic acid and paeonilactone A are other compounds that found in the same urine sampling time (8h). In negative ionization, flavonoids such as roseoside and cimicifugic acid E, phenolics; syringic and sinapic acid, sesquiterpene such as chloranosie B and unknown compounds such as tryptophane and echinothiophene were identified in the same sampling time. Table 4.18 showed the metabolites that identified in 8h.

Table 4.18: Metabolites in Human Urine that Identified in 8h

	Compounds (+H)	**Compounds (-H)**
Flavonoids		roseoside cimicifugic acid E
Alkaloids	polycanthine	
Phenolics	eugenyl glucoside	syringic sinapic acid
Sesquiterpene	pseudosantonin	chloranoside B
Others	geniposidic acid paeonilactone A	tryptophane echinothiophene

And there is only one known compound that appeared after 12h consuming Ajwa dates flesh which is methyl lucidenate Q (Nguyen et al., 2015) in positive ionization and eucommiol in negative ionization. Most urine metabolite appeared after 24h Ajwa dates flesh consumption. There are 32 urine metabolite were identified in positive ionization including alkaloids, phenol, amino acid, flavonoids, terpene glycoside and terpenoid. Other known compounds found to be appeared in 24h after Ajwa dates intake are ajugasterone C-2,3,20,22-diacetonide (Olennikov, 2018),

andropanolide (Sun et al., 2018), bruceine H (Yan et al., 2016), dehydroabietic acid (Gu et al., 2017), desmodimine (Wang et al., 2017b), glucosyringic acid, glutamine, heterodendrin, kusulactone, linustatin (Knutsen et al., 2017), methyl-α-D-fructofuranoside, methyl-β-D-fructofuranoside (Karla et al., 2017), morusimic acid F (Kamat et al., 2017), mudanpioside F (Liu et al., 2018), paeonisuffrone (Wang et al., 2017d), paeonolide (Viborg et al., 2017), pterodontriol D (Mai et al., 2017), ranunculin (Wang et al., 2017a), schizonepetoside E (Wagner et al., 2018), scutellone E, sibiricaphenone (Feng et al., 2018) and vitamin B5. Alkaloids that identified are coniferol (Lyu et al., 2018), gentianine (Fatima et al., 2018) and picrasidine P (Amaral et al., 2017).

Meanwhile other compounds are phenol; ginkgolic acid (Zhou et al., 2018), flavonoid; lutonarin (Lelciu et al., 2018), amino acid; tyrosine, terpene glycoside; trans-carveol-6-β-glucopyranoside and terpenoid; xanthatin. Negative ionization gives flavonoids; bruceine E; catalpol; fraxin and harpagide, alkaloid; delphatine, phenolic; 3,4-di-O-galloylquinic acid; 3,5-O-dimethyl gallic acid; cholic acid; glucuronic acid; isosilybin and linocinnamarin, amino acid; tyrosine; ascorbic acid and nimilinic acid, terpenoid; shionoside A; shionoside B and lactinolide.

Other compounds that identified in negative ionization for same sampling time (24h) are aloenin, andrographatoside, apocynin B, chrysanthemin, citric acid, dehydromorroniaglycone, martynoside A, mudanpioside G, neocuscutoside C, nigakilactone E, nortracheloside, nuezhenidic acid, portuloside A, vomicine, turpinionosides E, thymidine, tenuifoliside D, secologanoside, sebacic acid and sarmentosin. Table 4.19 presented the metabolites in human urine that appeared after 24h consumption of Ajwa dates flesh.

Table 4.19: Metabolites in Human Urine that Appeared after 24h Consumption of Ajwa Dates Flesh

	Compounds (+H)	Compounds (-H)
Flavonoids	lutonarin	bruceine E
		catalpol
		fraxin
		harpagide
Alkaloids		delphatine
	gentianine	
	picrasidine P	
Phenol	ginkgolic acid	3,4-di-O-galloylquinic acid
		3,5-O-dimethyl gallic acid
		cholic acid
		glucuronic acid
		isosilybin
		linocinnamarin
Amino Acid	tyrosine	
		tyrosine
Terpene Glycoside	trans-carveol-6-β-glucopyranoside	
Terpenoid	xanthatin	shionoside A
		shionoside B
		lactinolide
Unknown	ajugasterone C-2,3,20,22-diacetonide	aloenin
	andropanolide	andrographatoside
	bruceine H	apocynin B
		chrysanthemin
	dehydroabietic acid	
	desmodimine	citric acid
	glucosyringic acid	dehydromorroniaglycone
	glutamine	martynoside A
	heterodendrin	mudanpioside G
	kusulactone	neocuscutoside C
	linustatin	nigakilactone E
	methyl-α-D-fructofuranoside	nortracheloside
	methyl-β-D-fructofuranoside	nuezhenidic acid
	morusimic acid F	portuloside A
	mudanpioside F	vomicine
	paeonisuffrone	turpinionosides E

	paeonolide	thymidine
	pterodontriol D	tenuifoliside D
	ranunculin	secologanoside
	schizonepetoside E	sebacic acid
	scutellone E	sarmentosin
	sibiricaphenone	
	vitamin B5	

Appendix 22 illustrated the different pattern of chromatograms that indicated the variation of urine metabolite from 0h to 24 h in positive and negative ionization. It showed most compounds were identified in chromatogram of urine metabolite at 24h. Some peaks were spotted appeared and disappeared in the chromatogram. There are also peaks that belong to certain compounds remain in almost all the collection time (0h to 24h). The compounds named 6-hydroxykynurenic acid, adenosine, coixol, cuscohygrine, dictysine, evoxanthine, isoxanthohumol, lindelofine, magnocurarine, neokurarinol, oxymaistemonine, uridine and xanthosine.

Compared to urine metabolite identified in 0h, more urine metabolites are appeared after the consumption of Ajwa dates flesh. This study found that Ajwa dates contain lots of flavonoid, anthocyanidin, phenolic and sterol which expected gives the notable changes of metabolites in urine samples.

4.2.3.2 Statistical Analysis

One of the techniques in chemometric had been used in this study is Principal Components Analysis (PCA). The scores plot elucidating the urine metabolite and the loadings plot is the urine time collection. From Figure 4.10, urine metabolite that identified only in respective time collection has been highlighted. As illustrated, the scores and loadings plot pattern are similar. Urine metabolite that had been colored in scores plot are belongs to each collection time in loadings plot, accordingly. The

metabolite that scattered in between collection time was showing that metabolites had been identified in both or more collection time.

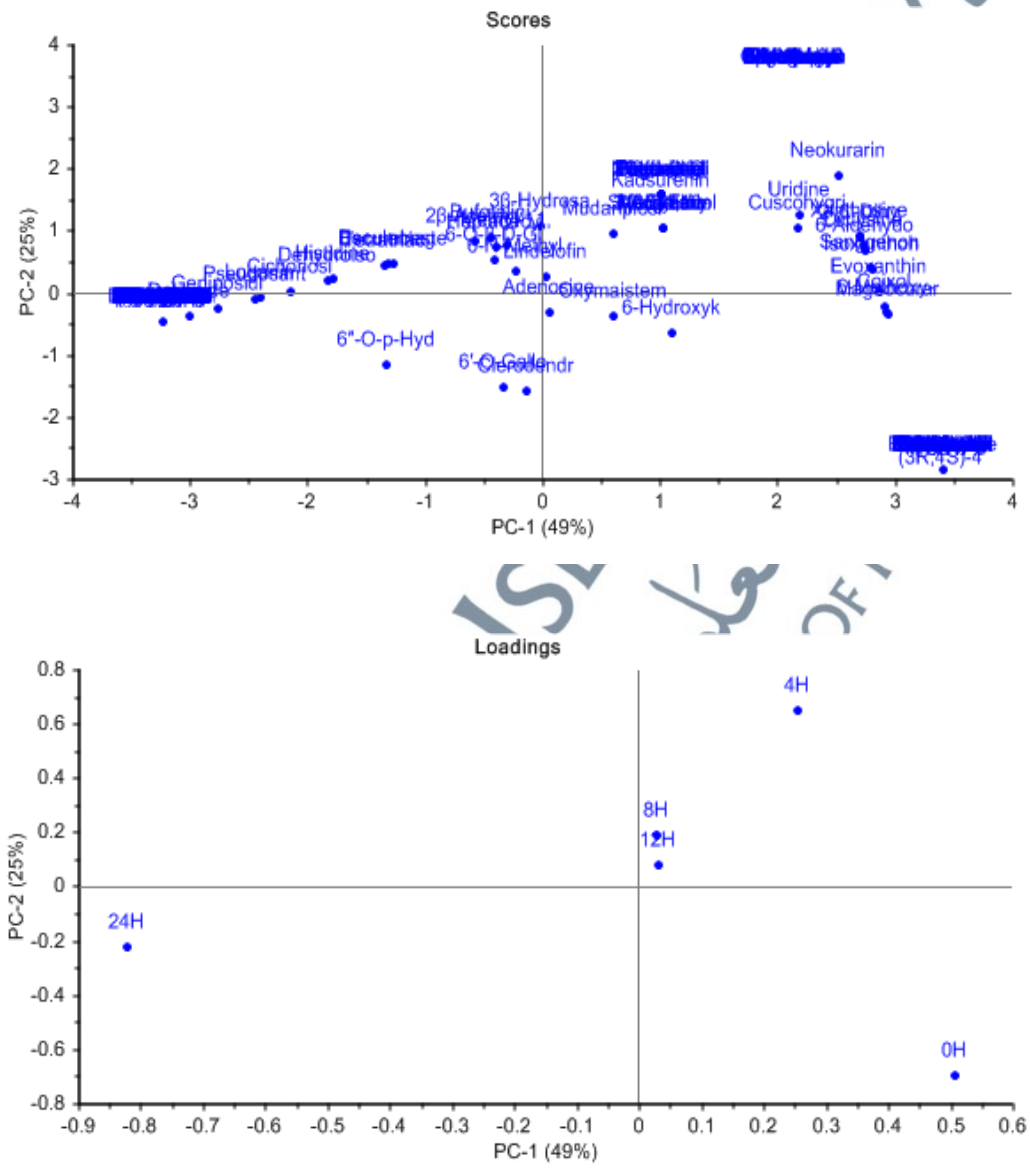

Figure 4.10: Scores and Loadings Plot of Urine Metabolites 24h after Consuming Ajwa Dates Flesh in Positive Ionization.

4.3 Summary

Dates flesh contains a number of potential pharmacological functions due to the presence of chemical compounds such as flavonoids, phenolics, alkaloids, and sterols. Many studies use the mixture of polar solvents extraction to extract targeted group of compounds, but in this study, by using three mixture designs in which the polarities of the mixtures of extraction were changed, resulted in more chemical compounds be extracted and identified using GC-MS. β-sitosterol and vitamin E can be identified merely in non-polar solvent. Most chemical compounds were extracted using polar or combination with polar solvent while longifolenaldehyde and nonadecyl pentafluoropropionate only detected in D5H extract. Other studies stated that longifolenaldehyde and nonadecyl pentafluoropropionate compounds in dates act as antifungals (Zhang et al., 2014) and antimicrobial (Renukadevi et al., 2011), respectively. To conclude, extraction solvent system of varying polarities differs significantly. In this study, LC-QTOF-MS analysis of Ajwa dates indicated that Ajwa dates contain flavonoids, alkaloids, sterols, and anthocyanin.

The ^{1}H-NMR analysis was conducted to study the profile of the metabolites change after the consumption of Ajwa dates. The effects of Ajwa dates on human body had been investigated in ^{1}H-NMR spectrum using Chenomx NMR suite 8.2 and were compared with the reference spectra in the Human Metabolome Database (HMDB). Phytochemicals in Ajwa dates influenced the changes of metabolites in human body in 24 hours after the Ajwa dates intake, based on PCA plots.

Samples urine (0h to 24h) from one volunteer was selected and analysis of the selected samples had been done using GC-MS and LC-QTOF-MS. GC-MS results on urine sample showed that extraction method does matter to extract the metabolites in urine samples. Data of acid base extraction exposed that this extraction method is not

suitable for urine metabolomics study. However, in LC-QTOF-MS, some phytochemicals including alkaloids, sterols, phenols and flavonoids were observed change according to urine time collection.

In conclusion, investigation on the urinary metabolites using NMR- based and MS-based could further confirm the beneficial effect of Ajwa dates in human health. The 24 hours urine collection allowed us to investigate the activity of gut microbiota in healthy people during day and night as it indicates the presence of biomarkers. Ajwa dates may possess the ability to reduce stress level as the level of biomarkers of depressive disorder; alanine, hippurate and citrate significantly decreased after the consumption of Ajwa dates. Obvious changes of the urinary metabolites are from 0 hour to 24 hours. Therefore, it indicates that the effect of Ajwa in human body is within 24 hours.