CHAPTER 4: RESULTS

4.1 Extraction of Samples

4.1.1 Water Content in Plant Samples

Water content in each 100g of samples showed in Table 4.1 below. All plant samples mainly consist of water. UKMR-2 plant sample contain highest percentage of water which is 90.28 % of the sample was water followed with UKMR-1 and Arab with percentage of water in sample were 89.55 % and 85.63 % respectively.

Table 4.1: Percentage of water content in each 100g of sample

Variety of *H. sabdariffa*	Water Content (%)
Arab	85.63 ± 0.13
UKMR-1	89.55 ± 0.17
UKMR-2	90.28 ± 0.06

4.1.2 Average Yield of Crude Extract

Table 4.2 shows the average yield of crude extract from 100 g of sample. The samples were extracted using distilled water. Arab variety gives the highest percentage of crude extract yield with 5.82g of crude extract was obtained from 100g of dried sample. 5.41g and 4.30g of crude extract were obtained from each 100g of UKMR-2 and UKMR-1 dried sample.

Table 4.2: Percentage of average crude extract yield from 100g of dried sample

Variety of *H. sabdariffa*	Crude extract (%)
Arab	5.82 ± 1.21
UKMR-1	4.30 ± 0.03
UKMR-2	5.41 ± 0.18

4.2 Antioxidant Activity Assay

4.2.1 DPPH Free Radical Scavenging Activity Assay

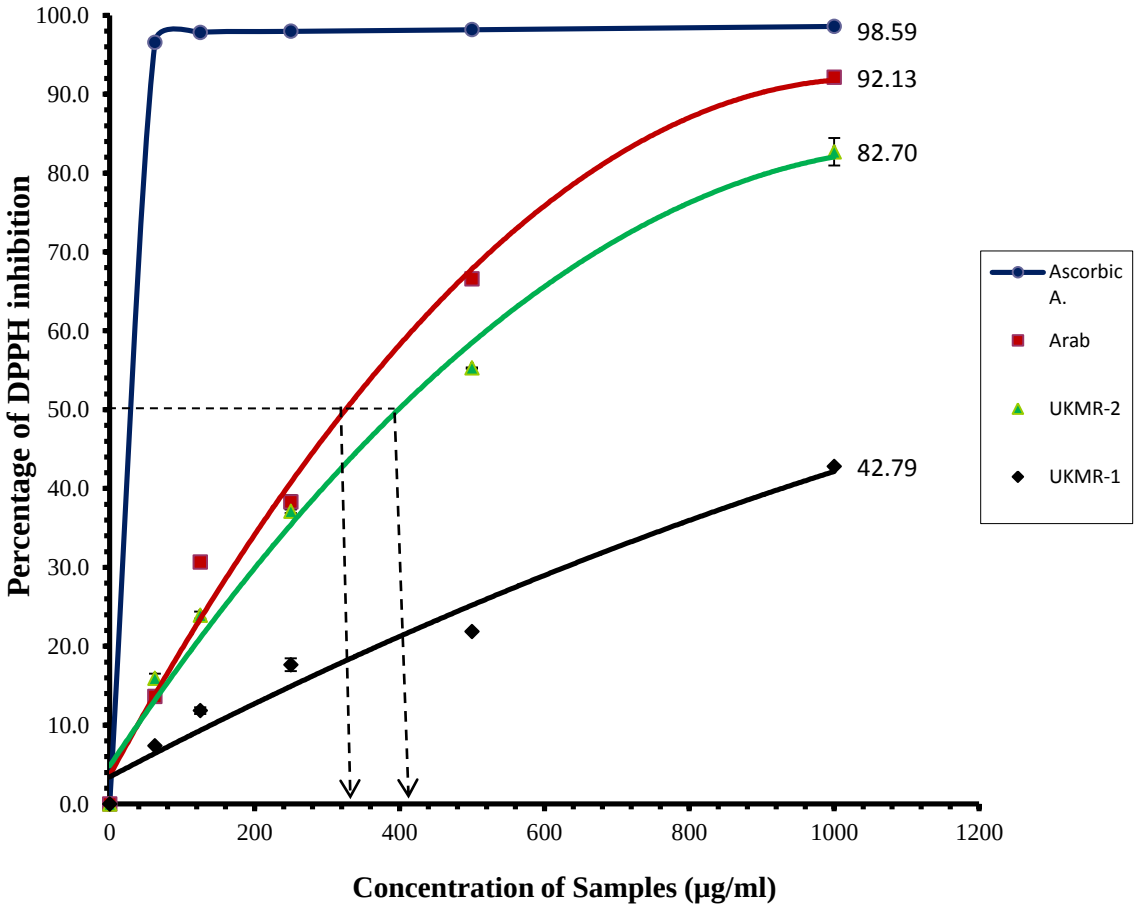
The potential antioxidant activity of three *Hibiscus sabdariffa* variety was determined on the basis of scavenging activity on the stable 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) free radicals. As these free radicals were scavenged by active compound in the sample crude extract, The DPPH color solution will bleach and causing decreasing in the photometric absorbance reading.

Table 4.3: Percentage of inhibition of plant samples and Ascorbic acid. Values are expressed as mean ± SE and n= 3.

Concentration of Crude Extract (µg/ml)	Percentage of Inhibition (%)			
	Ascorbic Acid	**Arab**	**UKMR-2**	**UKMR-1**
0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
62.5	96.56 ± 0.2	13.64 ± 0.4	15.96 ± 1.1	7.36 ± 0.0
125.0	97.81 ± 0.2	30.67 ± 1.3	23.94 ± 0.9	11.83 ± 0.8
250.0	97.96 ± 0.4	38.29 ± 1.7	37.13 ± 0.4	17.66 ± 1.6
500.0	98.16 ± 0.4	66.57 ± 0.7	55.28 ± 0.1	21.84 ± 0.1
1000.0	98.59 ± 0 .5	92.13 ± 0.3	82.70 ± 3.4	42.79 ± 0.0

The dose response curve of the DPPH radical scavenging assay of plant sample and ascorbic acid are illustrated in Figure 4.1. Aqueus extract from Arab variety exhibits the highest radical scavenging activity with IC_{50} = 462.0 µg/ml followed by UKMR-2 with IC_{50} 640.0 µg/ml.

Figure 4.1: DPPH radical scavenging assay dose-response curve of ascorbic acid, Arab, UKMR-1 and UKMR-2 samples. Values are expressed as mean ± SE and n=3.



4.2.2 Metal Chelating Assay

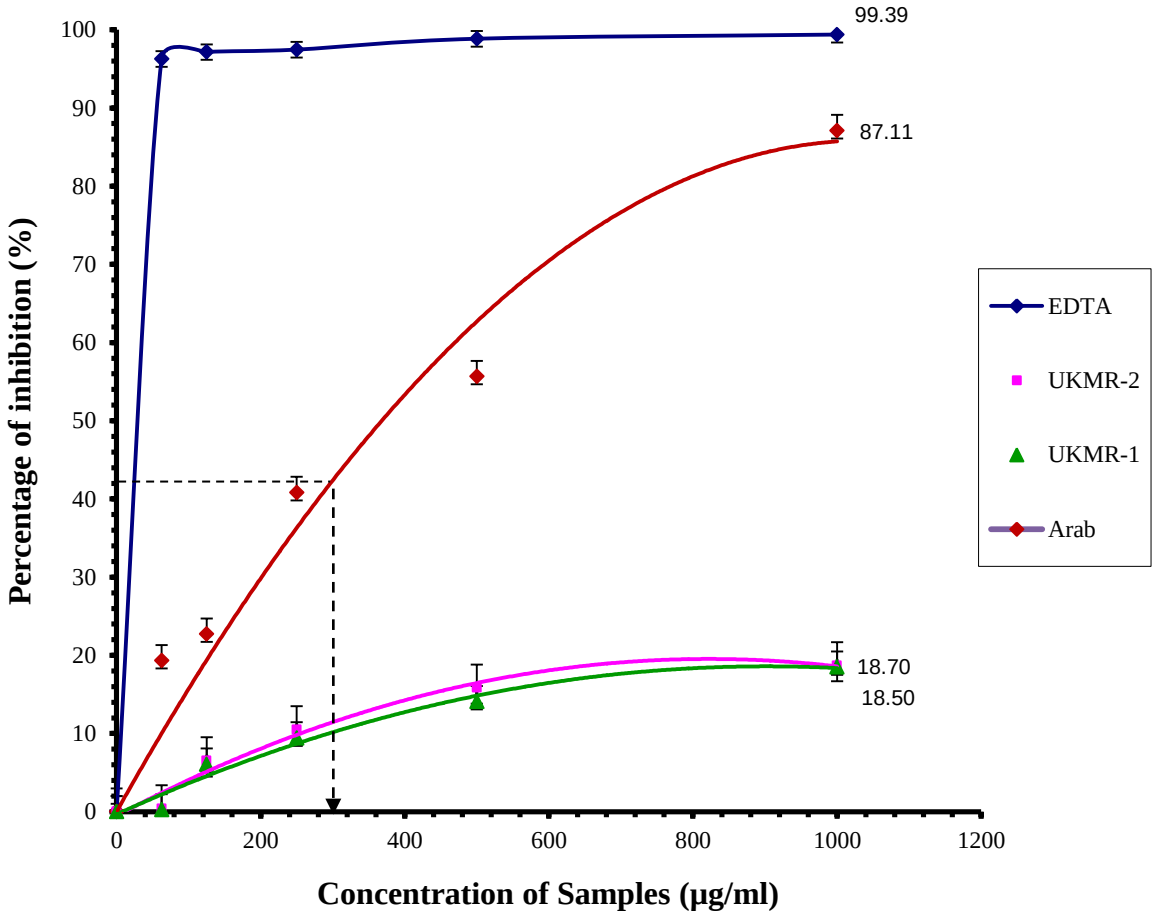
Antioxidant potential of *H. sabdariffa* from Arab, UKMR-1 and UKMR-2 varieties extracts was also evaluated through the ability of the extract to chelate Fe^{2+} ion. In this study, the red color formed by the interaction of ferrozine and Fe^{2+} ions is decreased by the action of metal chelator compounds that exist in the extract. Thus, the lower photometric reading of the reaction mixtures indicates the higher metal chelating activity.

Table 4.4: Percentage of inhibition of *H. sabdariffa* extract. Values are expressed as mean ± SE and n= 3.

Concentration of Crude Extract (µg/ml	Percentage of Inhibition (%)			
	EDTA	**Arab**	**UKMR-2**	**UKMR-1**
0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
62.5	96.27 ± 0.4	16.94 ± 2.4	0.38 ± 0.2	0.26 ± 0.2
125.0	97.15 ± 0.3	23.62 ± 1.3	6.51 ± 0.6	6.08 ± 1.0
250.0	97.46 ± 0.4	37.39 ± 0.6	10.50 ± 1.5	9.44 ± 0.3
500.0	98.86 ± 0.3	55.67 ± 0.2	15.84 ± 1.2	14.08 ± 0.3
1000.0	99.39 ± 0.2	87.11 ± 0.1	18.70 ± 0.2	18.5 ± 0.1

Figure 4.2 illustrates the dose-response curve of the metal chelating activities of three different varieties extracts of *H. sabdariffa* with EDTA as a standard reference. As shown in the figure below, the aqueus extract of Arab variety exhibit the highest chelating activity with IC_{50} = 370 µg/ml. While aqueus extract of UKMR-1 and UKMR-2 shows lower chelating activity.

Figure 4.2: Metal chelating assay dose-response curve of EDTA, Arab, UKMR-1 and UKMR-2 samples. Values are expressed as mean ± SE and n= 3.



4.2.3 Reducing Power Assay

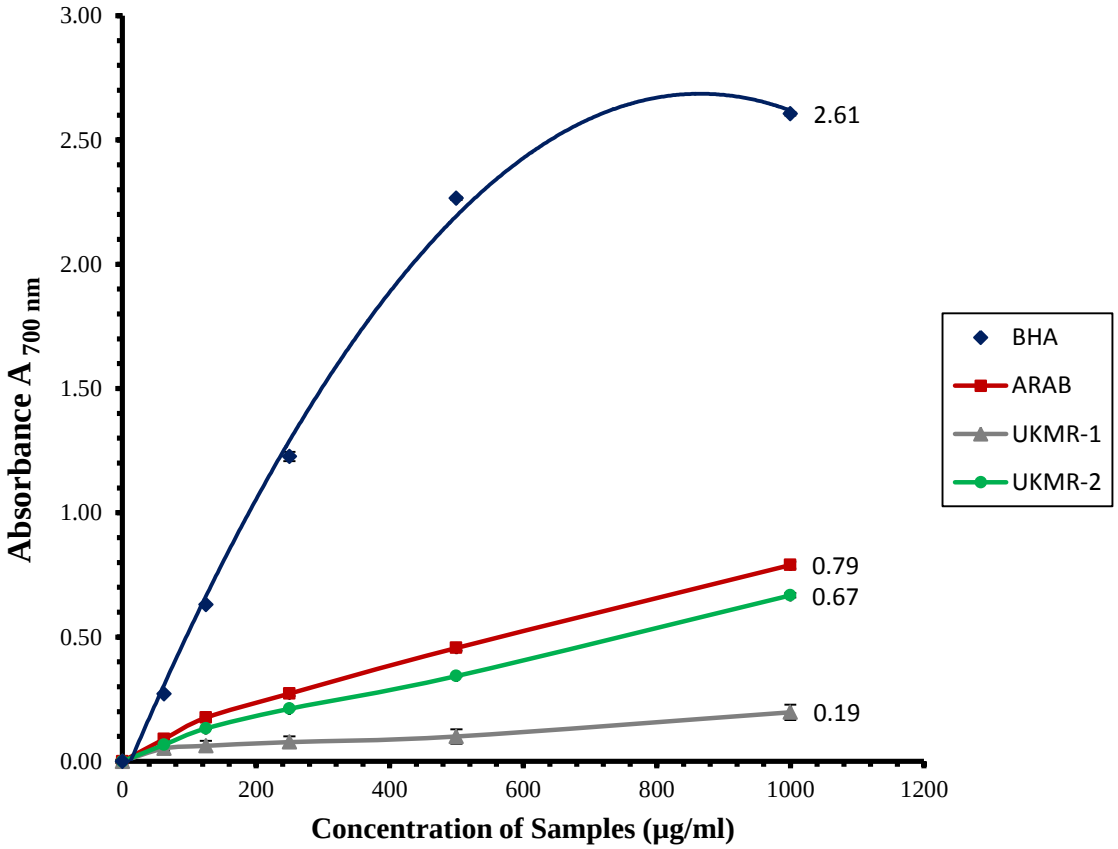
The antioxidant potential of the *H. sabdariffa* extracts also was evaluated through the ability of the active compounds in the extract to reduce Fe^{3+} in ferricyanide complex to ferrous/ Fe^{2+} form. The formation of ferrous/Fe^{2+} reflects the reducing power ability of the samples and can be monitored by measuring the formation of Pearl's Prussian blue using spectrophotometer at 700 nm wavelength.

Table 4.5: Photometric reading of the sample. Values are expressed as mean ± SE and n=3.

Concentration of Crude Extract (µg/ml)	Absorbance			
	BHA	**Arab**	**UKMR-2**	**UKMR-1**
0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
62.5	0.27 ± 0.0	0.09 ± 0.0	0.07 ± 0.2	0.05 ± 0.0
125.0	0.63 ± 0.1	0.18 ± 0.1	0.13 ± 0.2	0.06 ± 0.0
250.0	1.23 ± 0.0	0.27 ± 0.1	0.21 ± 0.3	0.07 ± 0.0
500.0	2.27 ± 0.0	0.47 ± 0.0	0.34 ± 0.3	0.10 ± 0.1
1000.0	2.61 ± 0.1	0.79 ± 0.3	0.67 ± 0.2	0.19 ± 0.1

Figure 4.3 presenting the dose-response curve of the reducing power activities Arab, UKMR-1 and UKMR-2 extracts with BHA as a standard reference. The figure below shows all plant extracts exhibit lower reducing power activities compared to BHA.

Figure 4.3: Reducing power assay dose-response curve of BHA, Arab, UKMR-1 and UKMR-2 samples. Values are expressed as mean ± SE and n= 3.



4.3 Toxicity Test

4.3.1 Brine Shrimp Lethality Assay

The toxicity effects of Arab, UKMR-1 and UKMR-2 of *H. sabdariffa* variety were investigated *in vivo* against the brine shrimp. All brine shrimps were survived when exposed to 10 µg/ml of Arab, UKMR-1 and UKMR-2 extract for 24 hours. The brine shrimps were also survived after 24 hours exposed to 100 µg/ml of UKMR-1 and UKMR-2 extract but died when exposed to Arab extract at same concentration and period of time. No brine shrimps were survived due to exposure to *H. sabdariffa* extracts at concentration 1000 µg/ml.

Table 4.6: Number of brine shrimp survived after 24 hours exposed to different concentration of plant extracts.

Concentration of Extract (µg/ml)	Total Brine Shrimp survived			
	ARAB	**UKMR-1**	**UKMR-2**	**Control**
10.00	10 ± 0.00	10 ± 0.00	10 ± 0.00	10 ± 0.00
25.00	10 ± 0.00	10 ± 0.00	10 ± 0.00	10 ± 0.00
50.00	10 ± 0.00	10 ± 0.00	10 ± 0.00	10 ± 0.00
100.00	6 ± 0.58	10 ± 0.00	10 ± 0.00	10 ± 0.00
250.00	3 ± 0.58	9 ± 0.58	6 ± 1.53	10 ± 0.00
500.00	0 ± 0.00	6 ± 1.00	2 ± 1.00	10 ± 0.00
1000.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	10 ± 0.00

The results present in Table 4.7 are the LC_{50} of Arab, UKMR-1 and UKMR-2 extracts. These extracts are considered toxic to brine shrimp if their LC_{50} < 1000 µg/ml. As shown in the table below, *H. sabdariffa* from Arab (LC_{50} = 146.9 µg/ml) variety exhibit higher toxicity effect on brine shrimp compared to UKMR-1 (LC_{50} = 503.6 µg/ml) and UKMR-2 (LC_{50} = 325.6 µg/ml). Thus *H. sabdariffa* from Arab variety may contain more bioactive compound than UKMR-1 and UKMR-2.

Table 4.7: LC_{50} values of H. sabdariffa variety extracts on brine shrimp lethality bioassay

Plant Sample Extracts	**Concentration of LC_{50} of Plant extracts (µg/ml)**			
	ARAB	**UKMR-1**	**UKMR-2**	**Control**
LC_{50} µg/ml	146.9	503.6	325.6	>1000 .00

4.3.2 Oral toxicity Test

4.3.2.1 Clinical Observation

All rats treated with 500 and 1000 mg/kg body weight/ day of Arab, UKMR-1 and UKMR-2 extracts were survived the 14 days oral dosing. There was no mortality attribut to any effect of plant samples. At the final of 14 days of oral toxicity study, there were no significant dose – related changes in the body weight of experimantal animal in all groups. Changes in experimental animal body weight during the study are illustrated in Figure 4.4. In-life observations also showed no fur appearance, change in skin, fur and eye color, mucous membranes, bizarre movement, convulsions, lacrimation and abnormal physical sign on experimental animals in all groups.

Figure 4.4: Body weight of rats treated with 1000 mg/kg body weight/ day of Arab, UKMR-1 and and UKMR-2 extracts.

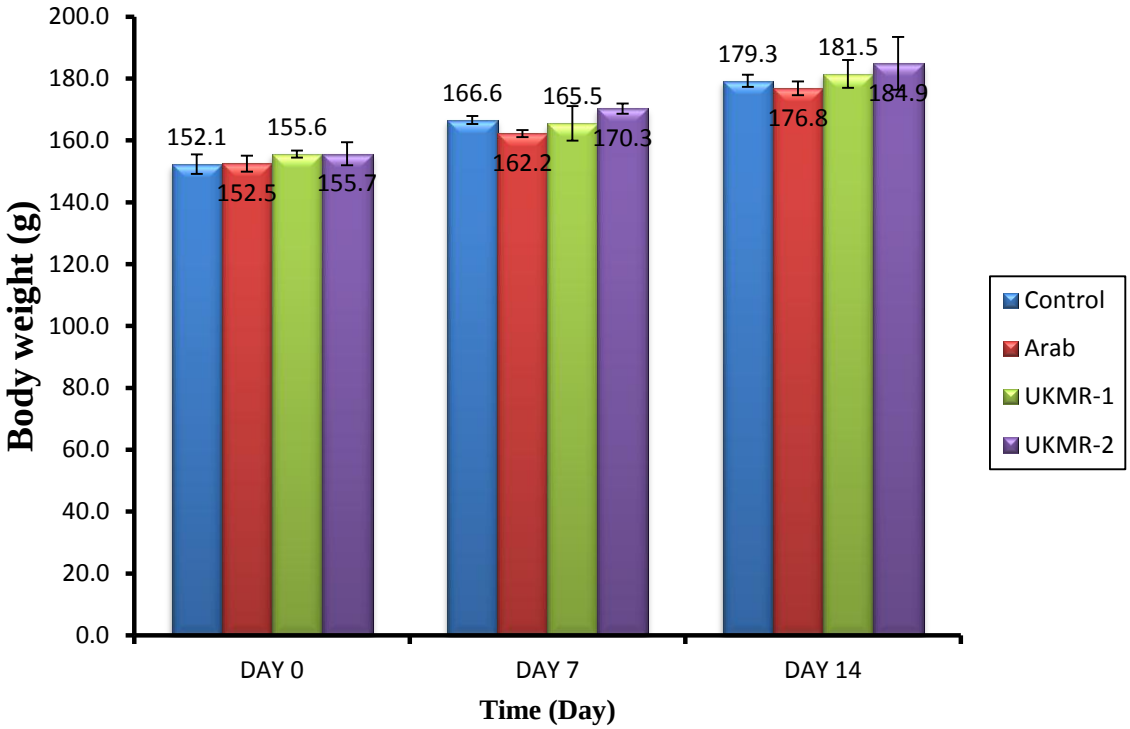
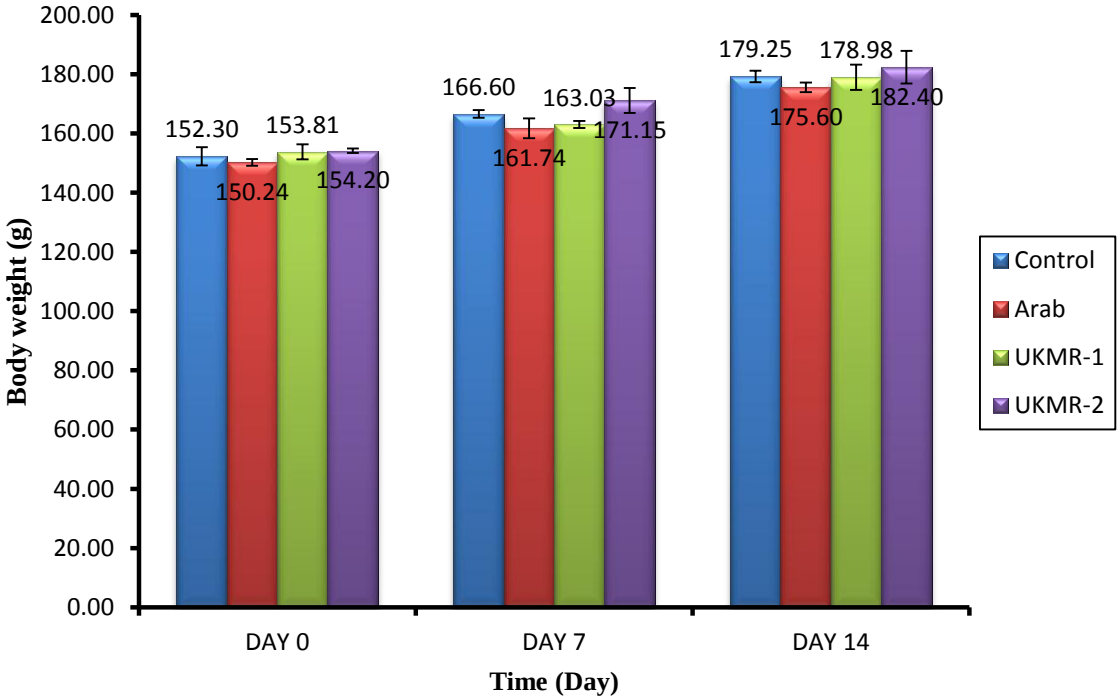


Figure 4.5: Body weight of the rats treated with 500 mg/kg body weight/ day of Arab, UKMR-1 and and UKMR-2 extracts.



4.3.2.2 Clinical Biochemistry Analysis

Results for liver function test on blood serum for rats treated with Arab, UKMR-1 and UKMR-2 for 1000 mg/kg body weight/day presented in Table 4.8. The average level of all parameters tested on blood serum for experimental animal treated with Arab and UKMR-2 showed no significant different compared to control group. Except the average level of total protein and globulin in blood serum of UKMR-1 treated group recorded a significant increasing ($p < 0.05$) compared to control group. While Alanine aminotransferase and Aspartate aminotransferase level in UKMR-1 treated group exhibit significant decreasing ($p < 0.05$) compared to control group.

Table 4.8: Serum values of liver function test for rats after 14 day oral administration of 1000 mg/kg body weight/ day plant sample. Values are expressed as mean ± SE and n = 6.

Group	LIVER FUNCTION TEST			
	Control	**Arab**	**UKMR-1 (n=6)**	**UKMR-2 (n=6)**
Total protein (g/L)	65.50 ± 0.75	67.50 ± 0.75	76.50 ± 0.18	66.25 ± 0.91
Albumin (g/L)	11.25 ± 0.30	12.25 ± 0.30	11.00 ± 0.37	11.75 ± 0.16
Globulin (g/L)	56.25 ± 0.70	55.00 ± 0.93	60.00 ± 0.37*	55.33 ± 0.15
Total bilirubin (umol/L)	2.00 ± 0.00	2.00 ± 0.00	2.17 ± 0.17	2.00 ± 0.00
Conjugated Bilirubin (umol/L)	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
Alanine aminotransferase (IU/L)	127.00 ± 10.80	137.00 ± 13.87	103.25 ± 2.09*	138.50 ± 6.78
Aspartate aminotransferase (IU/L)	461.00 ± 11.94	448.50 ± 8.91	143.50 ± 4.93*	416.25 ± 28.60
Alkaline phosphatase (IU/L)	211.75 ± 10.25	228.75 ± 8.87	88.00 ± 2.19	241.00 ± 11.00
G-Glutamyl Transferase (IU/L)	3.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00

*. The mean difference is significant compared to normal control at $p < 0.05$ level.

Table 4.9 shows the result of renal function test for rats treated with administration of 1000 mg/kg body weight/day plant samples. Clinical biochemistry analysis of renal function test on blood serum of Arab and UKMR-2 treated group showed no statistically significant changes in average level of parameters measured compared to control group. However, animal treated with UKMR-1 sample recorded significant different in Carbon dioxide, Anion gap and Creatinine level.

Table 4.9: Serum values of renal function test for rats after 14 day oral administration of 1000 mg/kg body weight/day plant sample. Values are expressed as mean ± SE and n=6.

Group	RENAL FUNCTION TEST			
	Control	**Arab**	**UKMR-1**	**UKMR-2**
Sodium (Na) (mmol/L)	136.83 ± 1.56	137.67 ± 0.76	138.50 ± 0.22	136.67 ± 0.88
Pottasium (K) (mmol/L	6.55 ± 0.51	6.58 ± 0.34	5.65 ± 0.25	6.6 5 ± 0.74
Chloride (mmol/L)	103.17 ± 0.70	101.67 ± 1.76	102.50 ± 0.22	104.00 ± 3.03
Carbon dioxide (mmol/L)	17.67 ± 2.04	20.88 ± 1.11	24.35 ± 0.83*	18.55 ± 1.02
Anion gap (mmol/L)	22.00 ± 1.13	21.83 ± 0.54	18.00 ± 0.50*	20.17 ± 0.31
Urea (mmol/L)	8.23 ± 0.71	7.85 ± 0.39	8.80 ± 0.36	8.07 ± 0.62
Creatinine (umol/L)	39.25 ± 1.45	39.33 ± 1.05	53.14 ± 0.16*	37.17 ± 3.90

*. The mean difference is significant compared to normal control at $p < 0.05$ level.

The values of liver function test parameter for all groups of rats are shown in Table 4.10. All parameter studied in this clinical biochemistry analysis showed statistically insignificant different ($p < 0.05$) when compared to control group except for albumin level in blood serum of animal treated with UKMR-1 aqueus extract. Albumin concentration in this group recorded significantly higher compared to control group.

Table 4.10: Serum values of liver function test for rats after 14 day oral administration of 500 mg/kg plant sample. Values are expressed as mean ± SE.

Group	LIVER FUNCTION TEST			
	Control	**Arab**	**UKMR-1**	**UKMR-2**
Total protein (g/L)	65.50 ± 0.75	66.25 ± 0.91	68.75 ± 2.26	65.50 ± 0.91
Albumin (g/L)	11.25 ± 0.30	11.75 ± 0.16	13.00 ± 0.68*	12.25 ± 0.30
Globulin (g/L)	56.25 ± 0.70	55.75 ± 0.30	54.50 ± 1.74	55.00 ± 0.93
Total bilirubin (umol/L)	2.00 ± 0.00	2.00 ± 0.00	3.00 ± 0.00	2.00 ± 0.00
Conjugated Bilirubin (umol/L)	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
Alanine aminotransferase (IU/L)	127.00 ± 10.79	130.00 ± 6.31	109.25 ± 1.74	111.50 ± 8.06
Aspartate aminotransferase (IU/L)	461.00 ± 11.94	434.75 ± 30.56	403.25 ± 50.44	448.50 ± 8.91
Alkaline phosphatase (IU/L)	211.75 ± 10.25	211.25 ± 6.63	210.25 ± 13.37	228.75 ± 8.87
G-Glutamyl Transferase (IU/L)	3.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00

*. The mean difference is significant compared to normal control at $p < 0.05$ level

Table 4.11 shows the values of studied parameter for renal function test for rats treated with administration of 500 mg/kg body weight/ day plant sample. The renal were function in normal condition as the biochemical analysis of blood serum shows the level of parameters were fall in normal range. Only sodium level in UKMR-1 treated group exhibit significantly higher compared to control group.

Table 4.11: Serum values of renal function test for rats after 14 day oral administration of 500 mg/kg plant sample. Values are expressed as mean ± SE and n = 6.

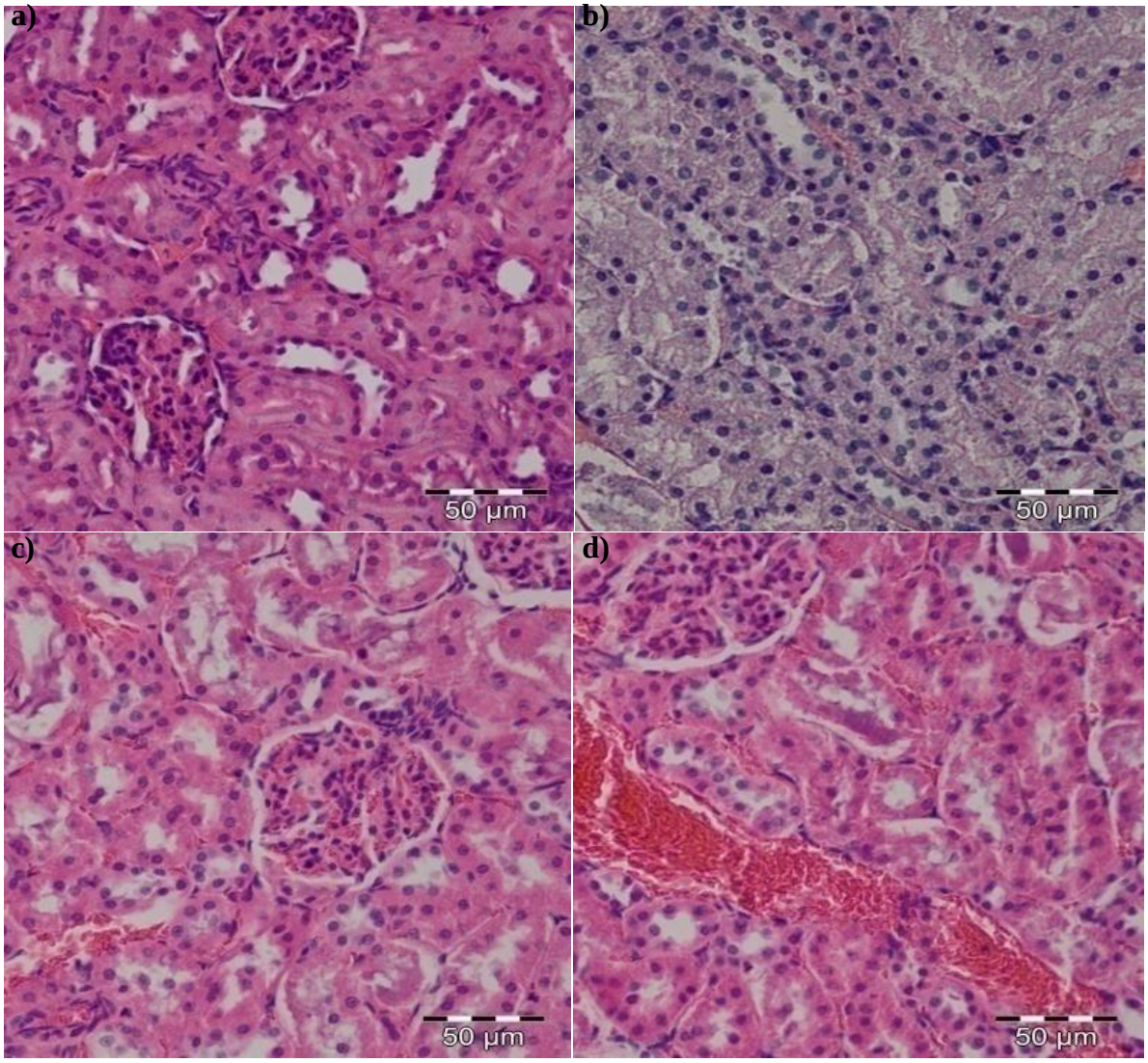
Group	RENAL FUNCTION TEST			
	Control (n=6)	**Arab (n=6)**	**UKMR-1 (n=6)**	**UKMR-2 (n=6)**
Sodium (Na) (mmol/L)	136.83 ± 1.56	137.50 ± 0.67	142.00 ± 0.73*	139.67 ± 0.42
Pottasium (K) (mmol/L	6.55 ± 0.51	5.87 ± 0.41	5.60 ± 0.20	5.12 ± 0.20
Chloride (mmol/L)	103.17 ± 0.70	103.00 ± 0.26	105. 67 ± 0.84	103.33 ± 0.71
Carbon dioxide (mmol/L)	17.67 ± 2.03	19.58 ± 0.73	21.04 ± 0.60	20.62 ± 0.74
Anion gap (mmol/L)	22.00 ± 1.13	19.67 ± 0.76	19.00 ± 0.37	19.67 ± 0.67
Urea (mmol/L)	8.23 ± 0.71	7.05 ± 0.66	9.97 ± 0.23	6.63 ± 0.36
Creatinine (umol/L)	39.25 ± 1.45	35.00 ± 1.90	54.00 ± 0.51	44.67 ± 0.67

* The mean difference is significant compared to normal control at $p < 0.05$ level.

4.3.2.3 Histopathology Analysis

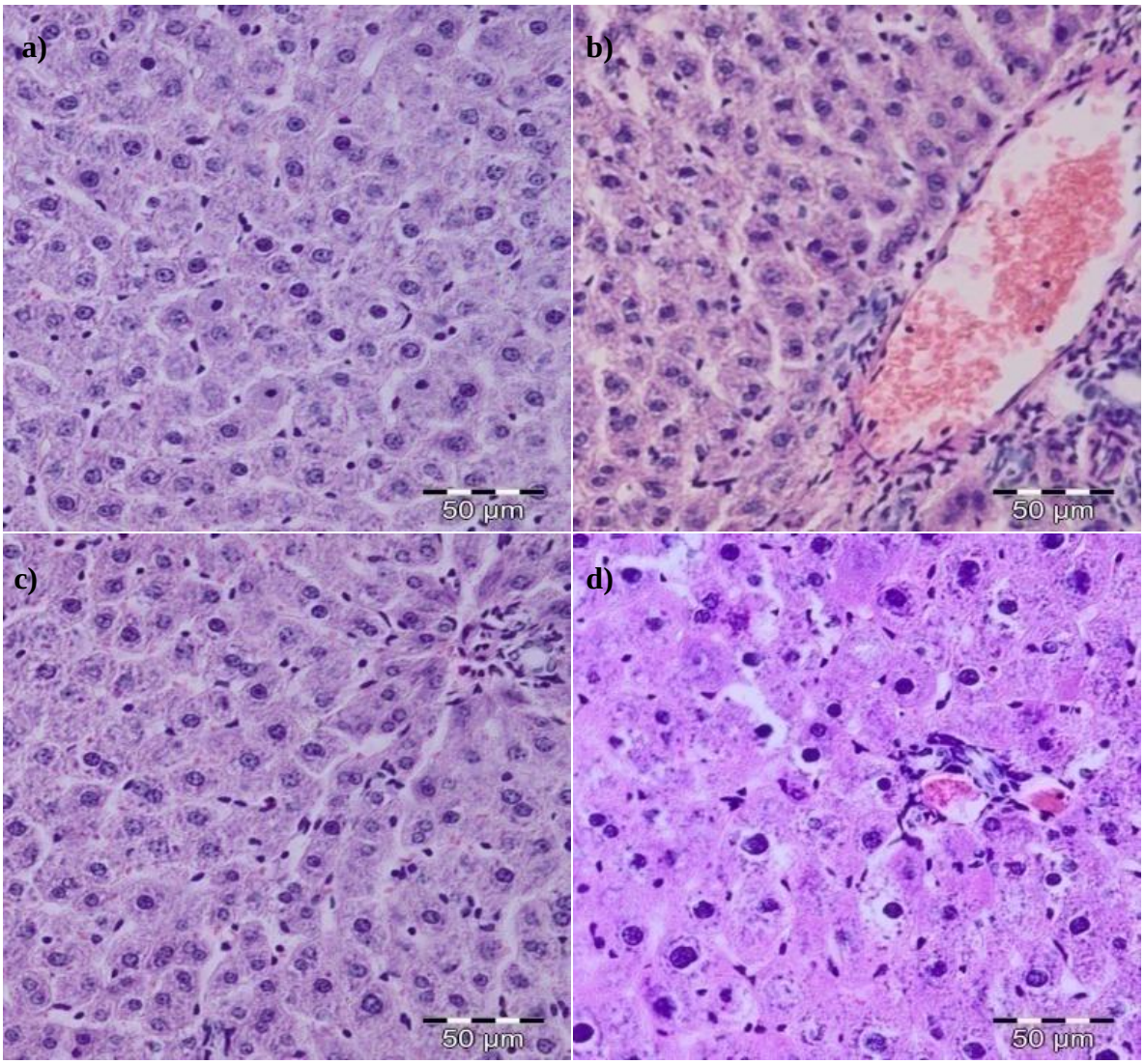
Histopathological examination of the kidney in rats treated with 1000 mg/ kg body weight/ day extracts of Arab, UKMR-1 and UKMR-2 groups revealed normal architecture in glomerulus and tubules. Cells structure in the treated groups also shows normal morphology and no different compared to normal group.

Figure 4.6: Microscopic images of a histological section (200x) of (a) a normal kidney, a kidney in rats treated with 1000 mg/ kg body weight/day of (b) Arab, (c) UKMR-1 and (d) UKMR-2 extracts.



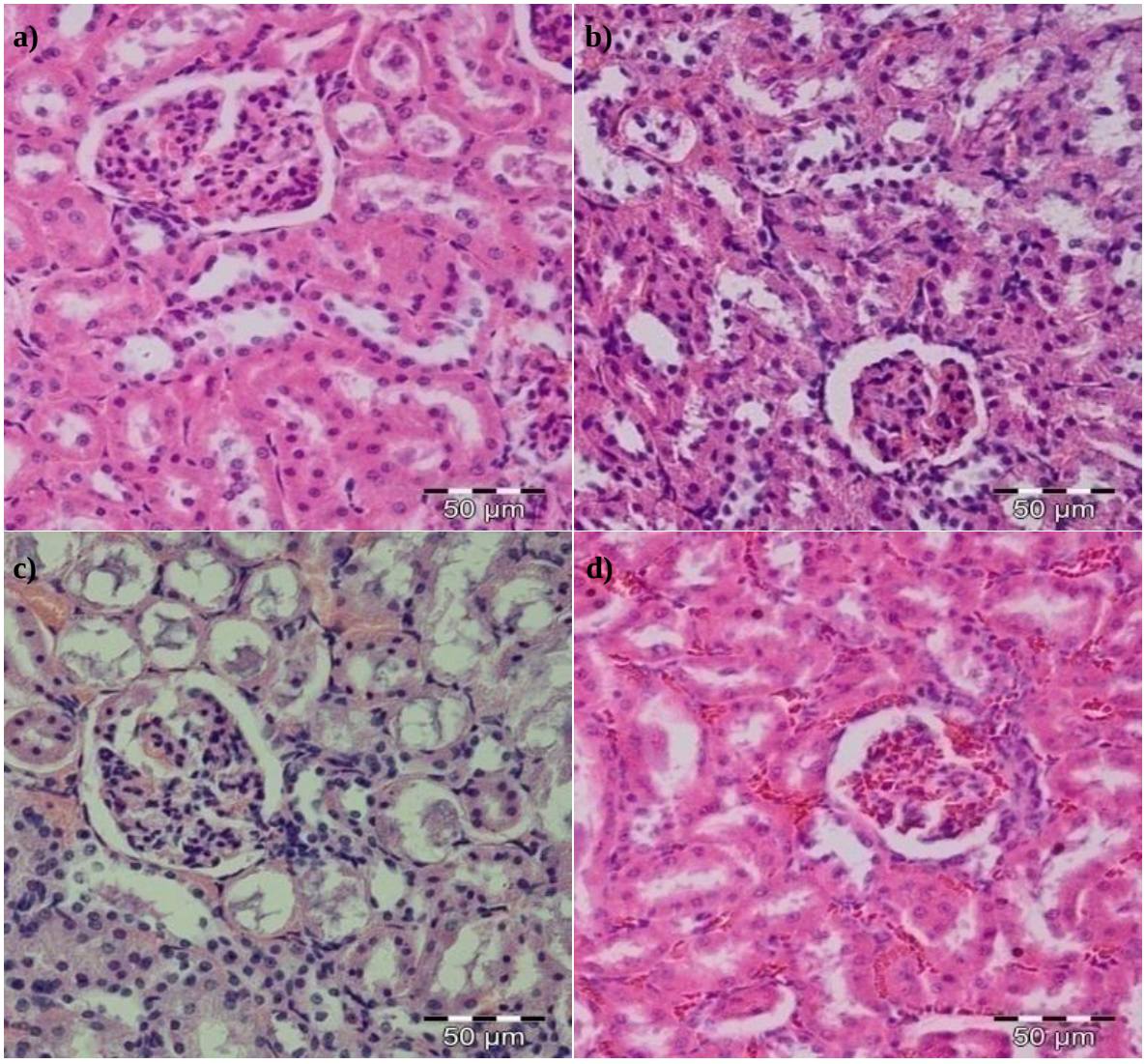
Liver sections from the rats were also prepared and stained with Hematoxylin and Eosin for visualization. Examination on liver sections from mice treated with 1000 mg/ kg body weight/ day extracts of Arab, UKMR-1 and UKMR-2 shows normal appearing hepatocytes and sinusoid spaces. There were no signs of liver disruption or malfunctioning such as necrotic or lesions cells in all treated groups.

Figure 4.7: Microscopic images of a histological section (200x) of (a) a normal liver, a liver in rats treated with 1000 mg/ kg body weight/day of (b) Arab, (c) UKMR-1 and (d) UKMR-2 extracts.



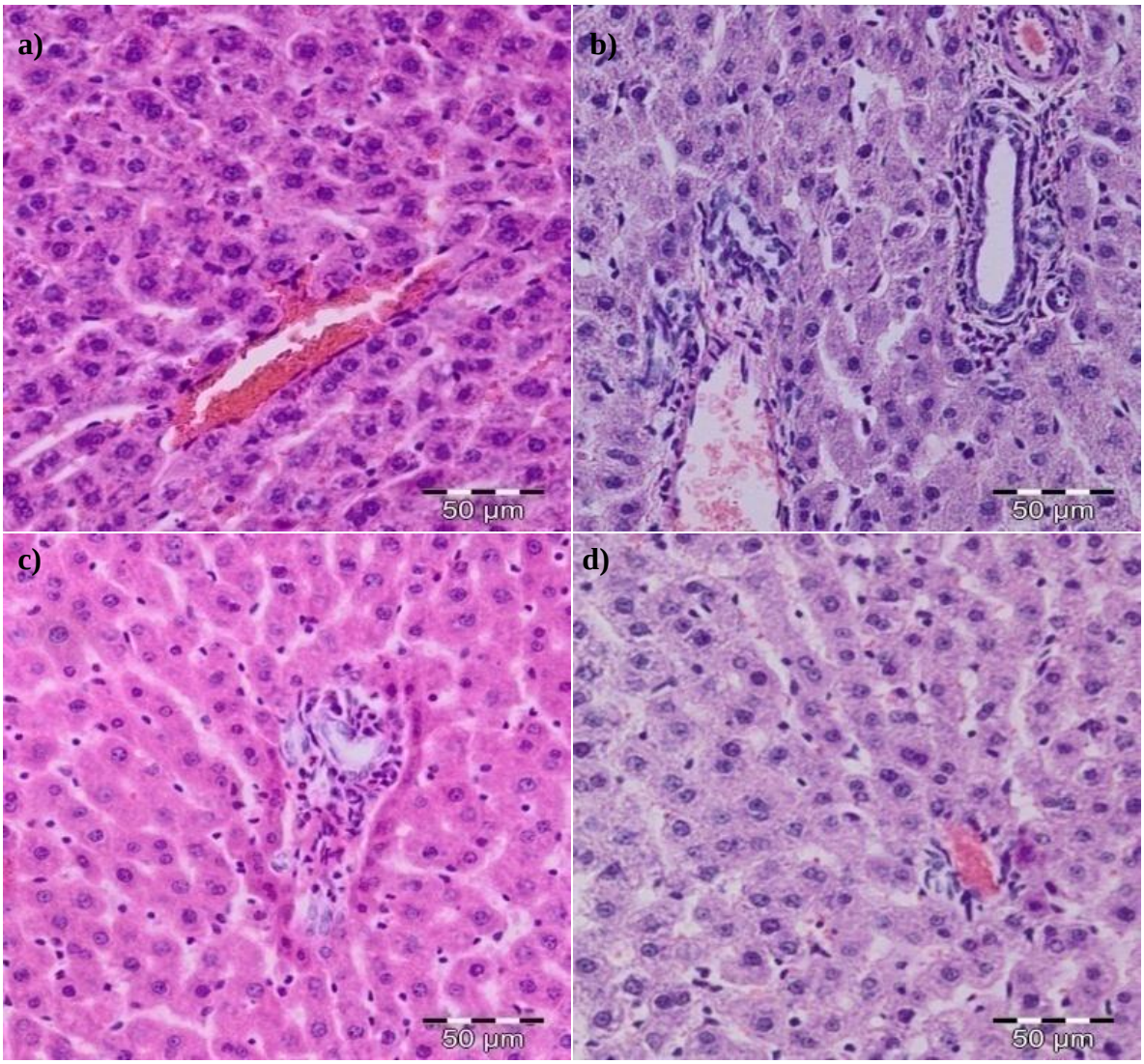
Analysis on microscopic images of a histological section of the kidney in rats treated with 500 mg/ kg body weight/ day extracts of Arab, UKMR-1 and UKMR-2 groups also shows normal architecture in glomerulus and tubules. Cells structure in the treated groups also shows normal morphology and no different compared to normal group.

Figure 4.8: Microscopic images of a histological section (200x) of (a) a normal kidney, a kidney in rats treated with 500 mg/ kg body weight/day of (b) Arab, (c) UKMR-1 and (d) UKMR-2 extracts.



Examination on liver sections stained with Hematoxylin and Eosin from rat treated with 1000 mg/ kg body weight/ day and 500 mg/ kg body weight/ day extracts of Arab, UKMR-1 and UKMR-2 shows normal appearing hepatocytes and sinusoid spaces. There were no signs of liver disruption or malfunctioning such as necrotic or lesions cells in all treated groups.

Figure 4.9: Microscopic images of a histological section (200x) of (a) a normal liver, a liver in rats treated with 500 mg/ kg body weight/day of (b) Arab, (c) UKMR-1 and (d) UKMR-2 extracts.

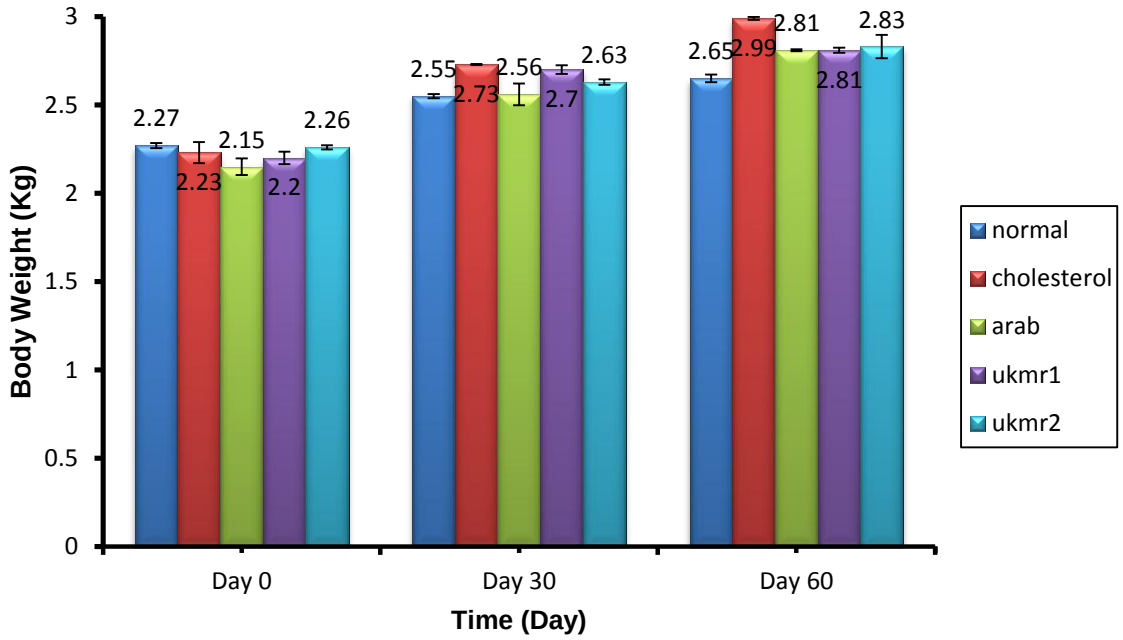


4.4 Hypercholesterolemic Assay

4.4.1 Body weight

The averages of experimental animal's body weight at beginning of the treatment were not significant among the group. After 60 days period of induction of hypercholesterolemia through feeding cholesterol diet, the increasing of body weight in experimental animal is significantly different among treated groups compared to normal control.

Figure 4.10: Body weight of rabbits in Normal control, Cholesterol control, Arab, UKMR-1 and UKMR-2 groups on day 0, 30th an 60th of experiment.



4.4.2 Triglycerides Measurement

Table below shows the effect of daily administration of aquous Arab, UKMR-1 and UKMR-2 extract on induced hypercholesterolemic rabbit for 60 day. Differences in initial triglyceride level (t = 0) were not statiscally significant between all group (P< 0.05). After 60 days of the induction period, triglycerides level in experimental animal in cholesterol, Arab, UKMR-1 and UKMR-2 groups were increase significantly compared to normal group.

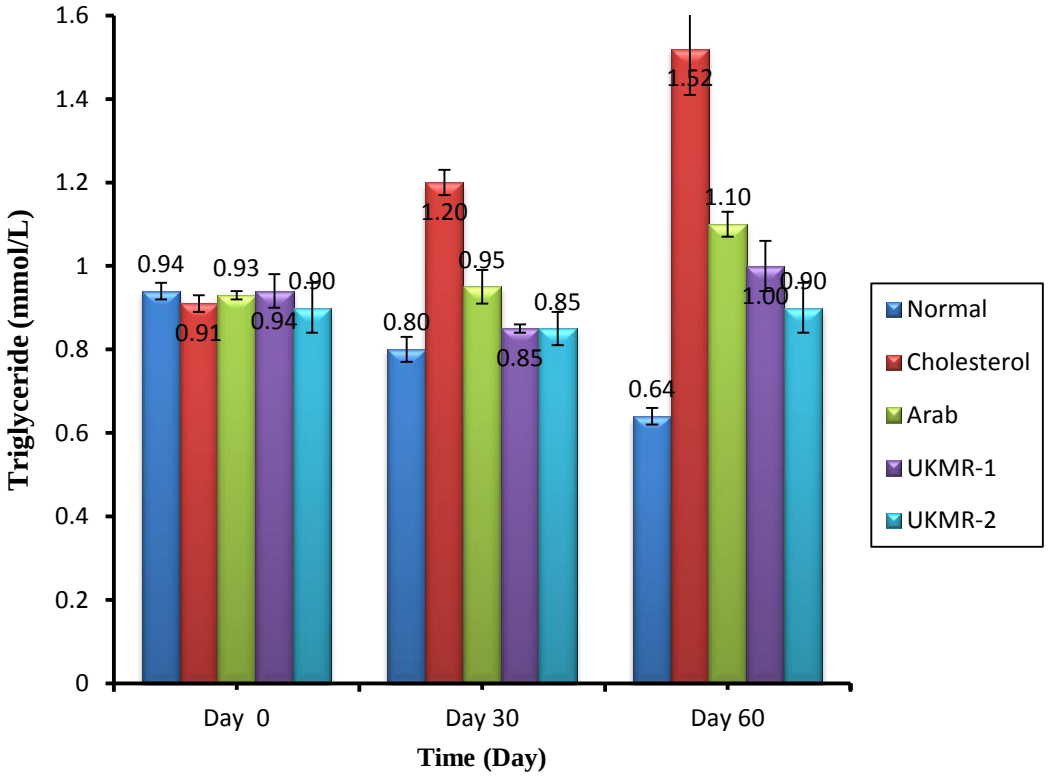
Table 4.12: Level of total triglyceride in blood serum of rabbits in various groups on 0, 30 and 60 days of treatment. Values are expressed as mean ± SE and n = 6.

Groups	Triglyceride Level (mmol/L)		
	Day 0	**Day 30**	**Day 60**
Normal	0.94 ± 0.02	0.80 ± 0.03	0.64 ± 0.02
Cholesterol	0.91 ± 0.02	1.20 ± 0.03*	1.52 ± 0.11*
Arab	0.93 ± 0.01	0.95 ± 0.04*	1.10 ± 0.03*
UKMR-1	0.94 ± 0.04	0.85 ± 0.01	1.00 ± 0.06*
UKMR-2	0.90 ± 0.06	0.85 ± 0.04	0.90 ± 0.06*

*. The mean difference is significant compared to normal control at p < 0.05 level.

Triglycerides level increased over the time period in all groups of experimetal animal fed with cholesterol diet. As shown in the figure below, triglyceride level in all groups of treated experimental animal increase significantly compared to normal group. Although giving 250mg/ kg body weight of Arab, UKMR-1 and UKMR-2 extract daily to induced hypercholesterolemia animal shows reduction in triglyceride level, but this reduction did not give any significant different compared to cholesterol group.

Figure 4.11: Triglyceride level in blood serum of experimental rabbits in various groups during 60 days of treatment. Values are expressed as mean ± SE and n = 6.



4.4.3 Total Cholesterol Measurement

The baseline level of total cholesterol (t=0) in the rabbits blood serum before the experiment was not significantly different within all groups. Without any treatment, total cholesterol level in the experimental animal was maintained at the low level throughout 60 days of the induction period. As shown in the table below, feeding the animal with 100 g/day diet with 10% of cholesterol induce total cholesterol level in blood serum markedly increase after 30 days and 60 days of experiment. These increasing of total cholesterol level in cholesterol, Arab, UKMR-1 and UKMR-2 group were very significant compared to the normal group.

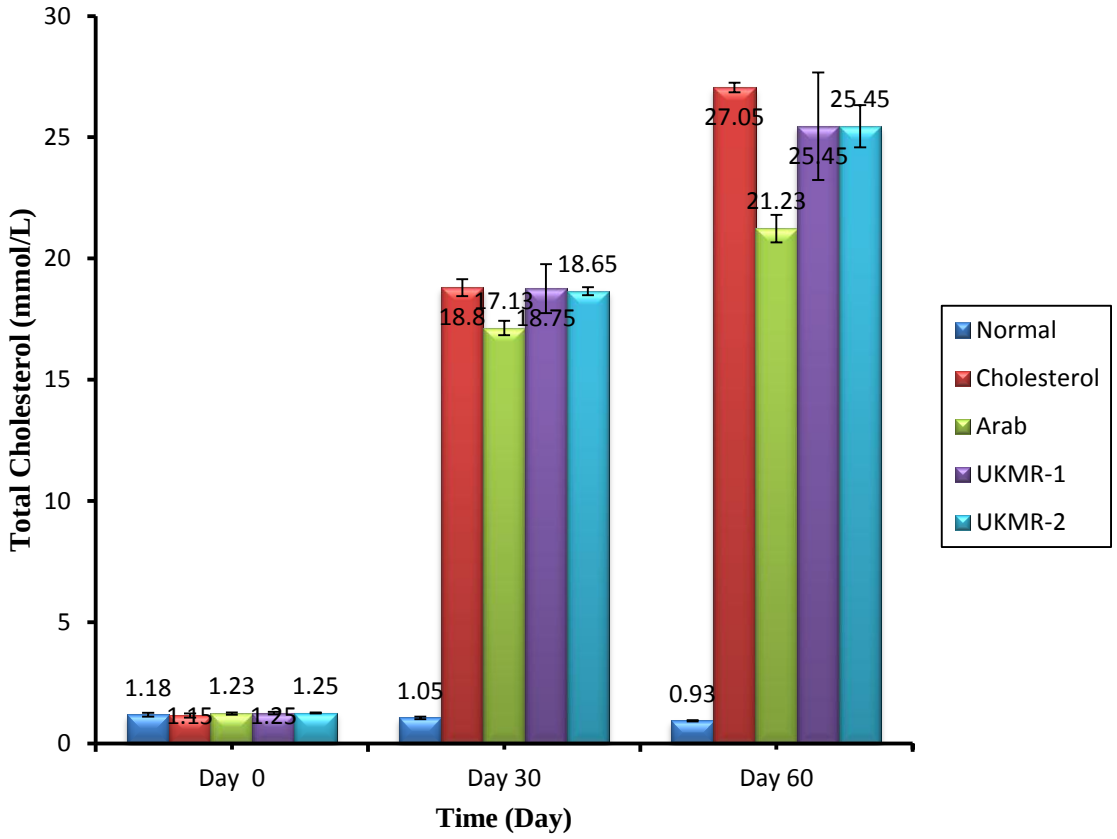
Table 4.12: Level of total cholesterol in blood serum of experimental rabbits in various groups on 0, 30 and 60 days of treatment. Values are expressed as mean ± SE and n = 6.

Groups	Total Cholesterol Level (mmol/L)		
	Day 0	**Day 30**	**Day 60**
Normal	1.18 ± 0.08	1.05 ± 0.05	0.93 ± 0.03
Cholesterol	1.15 ± 0.08	18.80 ± 0.35*	27.05 ± 0.20*
Arab	1.23 ± 0.05	17.13 ± 0.30*	21.23 ± 0.57*
UKMR-1	1.25 ± 0.05	18.75 ± 1.01*	25.45 ± 2.22*
UKMR-2	1.25 ± 0.02	18.65 ± 0.16*	25.45 ± 0.87*

*. The mean difference is significant compared to normal control at $p < 0.05$ level.

Figure below represent total cholesterol level in blood serum of experimental animals in normal, cholesterol, Arab, UKMR-1, and UKMR-2 groups. Total cholesterol level increased significantly over the time period in all groups of experimetal animal compared to normal group. Total cholesterol level in Arab, UKMR-1, and UKMR-2 groups increased but not as higher as in cholesterol group. However this reduction is not significantly different compared to cholesterol group.

Figure 4.13: Total cholesterol level in blood serum of experimental rabbits in various groups during 60 days of treatment. Values are expressed as mean ± SE and n= 6.



4.4.4 HDL Cholesterol Measurement

The level of HDL cholesterol in blood serum on day 0 of treatment was used as baseline because the different levels in all groups were not significant. In the period of induction of hypercholesterolemia through feeding 100g/day of 10% cholesterol diet to experimental animal, the increase of HDL cholesterol level in blood serum significantly different among all groups of the animal study compared to normal group.

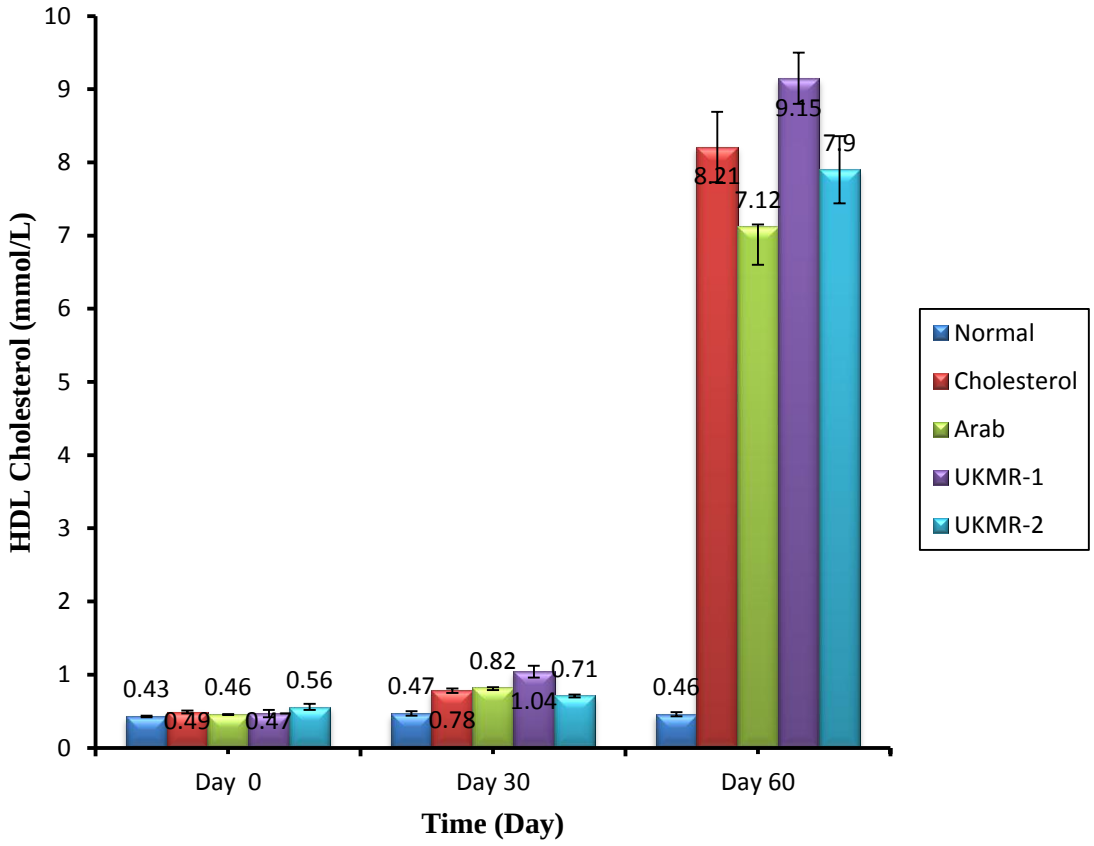
Table 4.14: Level of HDL cholesterol in blood serum of experimental rabbits in various groups on 0, 30 and 60 days of treatment. Values are expressed as mean ± SE and n =6.

Groups	HDL Cholesterol Level (mmol/L)		
	Day 0	**Day 30**	**Day 60**
Normal	0.43 ± 0.01	0.47 ± 0.03	0.46 ± 0.03
Cholesterol	0.49 ± 0.02	0.78 ± 0.03*	8.21 ± 0.48*
Arab	0.46 ± 0.01	0.82 ± 0.03*	7.12 ± 0.52*
UKMR-1	0.47 ± 0.05	1.04 ± 0.08*	9.15 ± 0.35*
UKMR-2	0.56 ± 0.04	0.71 ± 0.02*	7.90 ± 0.46*

*. The mean difference is significant compared to normal control at $p < 0.05$ level.

Without any treatment with Arab, UKMR-1 and UKMR-2 extracts, HDL cholesterol level in the hypercholesterolemic animal fed with cholesterol alone, was maintained at the high level throughout 6 weeks of the treatment period. As shown in Figure 4.15, administering 250mg/kg of Arab, UKMR-1 and UKMR-2 extracts caused decreasing levels of HDL cholesterol even though this decreasing did not give any significant different ($p < 0.05$) compared to cholesterol group.

Figure 4.15: HDL cholesterol level in blood serum of experimental rabbits in various groups during 60 days of treatment. Values are expressed as mean ± SE and n=6.



4.4.5 LDL Cholesterol Measurement

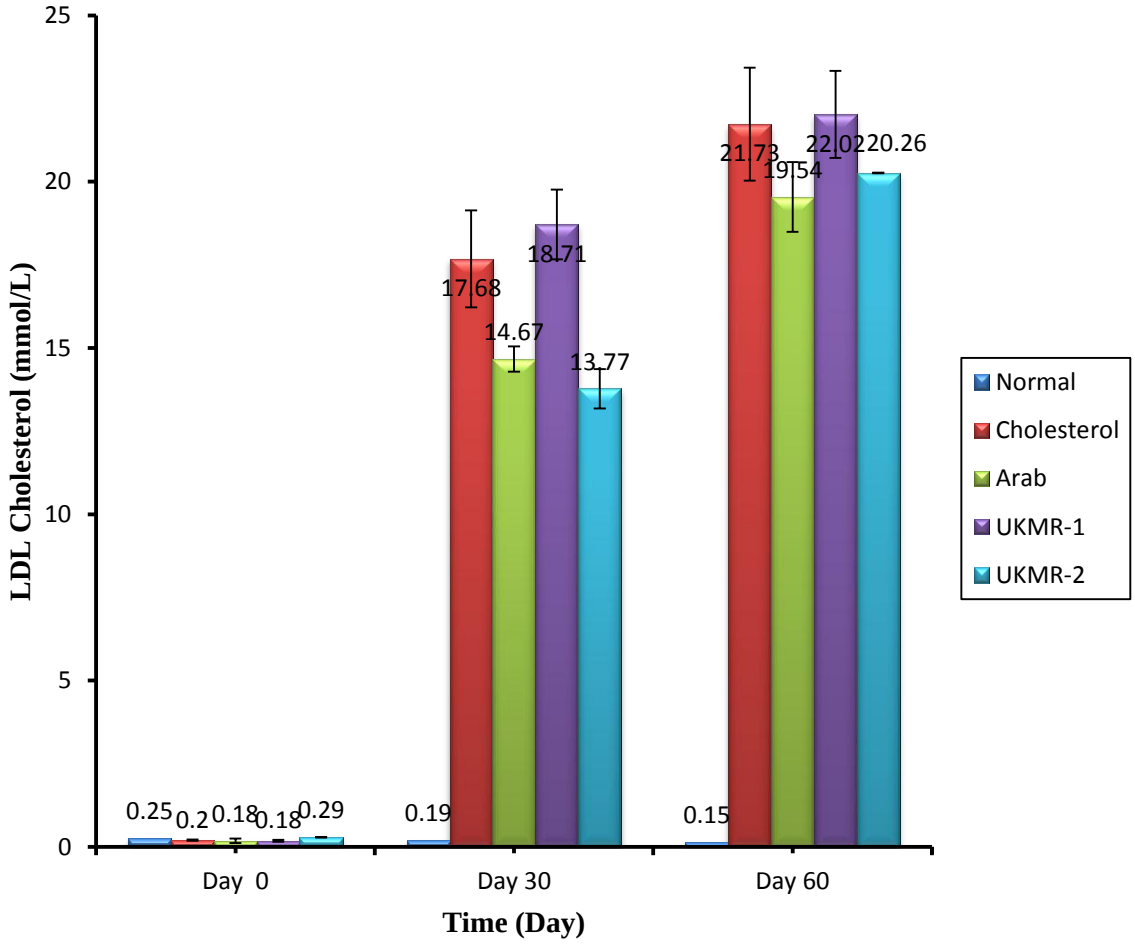
As shown in the table below, LDL cholesterol level in experimental rabbit blood serum also alleviate significantly through induction period. This alleviation is caused by the supplying the experimental animal with cholesterol diet. Without any treatment to the experimental animal, LDL level maintained at low level until day 60 of experiment in normal group.

Table 4.15: Level of LDL cholesterol in blood serum of experimental rabbits in various groups on 0, 30 and 60 days of treatment. Values are expressed as mean ± SE and n = 6.

Groups	LDL Cholesterol Level (mmol/L)		
	Day 0	**Day 30**	**Day 60**
Normal	0.25 ± 0.16	0.19 ± 0.00	0.15 ± 0.01
Cholesterol	0.20 ± 0.02	17.68 ± 1.46*	21.73 ± 1.70*
Arab	0.18 ± 0.07	14.67 ± 0.38*	19.54 ± 1.05*
UKMR-1	0.18 ± 0.03	18.71 ± 1.05*	22.02 ± 1.31*
UKMR-2	0.29 ± 0.01	13.77 ± 0.59*	20.26 ± 0.01*

*. The mean difference is significant compared to normal control at $p < 0.05$ level.

Figure 4.16: LDL cholesterol level in blood serum of experimental rabbits in various groups during 60 days of treatment. Values are expressed as mean ± SE and n= 6.

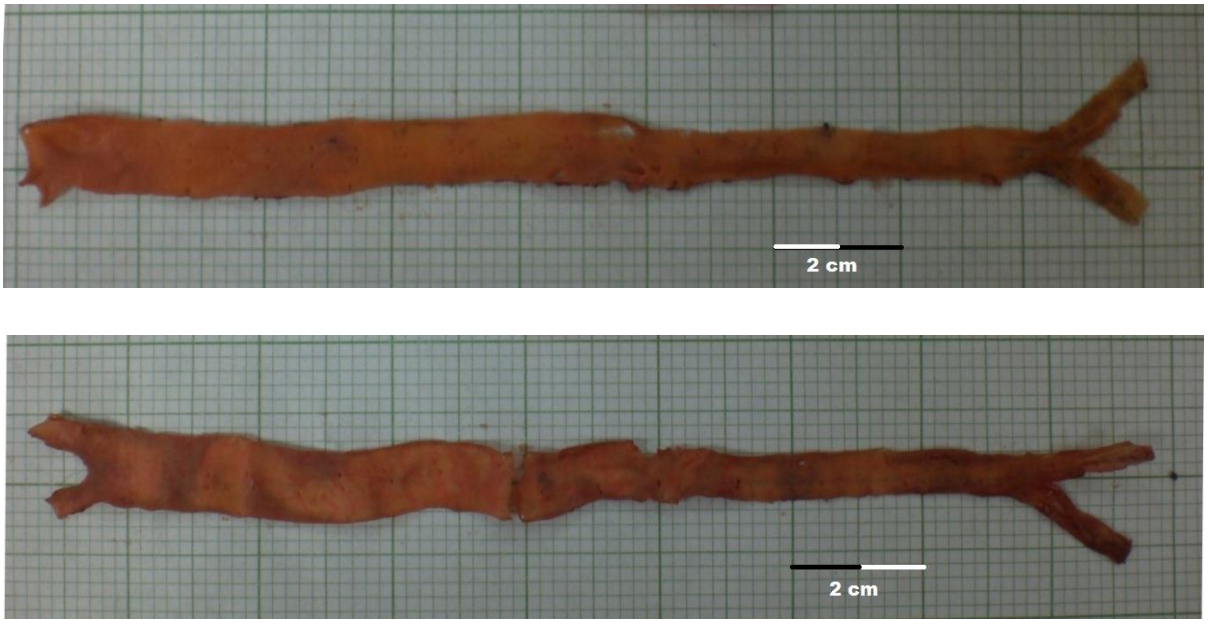


4.4.6 Atherosclerotic Plagues Analysis

4.4.6.1 Atherosclerotic Plagues Analysis for Normal Group

Representative photographs of the atherosclerotic changes on the endothelial surface of the aorta from the normal groups are shown in the picture below. Atherosclerotic plaques were not observed on the endothelial surface of the aorta in this group.

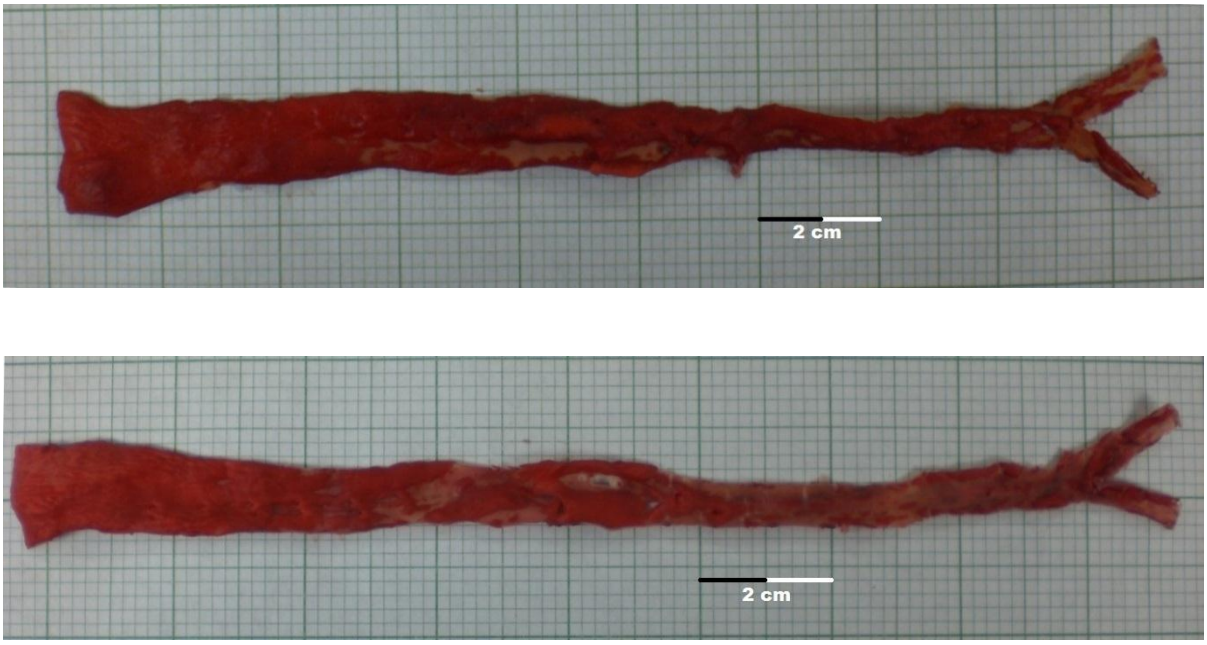
Figure 4.17: Intimal surface of the aortas from normal control group.



4.4.6.2 Atherosclerotic Plagues Analysis for Cholesterol Group

Picture below shows the atheromatous plaques on the endothelial surface of the aorta from the cholesterol control groups. Approximately 67.11% ± 0.37 of the endothelial surface of the aorta was covered with atheromatous plague after the animals were 60 day induced with hypercholesterolemic condition.

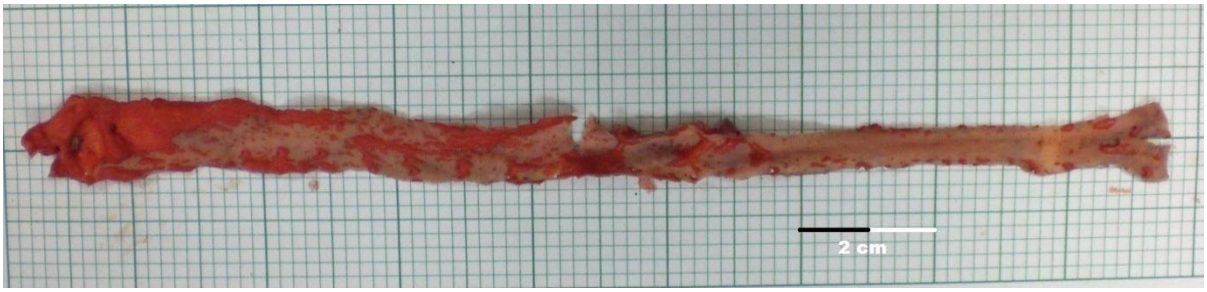
Figure 4.18: Intimal surface of the aortas from cholesterol control group of rabbits showing sudan IV stainable lipid deposit. Lipid deposits are stained brick red.

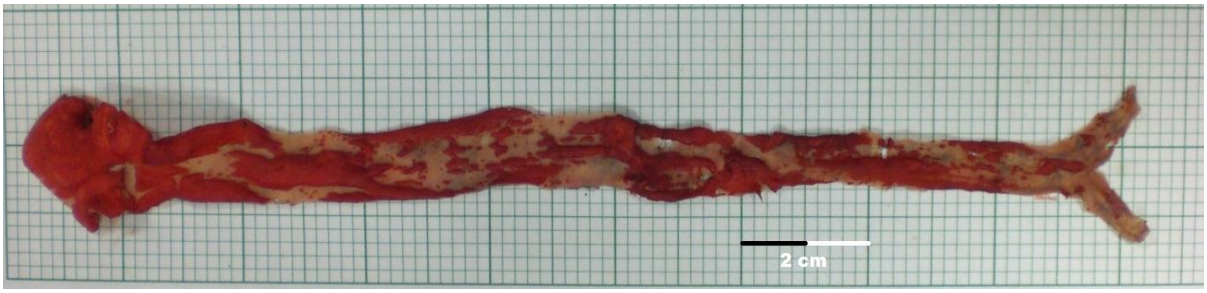


4.4.6.3 Atherosclerotic Plagues Analysis for Arab Variety Group

Picture below shows the atheromatous plaques on the endothelial surface of the aorta from the Arab groups. The aorta of the induced-hypocholesterolemic animals treated with Arab vareity of *H. sabdariffa* shows 62.68 % ± 3.44 of the aorta surface was covered with atheromatous plague.

Figure 4.19: Intimal surface of the aortas from Arab extract treated group of rabbits showing sudan IV stainable lipid deposit. Lipid deposits are stained brick red.

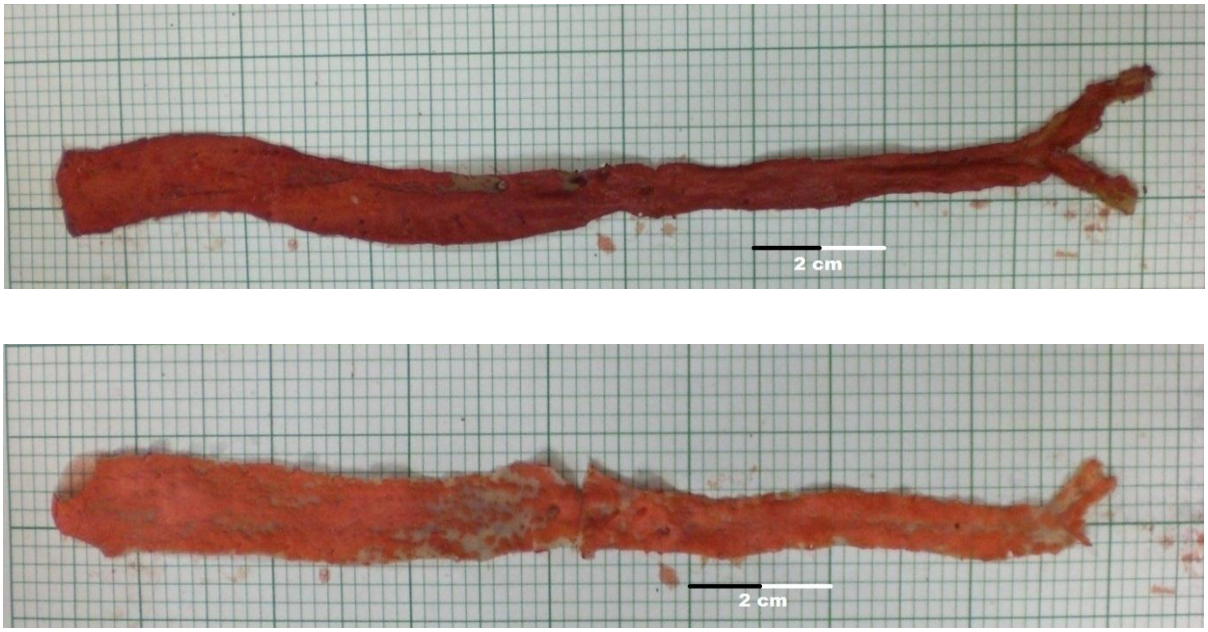




4.4.6.3 Atherosclerotic Plagues Analysis for UKMR-1 Variety Group

The endothelial surface of the aorta from UKMR-1 group animals shows the atheromatous plaques was developed on the intimal surface of the aortas. Nearly 68.48 % ± 0.19 of the Intimal surface of the aorta was covered with atheromatous.

Figure 4.20: Intimal surface of the aortas from UKMR-1 group of rabbits showing sudan IV stainable lipid deposit.



4.4.6.5 Atherosclerotic Plagues Analysis for UKMR-2 Variety Group

Approximately 64.46 % ± 2.19 of the endothelial surface of the aorta from UKMR-2 group animals was covered with the atheromatous plague. Picture below shows the atheromatous plague clearly can be seen develop on the Intimal surface of the aorta.

Figure 4.21: Intimal surface of the aortas from UKMR-2 group of rabbits showing sudan IV stainable lipid deposit.

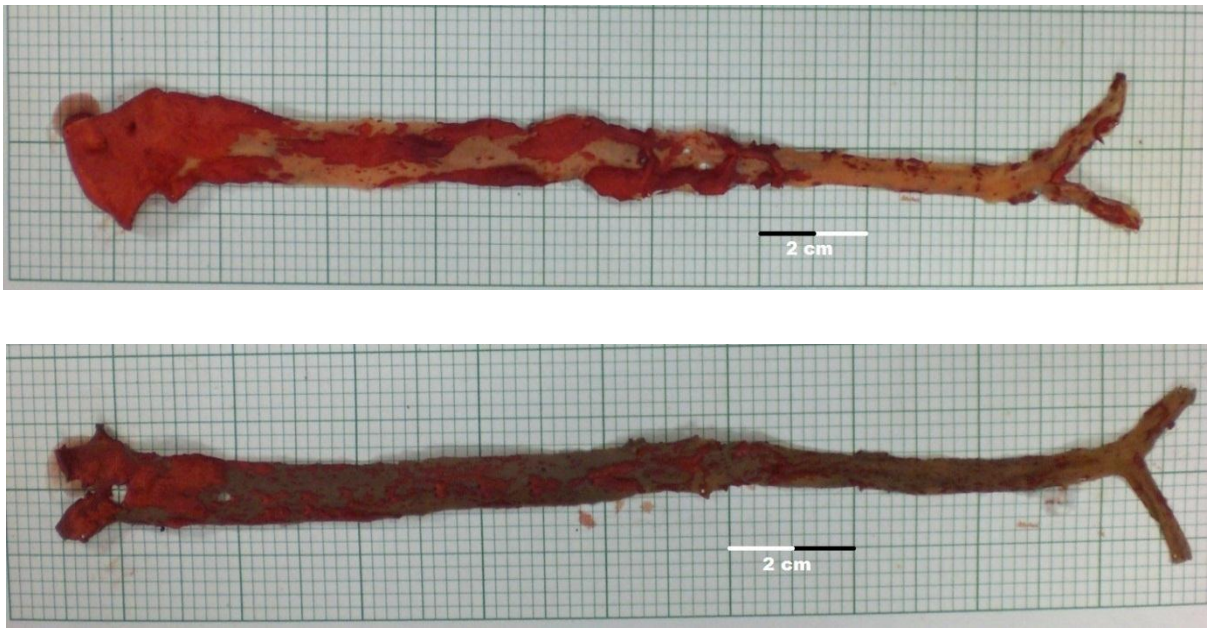


Table below shows the percentage of the intimal surface of the aortas covered with atheromatous plague from different groups of rabbits. The elavation of serum cholesterol level resulted from daily administration of cholesterol in animals diet induced the development of atherosclerosis. This was proved by the present of huge percentage of atheromatous plague in cholesterol control, Arab, UKMR-1 and UKMR-2 treated animals but not in normal control animals.

Table 4.16: Percentage of intimal surface covered by atherosclerosis in different groups of rabbits

Group	Percentage of Intimal Surface Covered by Atherosclerosis (%)
Normal Control	0.00 ± 0.00
Cholesterol Control	67.11 ± 0.37*
Arab	62.68 ± 3.44*
UKMR-1	68.48 ± 0.19 *
UKMR-2	64.46 ± 2.19*

*. The mean difference is significant compared to normal control at $p < 0.05$ level.

4.5 Phytochemical Analysis

4.5.1.1 Thin layer Chromatography (TLC) of Arab Variety

The results obtained from Thin Layer Chromatography (TLC) analysis for Arab variety showed in the table below. There were seven spots were produced on the TLC plate with color varying from red, blue, violet and yellow. Among the spots produced, four were determined derived from phenol group (R_f = 7.7, 57.7 and 71.8) and flavonoids group (R_f = 83.3).

Table 4.17: R_f values and visualization of chromatogram of Arab variety on TLC plate (Eluent: acetone: methanol = 3:7)

Label Compounds	R_f x100	Colour		Dragendorff	Vanillin	Anisaldehyde-sulphuric acid	Iodine Vapour	Comment
		Visible light	UV-light					
compound 1	7.7	Red	-	-	Red	-	-	Phenolic compound (probably anthocyanins)
compound 2	10.3	Blue	-	-	-	-	-	Unknown
compound 3	11.5	Blue	-	-	-	-	-	Unknown
compound 4	20.5	Blue	-	-	-	-	-	Unknown
compound 5	57.7	Blue	-	-	Red	-	-	Phenolic compound (probably anthocyanins)
compound 6	71.8	Violet	-	-	Red	-	-	Phenolic compound (probably anthocyanins)
compound 7	83.3	Yellow	-	-	-	Pink	-	Flavonoids

4.5.1.2 Thin layer Chromatography (TLC) of UKMR-1 Variety

As shown in Table 4.18, there were seven spots were detected on the TLC plate with R_f values varying from 5.06 to 83.5. Five out of seven (R_f = 5.06, 7.6, 16.5, 65.8 and 81.0) spots were react to vanillin and revealed the present of phenol compounds. A yellow spot with R_f value = 83.5 reacted to Anisaldehyde-sulphuric acid and produced pink color was determined from flavonoids group.

Table 4.18: R_f values and visualization of chromatogram of UKMR-1 variety on TLC plate (Eluent: acetone: methanol = 3:7)

Label Compounds	R_f x100	Colour		Dragendorff	Vanillin	Anisaldehyde-sulphuric acid	Iodine Vapour	Comment
		Visible light	UV-light					
compound 1	5.06	Red	-	-	Red	-	-	Phenolic compound (probably anthocyanins)
compound 2	7.6	Red	-	-	Red	-	-	Phenolic compound (probably anthocyanins)
compound 3	16.5	Red	-	-	Red	-	-	Phenolic compound (probably anthocyanins)
compound 4	58.2	Blue	-	-	-	-	-	Phenolic compound (probably anthocyanins)
compound 5	65.8	Violet	-	-	Red	-	-	Phenolic compound (probably anthocyanins)
compound 6	81.0	Violet	-	-	Red	-	-	Phenolic compound (probably anthocyanins)
compound 7	83.5	Yellow	-	-	-	Pink	-	Flavonoids

4.5.1.3 Thin Layer Chromatography (TLC) of UKMR-2 Variety

Thin Layer Chromatography visualization of UKMR-2 variety is representing in the table below. UKMR-2 variety extract migrate on TLC plate to produced eight spots with red, violet, blue, orange and yellow color and R_f value varying from 3.8 to 68.4. Six spots with R_f value from 3.8 to 41.8 were gave positive result for phenol compound when tested with vanillin solution. While a yellow and orange spots with R_f value 68.4 and 55.7 were determined from flavonoids group.

Table 4.19: R_f values and visualization of chromatogram of UKMR-2 variety on TLC plate (Eluent: acetone: methanol = 3:7)

Label Compounds	R_f x100	Colour		Dragendorff	Vanillin	Anisaldehyde-sulphuric acid	Iodine Vapour	Comment
		Visible light	UV-light					
compound 1	3.8	Red	-	-	Red	-	-	Phenolic compound (probably anthocyanins)
compound 2	7.6	Red	-	-	Red	-	-	Phenolic compound (probably anthocyanins)
compound 3	8.9	Red	-	-	Red	-	-	Phenolic compound (probably anthocyanins)
compound 4	12.7	Violet	-	-	Red	-	-	Phenolic compound (probably anthocyanins)
compound 5	15.2	Violet	-	-	Red	-	-	Phenolic compound (probably anthocyanins)
compound 6	41.8	Blue	-	-	-	-	-	Unknown
compound 7	55.7	Orange	-	-	-	Pink	-	Flavonoids
compound 8	68.4	Yellow	-	-	-	Pink	-	Flavonoids

4.5.2 LC-MS/MS Analysis

4.5.2.1 LC-MS/MS Analysis of Arab Variety

Initial phytochemical analysis using LCMS/MS of *H. sabdariffa* from Arab variety revealed the present of several chemical compounds mostly from flavonoids group. Six different compounds were detected from aqueous extract of Arab variety which are gossypetin-3-O-glu-7-O-xylo, herbacetin-8-O-xylo-3-O-glu, delphinidin, delphinidin-3-sambubioside**,** cyanidin 3-sambubioside, and kaempferol-3-O-rutinoside.

Figure 4.22: Full chromatograms of Arab variety in the positive mode

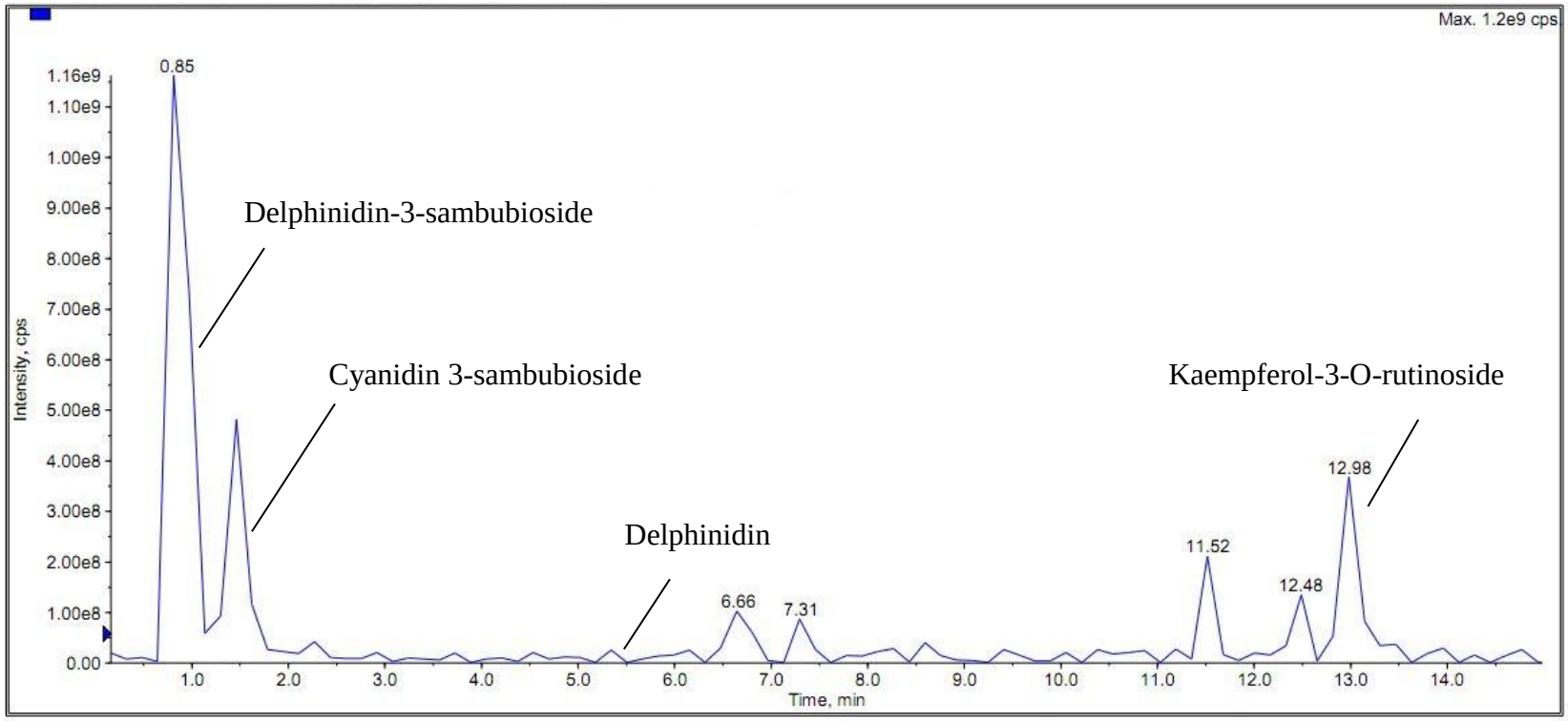


Figure 4.23: Fragmentation of Delphinidin: (A) Full chromatograms of Arab variety in the positive mode. (B) Fragmentation of peak at retention time 5.666 min results in a MS spectrum with m/z 303.0 dominating.

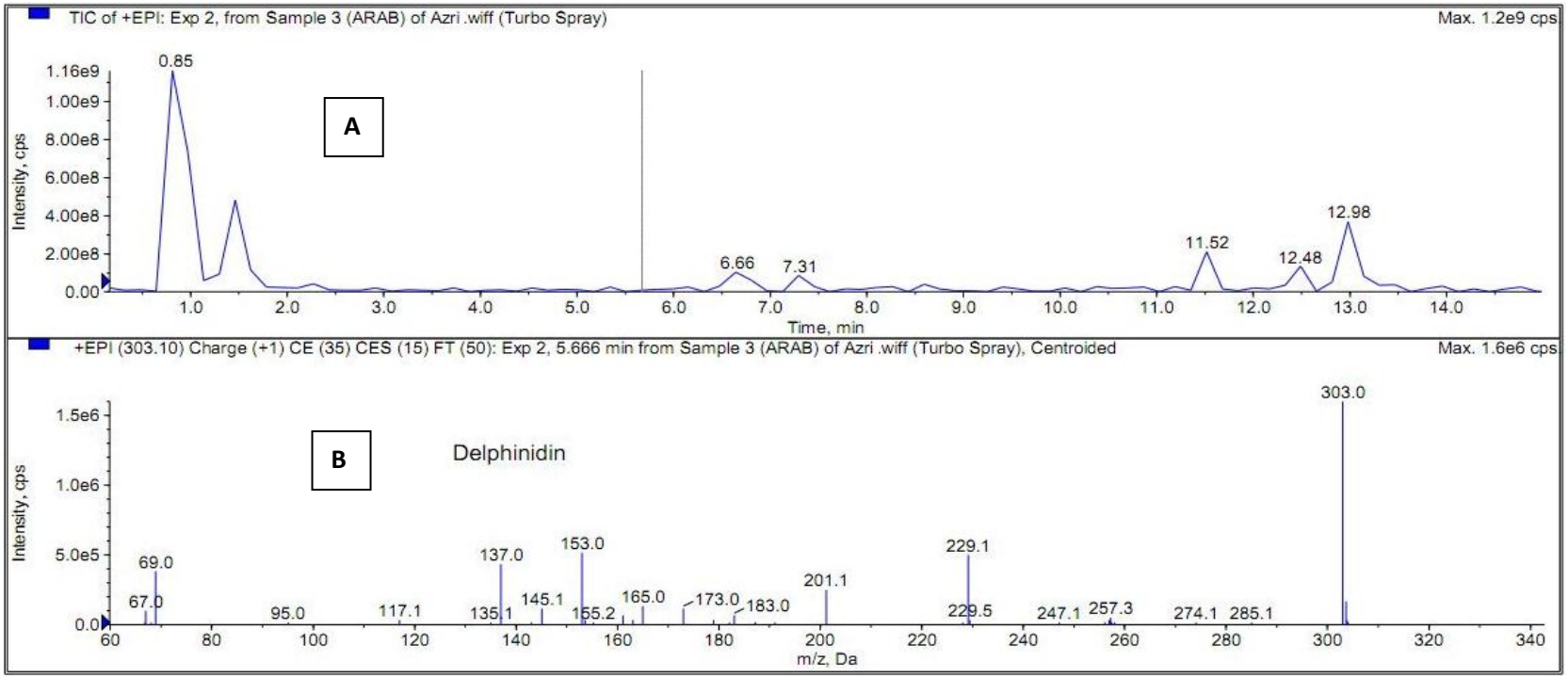


Figure above shows full scan (positive mode) of Arab variety with delphinidin MS/MS fragmentation. The retention time for delphinidin was 5.666 minute. The mass spectrum fragmentation of delphinidin was displayed the following major peak at m/z: 303.0, 229.1, 201.1, 153.0, 137.0 and 69.0. The count per second (cps) of the tallest peak was 1.6e6 cps.

The mass spectrum fragmentation in Figure 4.23 was appearing as large peak at m/z 303.1. The product ion spectrum of the ion (m/z 303.1) showed fragment ions at m/z 285.1 $[C_{15}H_9O_6]^+$ (loss of water), m/z 257.3 $[C_{14}H_9O_5]^+$ (loss of CO) and m/z 229.1 $[C_{13}H_9O4]^+$ (loss of another CO). The molecular ion then shows fragmentation at m/z 201.1 $[C_{13}H_9O_4]^+$ (lost of CO), m/z 153 $[C_{12}H_4O_3]^+$ (lost of C and two water molecules), m/z 137 $[C_{12}H_4O_2]^+$ (lost of O). Thus, by looking at the fragmentation pattern, the LCMS/MS chromatogram shows a peak at retention time 5.666 minute was identified as delphinidin based on ACD/Labs advanced chemometrics mass fragmentations predictive software. The chemical formula for delphinidin is $C1_5H_{11}O_7$. The molecular weight is 303.24 g/mol. delphinidin, which belongs to the anthocyanidins is highly water-soluble and is easily degraded by hydrolysis.

Figure 4.24: Molecular structure of delphinidine

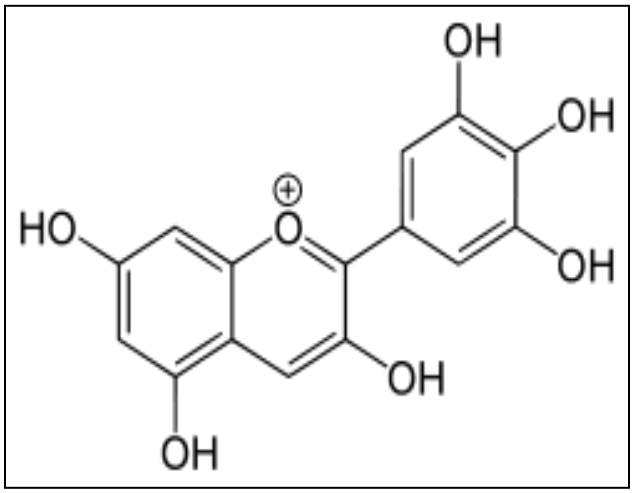


Figure 4.25: Fragmentation of delphinidin-3-sambubioside: (A) Full chromatograms of Arab variety in the positive mode. (B) Fragmentation of peak at retention time 0.809 min results in a MS spectrum with m/z 597.0 dominating.

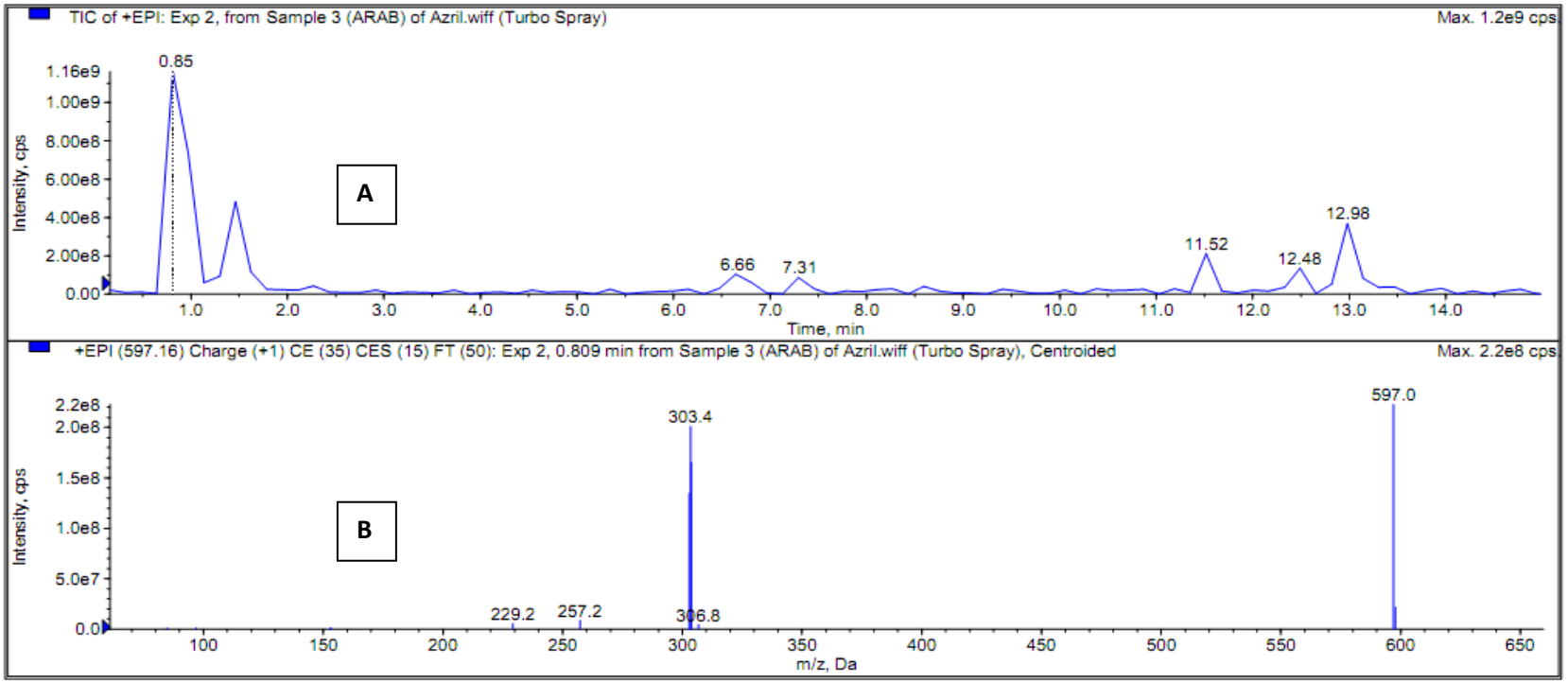


Figure above shows full scan (positive mode) of Arab variety with delphinidin-3-sambubioside MS/MS fragmentation. The retention time for Delphinidin-3-sambubioside was 0.809 minute. The mass spectrum fragmentation of delphinidin-3-sambubioside was displayed the following major peak at m/z: The count per second (cps) of the tallest peak was cps.

The mass spectrum fragmentation in Figure 4.23 was appearing as large peak at m/z 597.0. The product ion spectrum of the ion (m/z 597.0) [$C_{26}H_{29}O_{16}$] shows fragment at m/z 303.4 [$C_{15}H_{11}O_7$] $^+$ indicate the molecular ion has lost of $C_{11}H_{18}O_9$ molecule. The ion spectrum also showed fragment ions at m/z 257.2 [$C_{14}H_9O_5$] $^+$ indicate the molecular ion lost of CO and water H_2O molecule. The ion was then produce fragment at m/z 229.2 [$C_{13}H_9O_4$] $^+$ due to lost of CO molecule. The molecular ion fragmentation of LCMS/MS chromatogram shows a peak at retention time 0.809 minute was identified as delphinidin-3-sambubioside based on ACD/Labs advanced chemometrics mass fragmentations predictive software. The chemical formula for delphinidin-3-sambubioside is $C_{26}H_{29}O_{16}$. The molecular weight for delphinidin-3-sambubioside is 597.5 g/mol.

Figure 4.26: Chemical structure of delphinidin-3-sambubioside

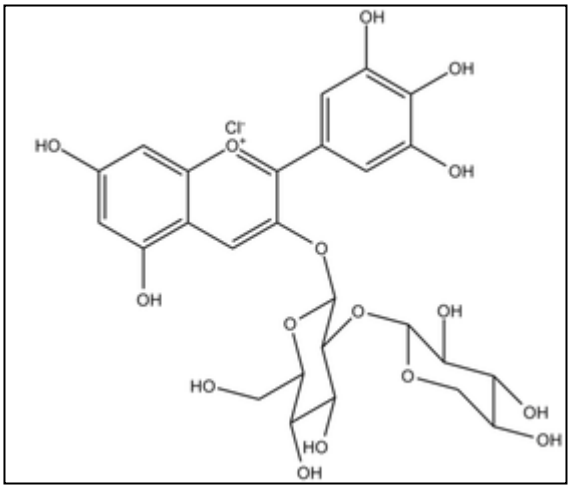


Figure 4.27: Fragmentation of cyanidin-3-glucoside: (A) Full chromatograms of Arab variety in the positive mode. (B) Fragmentation of peak at retention time 1.460 min results in a MS spectrum with m/z 287.0 dominating.

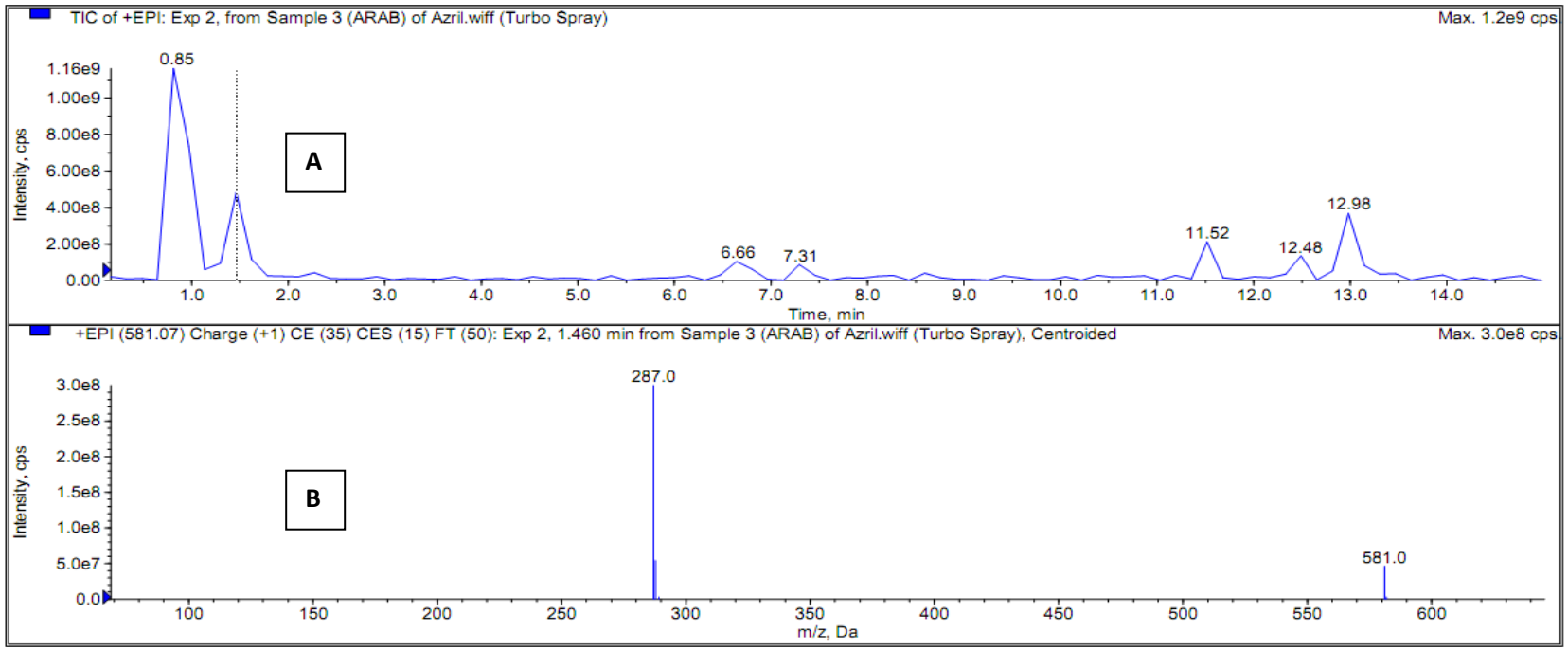


Figure above shows full scan (positive mode) of Arab variety with cyanidin-3- glucoside MS/MS fragmentation. The retention time for cyanidin-3-sambubioside was 1.460 minute. The mass spectrum fragmentation of cyanidin-3- glucoside was displayed the following major peak at m/z: 581.0 and 287.0. The count per second (cps) of the tallest peak was 3.0e8 cps.

The chromatogram peak at retention time 1.460 in Figure 4.27 was shows largest fragmentation at m/z 581.1. The product ion spectrum of the ion (m/z 581.1) showed fragment at m/z 287.0 $[C_{15}H_7O_4Cl]^+$ due to lost of $C_{11}H_{22}O_{11}$ molecule. The LCMS/MS chromatogram peak at retention time 1.460 minute was identified as cyanidin-3-sambubioside based on ACD/Labs advanced chemometrics mass fragmentations predictive software. The chemical formula for cyanidin 3-glucoside is $C_{26}H_{29}O_{15}Cl$ and its molecular weight is 581.15 g/mol. cyanidin 3-glucoside is belongs to the anthocyanins, which is a class of natural chemicals belonging to the flavonoids.

Figure 4.28: Chemical structure of cyanidin 3-glucoside

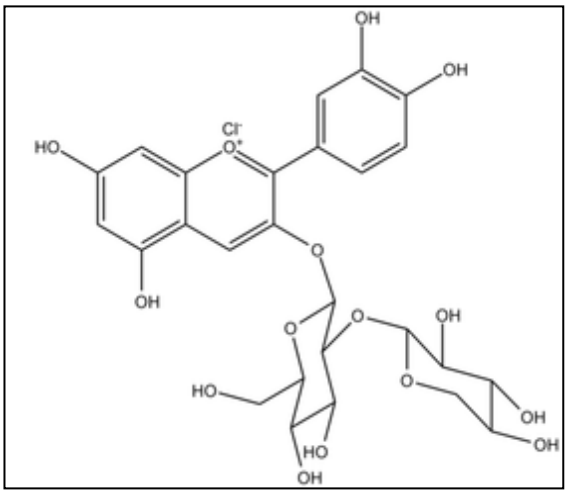


Figure 4.29: Fragmentation of kaempferol-3-O-rutinoside: (A) Full chromatograms of Arab variety in the positive mode. (B) Fragmentation of peak at retention time 12.981 min results in a MS spectrum with m/z 593.2 dominating.

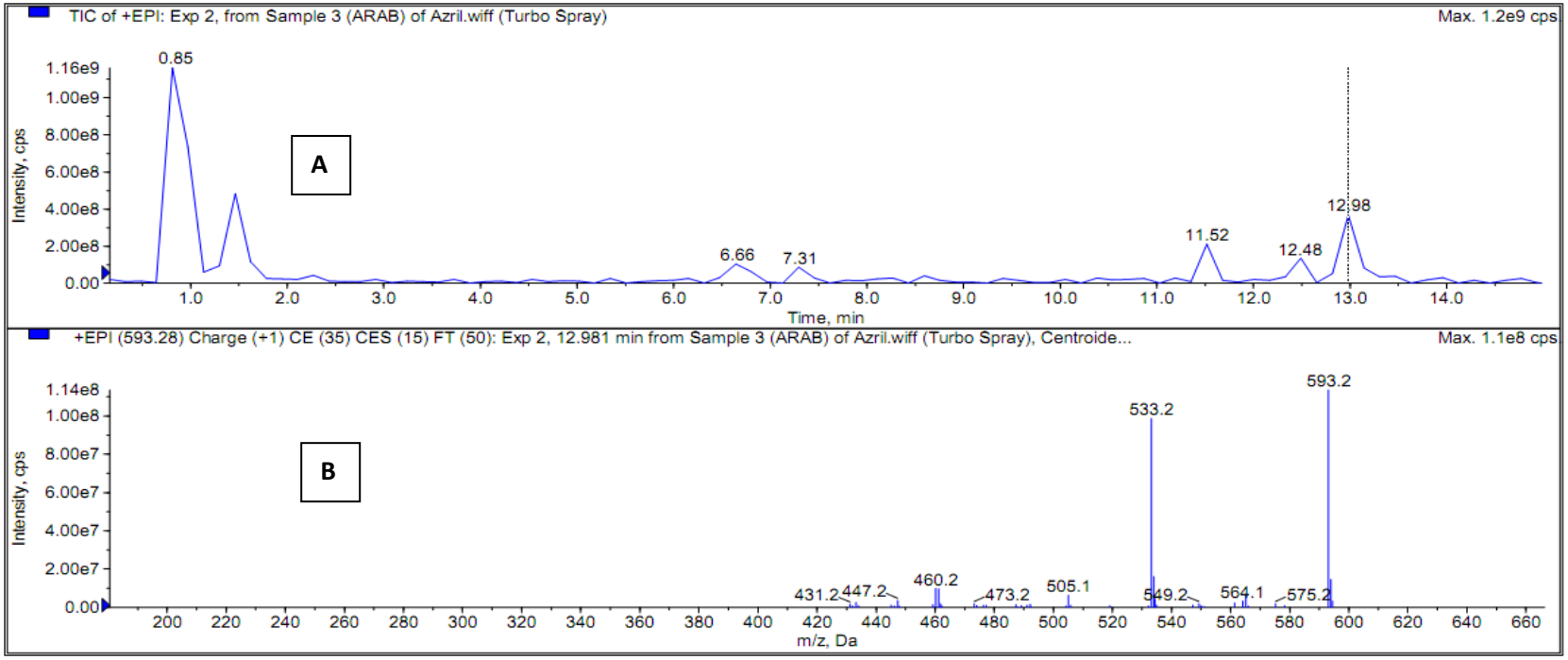


Figure above shows full scan (positive mode) of Arab variety with kaempferol-3-O-rutinoside MS/MS fragmentation. The retention time for kaempferol-3-O-rutinoside was 12.981 minute. The mass spectrum fragmentation of kaempferol-3-O-rutinoside was displayed the following major peak at m/z: 593.2, 533.2, 505.1 and 460.2. The count per second (cps) of the tallest peak was 1.1e8 cps.

Figure 4.29 shows LCMS/MS chromatogram for kaempferol-3-O-rutinoside. The chemical formula for kaempferol-3-O-rutinoside is $C_{27}H_{30}O_{15}$ and its molecular weight is 594.52 g/mol. The mass spectrum fragmentation of kaempferol-3-O-rutinoside was appearing as large peak at m/z 593.2. The product ion spectrum of the kaempferol-3-O-rutinoside ion (m/z 593.2) showed fragment ions at m/z 575.2 $[C_{27}H_{28}O_{15}]^+$ due to lost of water molecule, m/z 533.2 $[C_{25}H_{27}O_{14}]^+$ (lost of C_2OH), m/z 505.1 $[C_{24}H_{27}O_{13}]^+$ (lost of CO) and m/z 460.2 $[C_{23}H_{26}O_{11}]^+$ due lost of COOH molecule.

Figure 4.30: Chemical structure of kaempferol-3-O-rutinoside

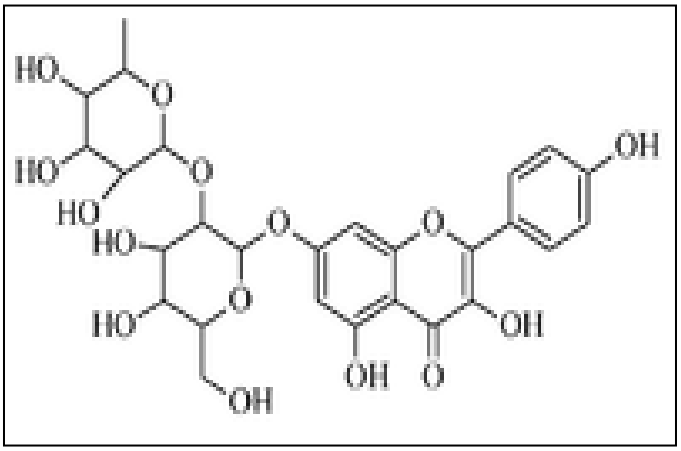


Figure 4.31: Full chromatograms of Arab variety in the negative mode.

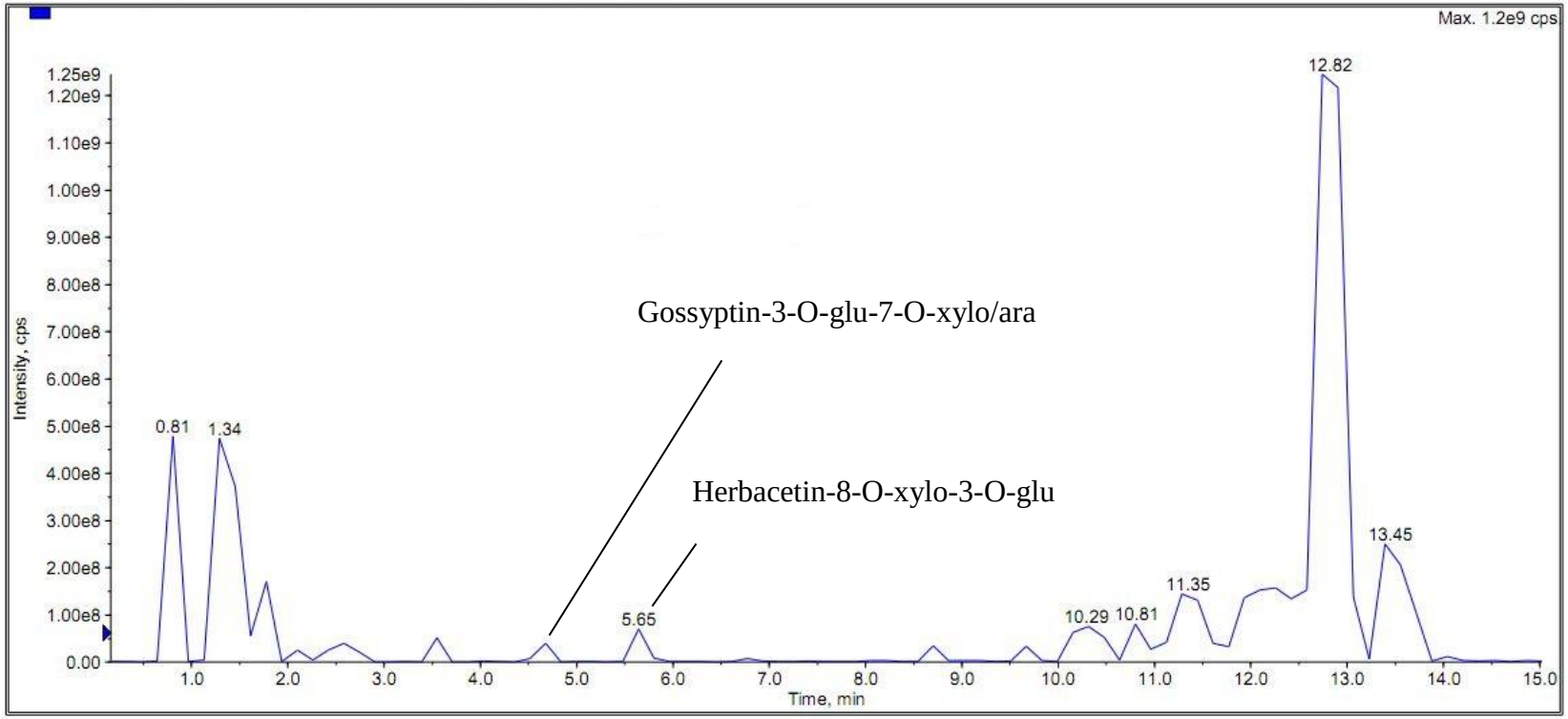


Figure 4.32: Fragmentation of gossyptin-3-O-glu-7-O-xylo/ara: (A) Full chromatograms of Arab variety in the negative mode. (B) Fragmentation of peak at retention time 4.675 min results in a MS spectrum with m/z 316.0 dominating.

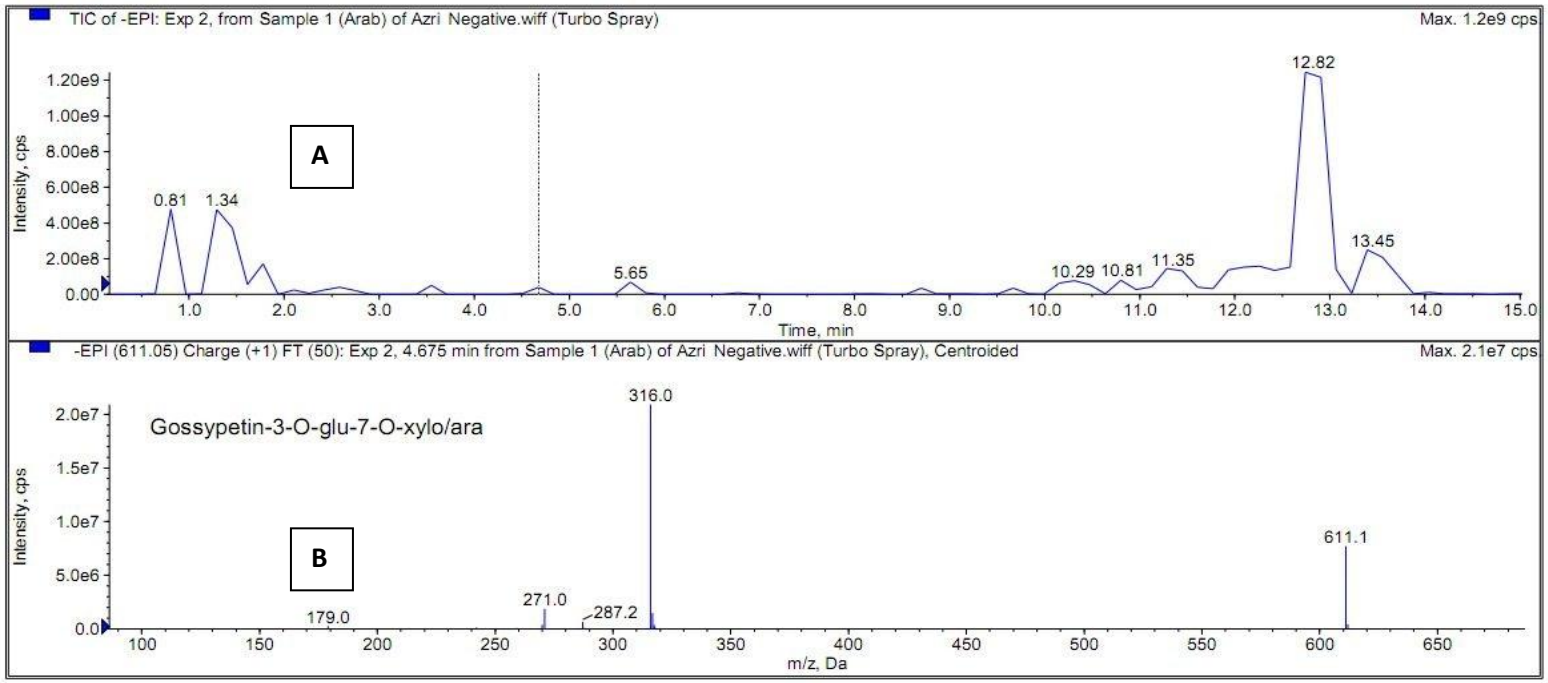
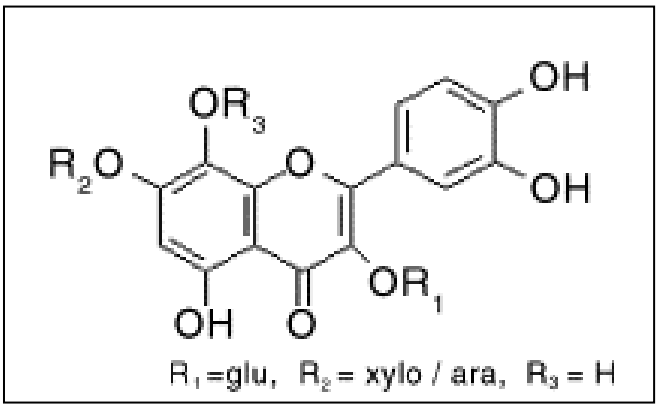


Figure above shows full scan (negative mode) of Arab variety with gossyptin-3-O-glu-7-O-xylo/ara MS/MS fragmentation. The retention time for gossyptin-3-O-glu-7-O-xylo/ara was 4.675 minute. The mass spectrum fragmentation of gossyptin-3-O-glu-7-O-xylo/ara was displayed the following major peak at m/z: 611.1, 316.0, and 271.0. The count per second (cps) of the tallest peak was 2.1e7 cps.

The LCMS/MS chromatogram shows a peak at retention time 4.675 minute was identified as gossyptin-3-O-glu-7-O-xylo/ara based on ACD/Labs advanced chemometrics mass fragmentations predictive software. The chemical formula for gossyptin-3-O-glu-7-O-xylo/ara is $C_{27}H_{32}O_{16}$. The molecular weight for gossyptin-3-O-glu-7-O-xylo/ara is 612.15 g/mol.

Figure 4.33: Chemical structure of gossyptin-3-O-glu-7-O-xylo/ara



The molecular ion of gossyptin-3-O-glu-7-O-xylo/ara appears as large peak at m/z 611.1. The ion spectrum showed fragment ions at m/z 316.0 $[C_{15}H_3O_7]^+$ indicate the molecular ion of gossyptin-3-O-glu-7-O-xylo/ara has lost of $C_{12}H_{29}O_9$ molecule. The ion spectrum also showed fragment ions at m/z 287.2 $[C_{14}H_6O_5]^+$ indicate the molecular ion lost of CO molecule. The ion was then produce fragment at m/z 271.0 $[C_{14}H_6O_4]^+$ due to lost of O.

Figure 4.34: Fragmentation of herbacetin-8-O-xylo-3-O-glu: (A) Full chromatograms of Arab variety in the negative mode. (B) Fragmentation of peak at retention time 5.642 min results in a MS spectrum with m/z 300.0 dominating.

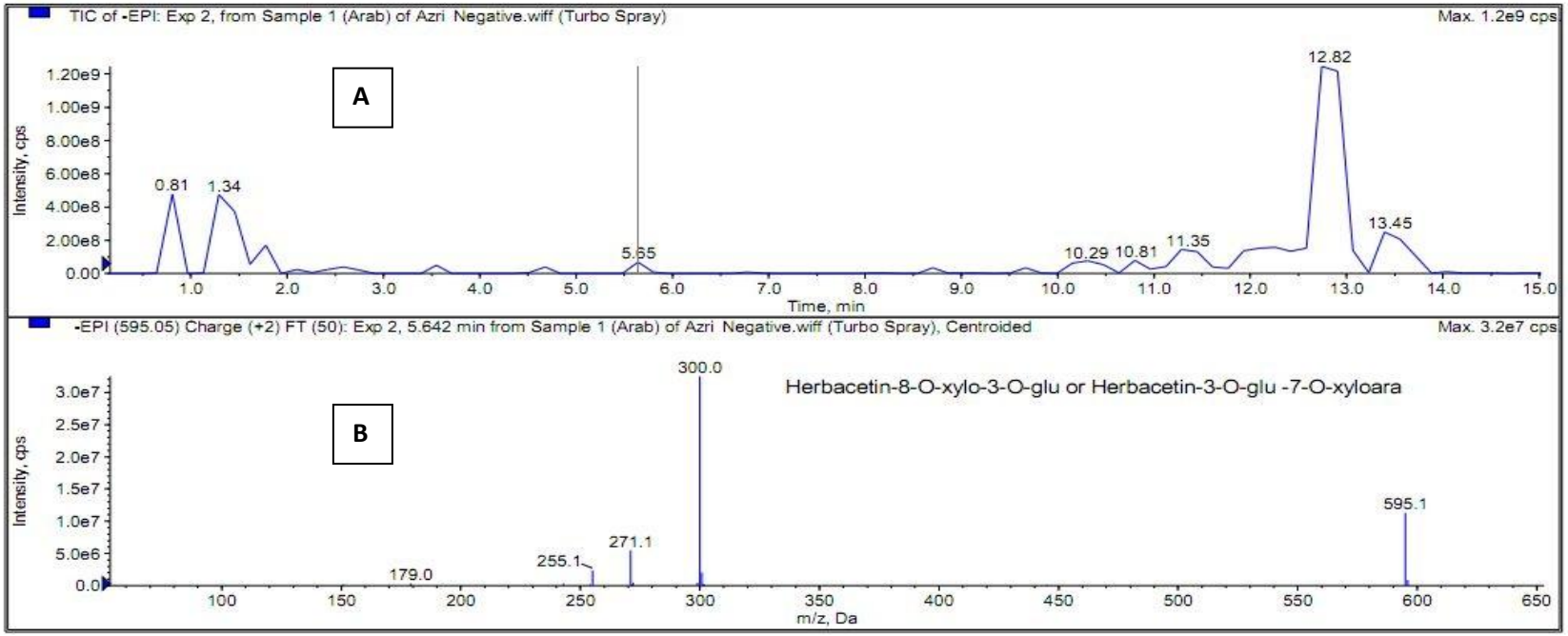
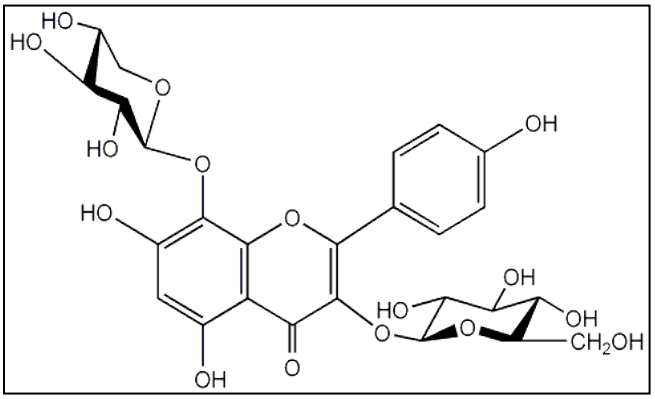


Figure above shows full scan (negative mode) of Arab variety with herbacetin-8-O-xylo-3-O-glu MS/MS fragmentation. The retention time for herbacetin-8-O-xylo-3-O-glu was 5.642 minute. The mass spectrum fragmentation of herbacetin-8-O-xylo-3-O-glu was displayed the following major peak at m/z: 595.1, 300.0, 271.1, 255.1 and 179.0. The count per second (cps) of the.tallest peak was 3.2e7 cps.

The LCMS/MS chromatogram shows a peak at retention time 5.642 minute was identified as herbacetin-8-O-xylo-3-O-glu based on ACD/Labs advanced chemometrics mass fragmentations predictive software. The chemical formula for herbacetin-8-O-xylo-3-O-glu is $C_{26}H_{28}O_{16}$. The molecular weight is 596.13 g/mol. Herbacetin-8-O-xylo-3-O-glu also known as Rhodalidin.

Figure 4.35: Molecular structure of herbacetin-8-O-xylo-3-O-glu



The molecular ion of herbacetin-8-O-xylo-3-O-glu appears as large peak at m/z 595.1. The ion spectrum shows fragment ions at m/z 300.0 $[C_{15}H_8O_7]^+$ indicate the molecular ion of herbacetin-8-O-xylo-3-O-glu has lost of $C_9H_{10}O_9$ molecule. The ion spectrum also showed fragment ions at m/z 271.1 $[C_{14}H_8O_6]^+$ indicate the molecular ion lost of CO molecule. The ion was then produce fragment at m/z 255.1 $[C_{14}H_6O_4]^+$ due to lost of O.

4.9.2 LC-MS/MS Analysis of UKMR-1 Variety

LCMS/MS phytochemical analysis of *H. sabdariffa* from UKMR-1 variety detected the present of several chemical compounds mostly from flavonoids group. Six different compounds were detected from aqueous extract of Arab variety which are delphinidin-3-sambubioside**,** hibiscetine, delphinidin, gossypetin, herbacetin-8-O-xylo-3-O-glu, and quercetin rutinoside.

Figure 4.36: Full chromatograms of UKMR-1 variety in the positive mode.

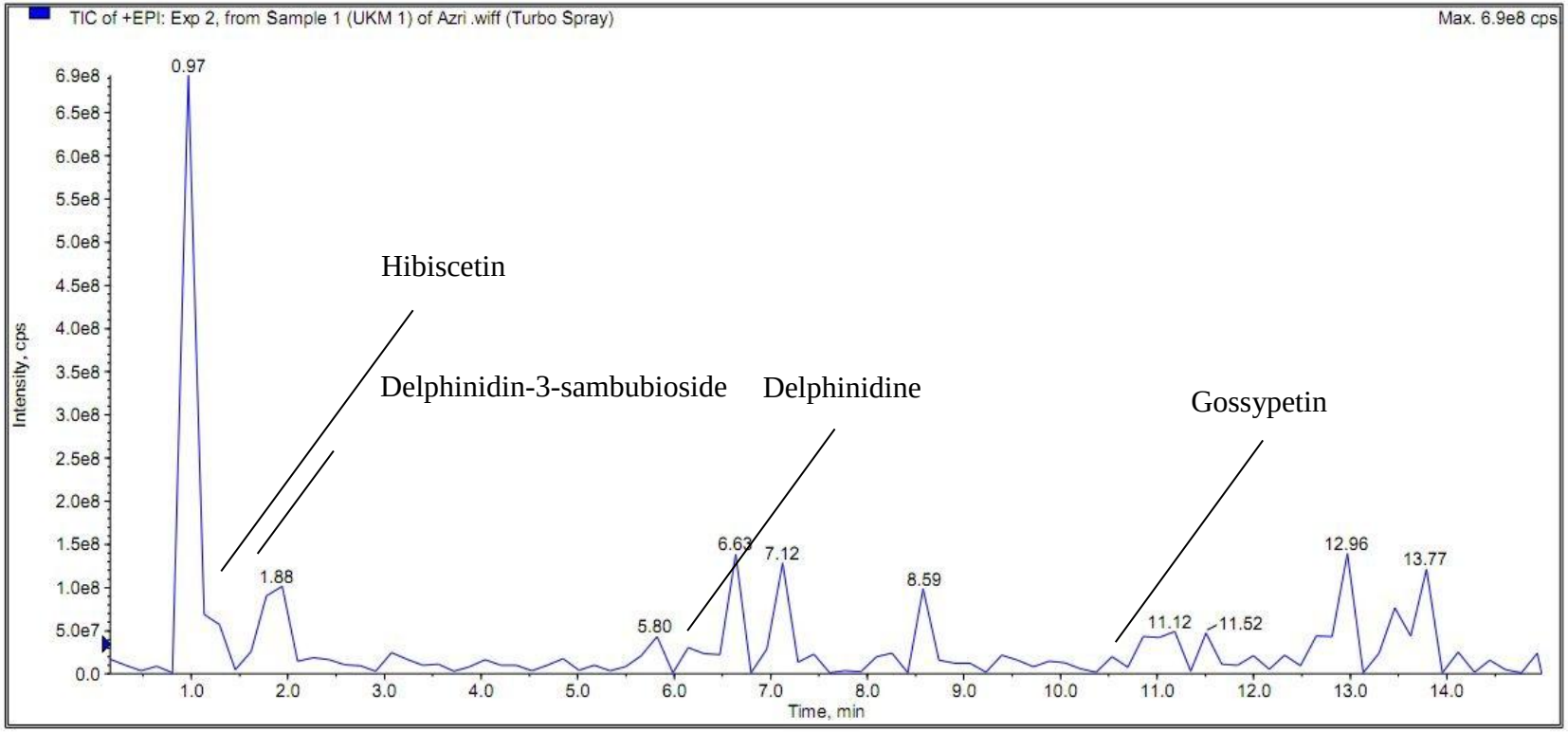


Figure 4.37: Fragmentation of delphinidin-3-sambubioside: (A) Full chromatograms of UKMR-1 variety in the positive mode. (B) Fragmentation of peak at retention time 1.779 min results in a MS spectrum with m/z 303.0 dominating

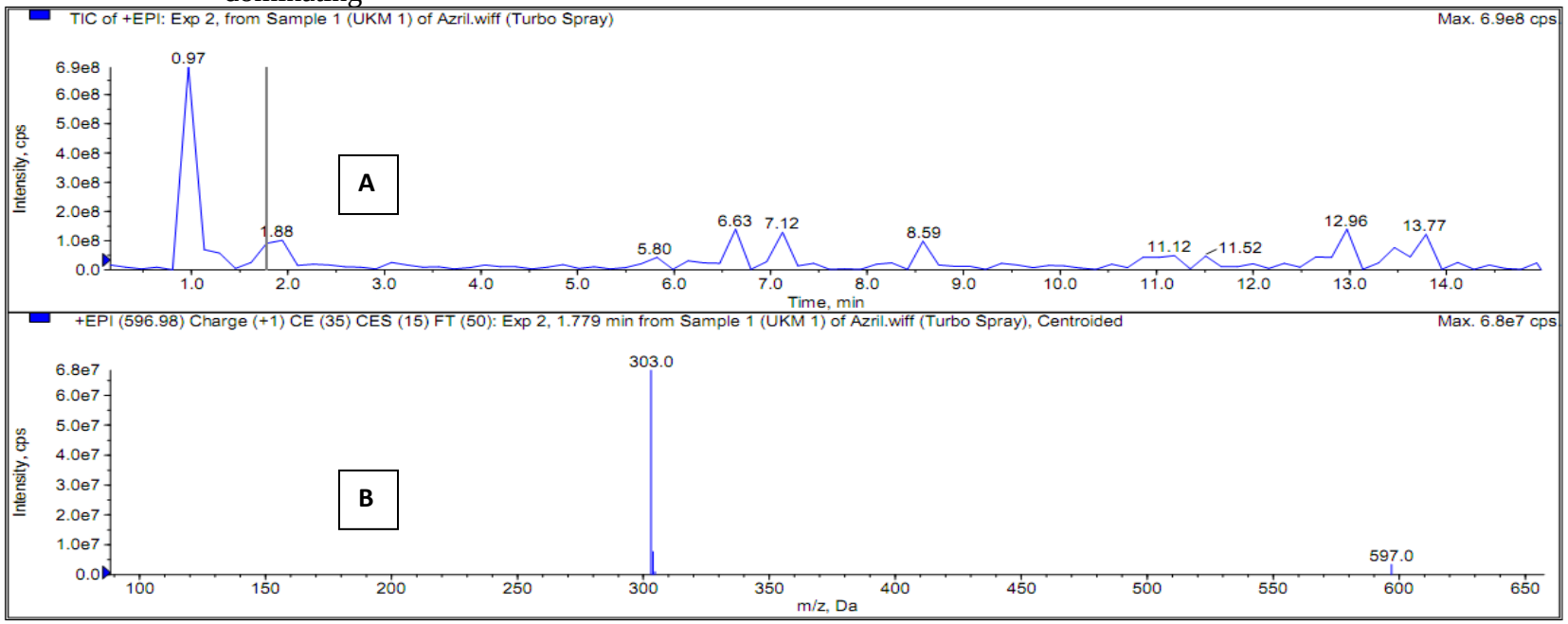
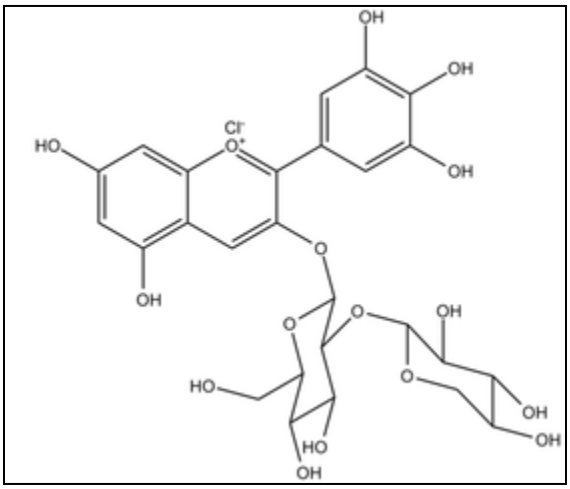


Figure above shows full scan (positive mode) of UKMR-1 variety with Delphinidin-3-sambubioside MS/MS fragmentation. The retention time for delphinidin-3-sambubioside was 1.779 minute. The mass spectrum fragmentation of delphinidin-3-sambubioside was displayed the following major peak at m/z: 597.0 and 303.0. The count per second (cps) of the tallest peak was 6.8e7 cps.

The LCMS/MS chromatogram shows a peak at retention time 0.809 minute was identified as delphinidin-3-sambubioside base on ACD/Labs advanced chemometrics mass fragmentations predictive software. The chemical formula for Delphinidin-3-sambubioside is $C_{26}H_{29}O_{16}$. The molecular weight for delphinidin-3-sambubioside is 597.5 g/mol.

Figure 4.38: Molecular structure of delphinidin-3-sambubioside



The molecular ion of delphinidin-3-sambubioside appears as large peak at m/z 597.0. The ion spectrum showed fragment ions at m/z 303.4 $[C_{15}H_{11}O_7]^+$ indicate the molecular ion of delphinidin-3-sambubioside has lost of $C_{11}H_{18}O_9$ molecule. The ion spectrum also showed fragment ions at m/z 257.2 $[C_{14}H_9O_5]^+$ indicate the molecular ion lost of CO and water H_2O molecule. The ion was then produce fragment at m/z 229.2 $[C_{13}H_9O_4]^+$ due to lost of CO molecule.

Figure 4.39: Fragmentation of hibiscetin: (A) Full chromatograms of UKMR-1 variety in the positive mode. (B) Fragmentation of peak at retention time 1.46 min results in a MS spectrum with m/z 377.0 dominating.

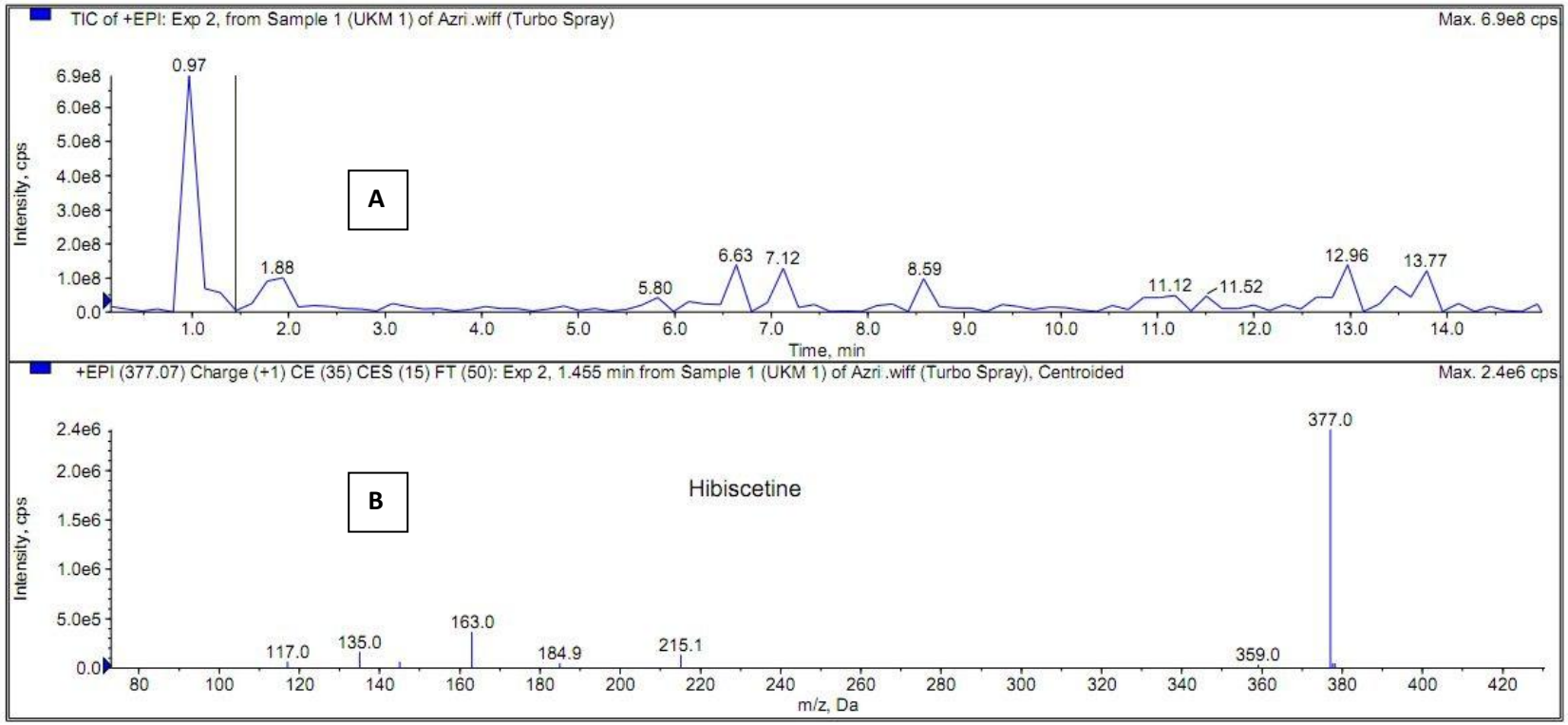


Figure above shows full scan (positive mode) of UKMR-1 variety with hibiscetin MS/MS fragmentation. The retention time for hibiscetin was 1.455 minute. The mass spectrum fragmentation of hibiscetin was displayed the following major peak at m/z: 377.0, 215.1, 163.0 and 135.0. The count per second (cps) of the tallest peak was 2.4e6 cps.

The mass spectrum fragmentation in Figure above was appearing as large peak at m/z 377.0. The product ion spectrum of the ion (m/z 377.0) showed fragment ions at m/z 359.0 $[C_{18}H_{14}O_8]^+$ (loss of water), m/z 215.1 $[C_{12}H_{14}O_8]^+$ (loss of two C_6). Thus, by looking at the fragmentation pattern, the LCMS/MS chromatogram shows a peak at retention time 1.455 minute was identified as hibiscetin based on ACD/Labs advanced chemometrics mass fragmentations predictive software. The chemical formula for hibiscetin is $C_{18}H_{16}O_9$. The molecular weight is 376 g/mol.

Figure 4.40: Chemical structure for hibiscetin

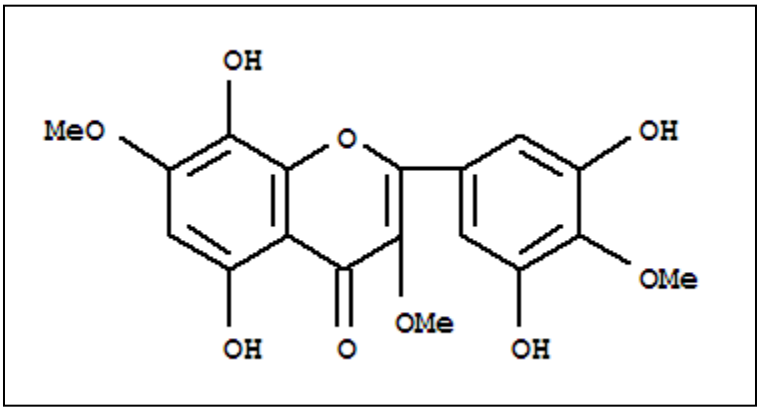


Figure 4.41: Fragmentation of delphinidin: (A) Full chromatograms of UKMR-1 variety in the positive mode. (B) Fragmentation of peak at retention time 6.15 min results in a MS spectrum with m/z 303.1 dominating

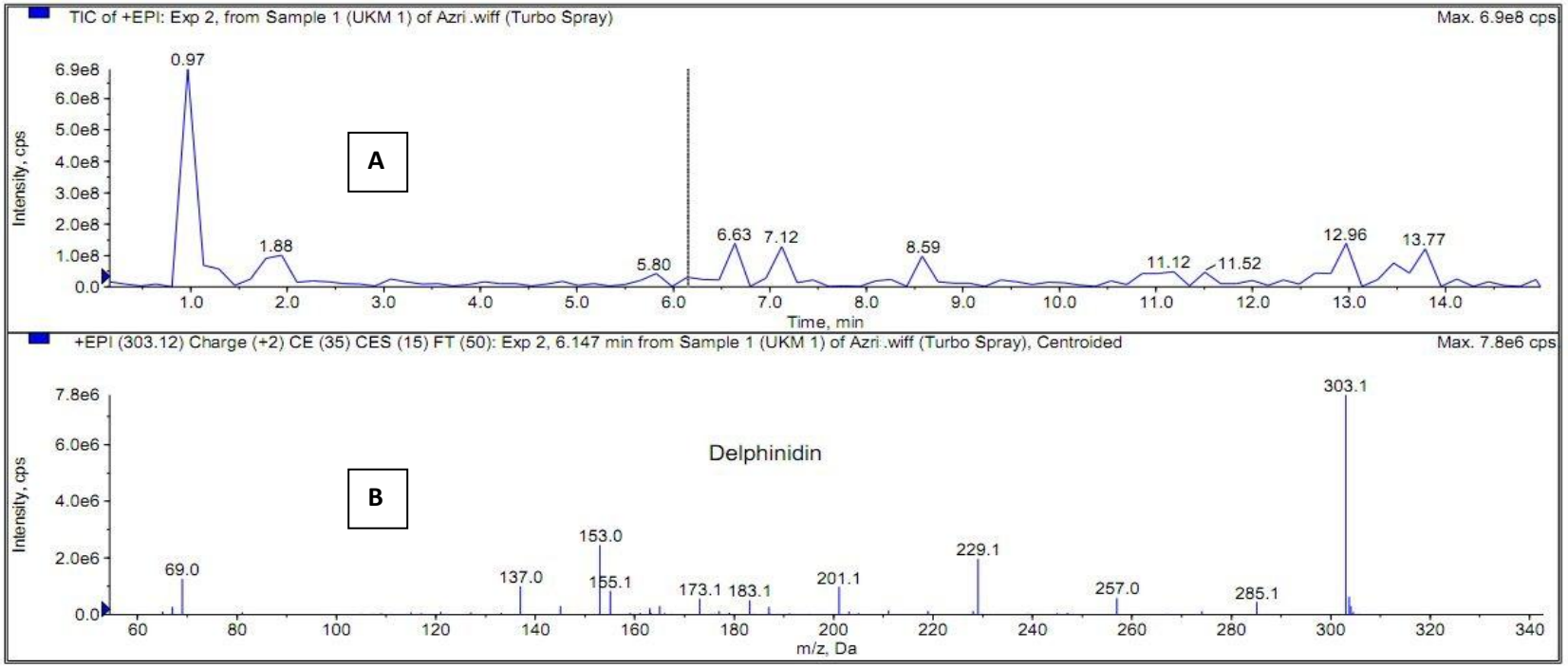


Figure above shows full scan (positive mode) of UKMR-1 variety with delphinidin MS/MS fragmentation. The retention time for delphinidin was 6.147 minute. The mass spectrum fragmentation of delphinidin was displayed the following major peak at m/z: 303.1, 229.1, 153.0 and 69.0. The count per second (cps) of the tallest peak was 7.8e6 cps.

The mass spectrum fragmentation in figure above was appearing as large peak at m/z 303.1. The product ion spectrum of the ion (m/z 303.1) showed fragment ions at m/z 285.1 $[C_{15}H_9O_6]^+$ (loss of water), m/z 257.3 $[C_{14}H_9O_5]^+$ (loss of CO) and m/z 229.1 $[C_{13}H_9O4]^+$ (loss of another CO). The molecular ion then shows fragmentation at m/z 201.1 $[C_{13}H_9O_4]^+$ (lost of CO), m/z 153 $[C_{12}H_4O_3]^+$ (lost of C and two water molecules), m/z 137 $[C_{12}H_4O_2]^+$ (lost of O). Thus, by looking at the fragmentation pattern, the LCMS/MS chromatogram shows a peak at retention time 6.147 minute was identified as delphinidin based on ACD/Labs advanced chemometrics mass fragmentations predictive software. The chemical formula for delphinidin is $C1_5H_{11}O_7$. The molecular weight is 303.24 g/mol. Delphinidin, which belongs to the anthocyanidins is highly water-soluble and is easily degraded by hydrolysis.

Figure 4.42: Molecular structure of delphinidine

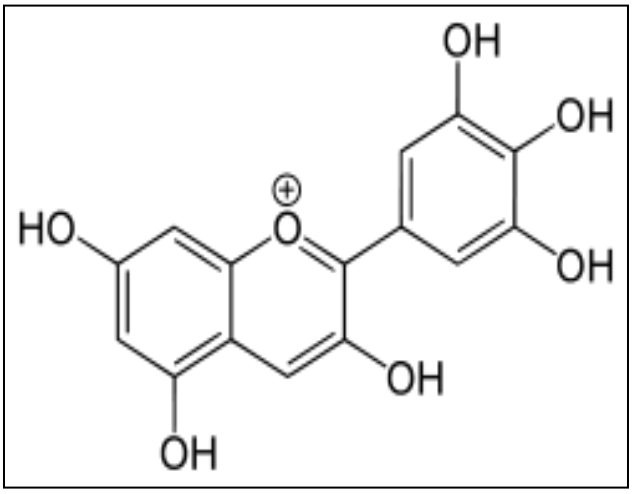


Figure 4.43: Fragmentation of gossypetin: (A) Full chromatograms of UKMR-1 variety in the positive mode. (B) Fragmentation of peak at retention time 10.70 min results in a MS spectrum with m/z 319.2 dominating.

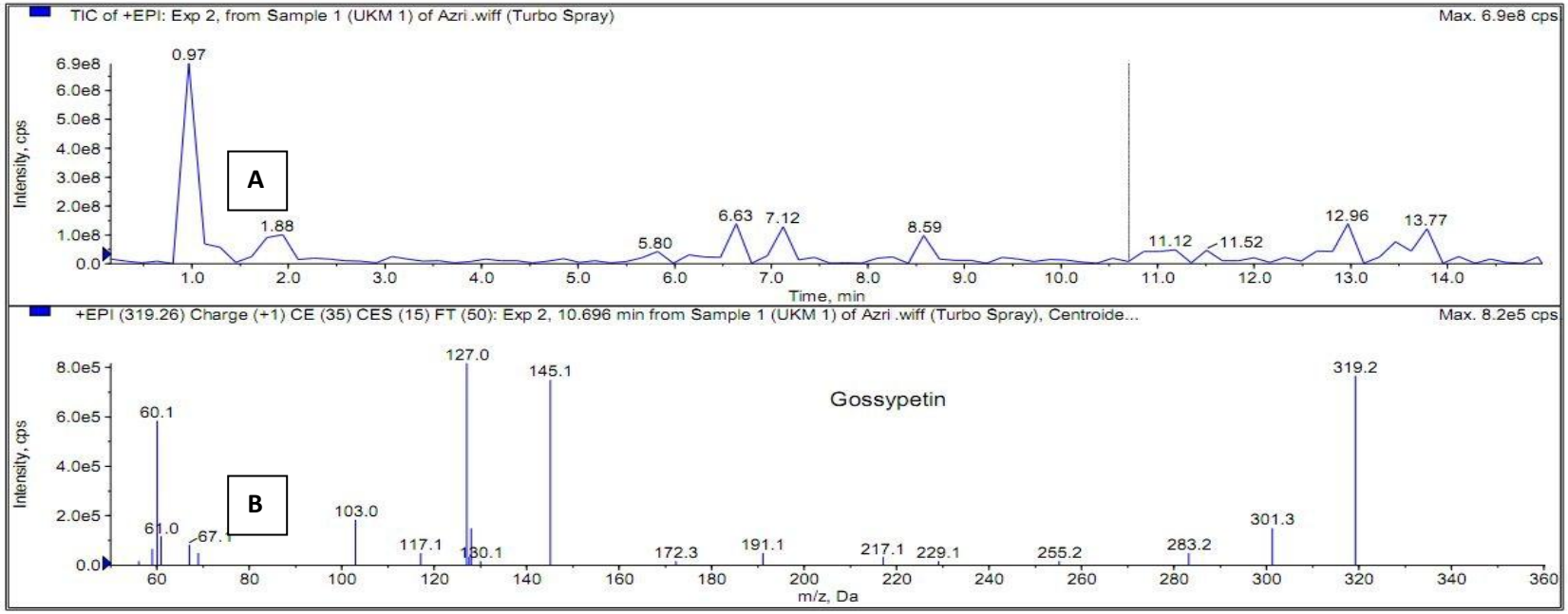
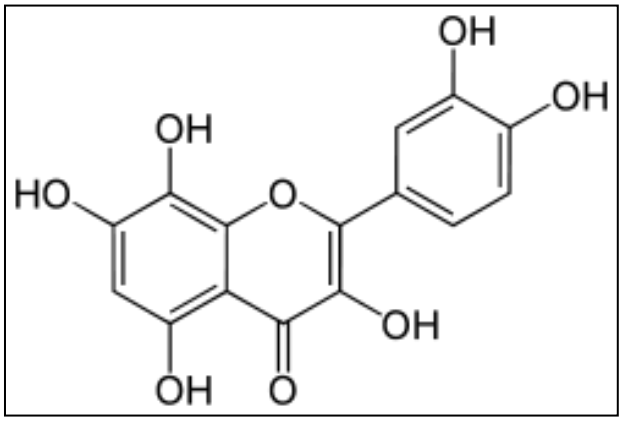


Figure above shows full scan (positive mode) of UKMR-1 variety with gossypetin MS/MS fragmentation. The retention time for Gossypetin was 10.696 minute. The mass spectrum fragmentation of gossypetin was displayed the following major peak at m/z: 319.2, 145.1, 127.0, and 60.1. The count per second (cps) of the tallest peak was 8.2e5 cps.

The LCMS/MS chromatogram shows a peak at retention time 10.696 minute was identified as Gossypetin based on ACD/Labs advanced chemometrics mass fragmentations predictive software. The chemical formula for Gossypetin is $C_{15}H_{10}O_8$. The molecular weight for gossypetin is 318.24 g/mol

Figure 4.44: Molecular structure of gossypetin



The molecular ion of gossypetin appears as large peak at m/z 319.2. The ion spectrum showed fragment ions at m/z 301.3 $[C_{15}H_9O_7]^+$ indicate the molecular ion of Gossypetin has lost of water molecule. The ion spectrum also showed fragment ions at m/z 283.2 $[C_{15}H_6O_6]^+$ indicate the molecular ion lost of additional water molecule. The ion was then produce fragment at m/z 255.2 $[C_{14}H_6O_5]^+$ due to lost of CO.

Figure 4.45: Full chromatograms of UKMR-1 variety in the negative mode.

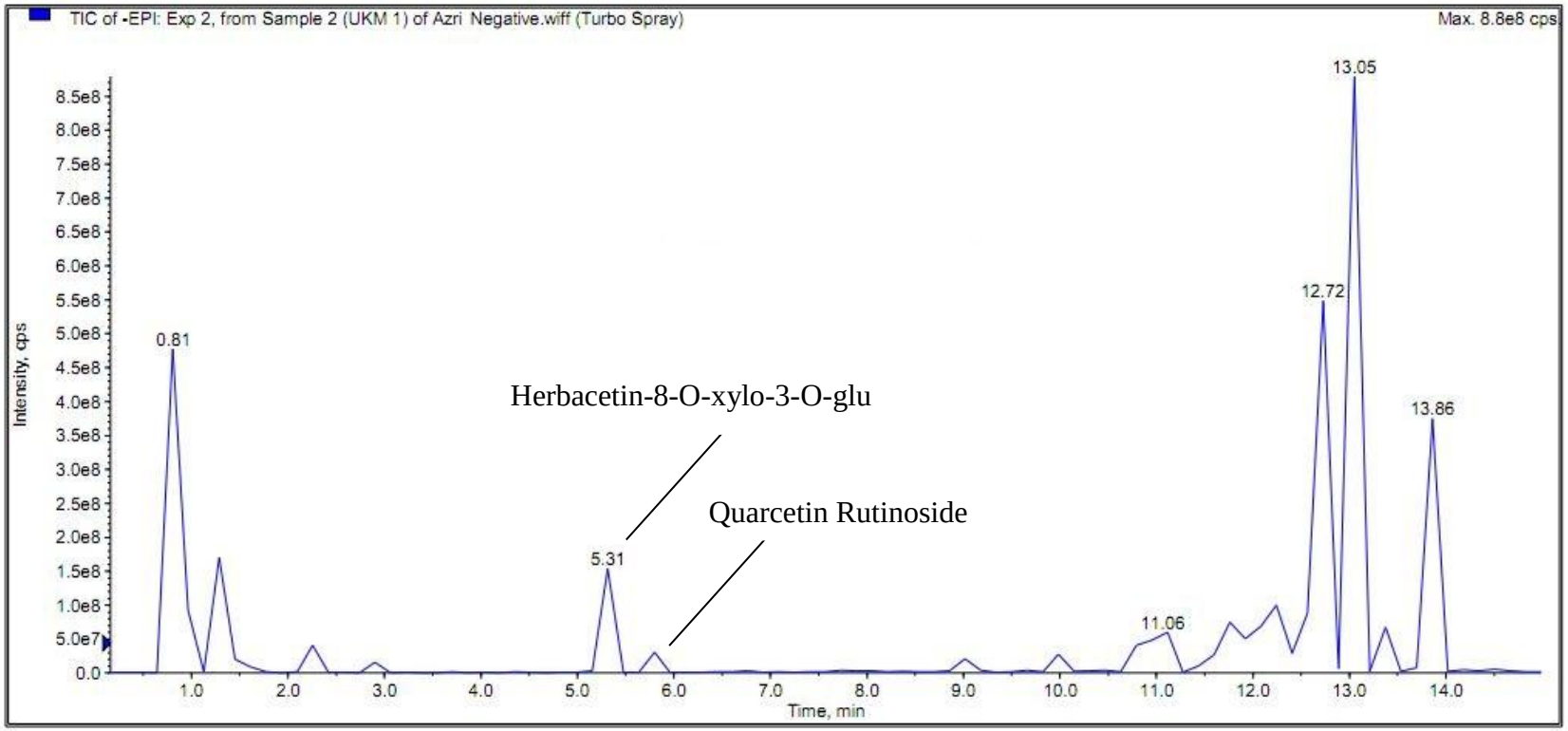


Figure 4.46: Fragmentation of herbacetin-8-O-xylo-3-O-glu: (A) Full chromatograms of UKMR-1 variety in the negative mode. (B) Fragmentation of peak at retention time 5.315 min results in a MS spectrum with m/z 595.1 dominating.

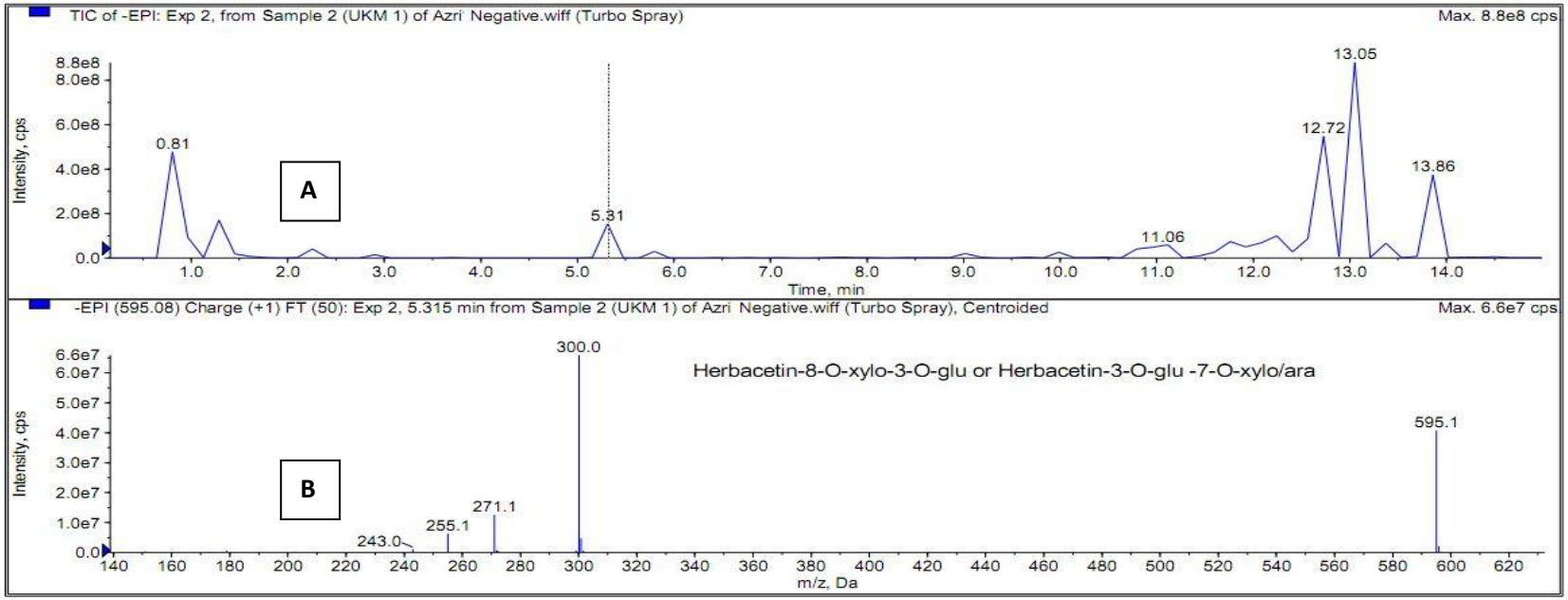
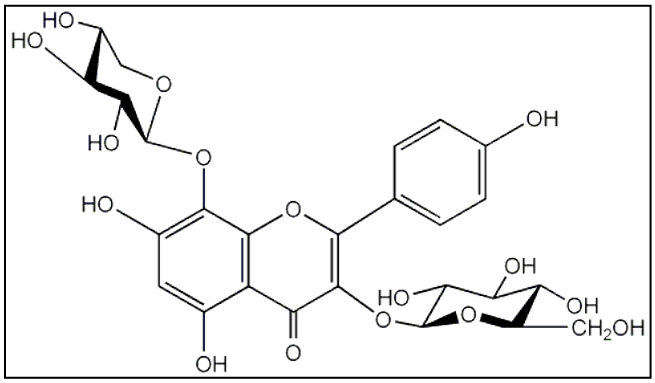


Figure above shows full scan (negative mode) of UKMR-1 variety with herbacetin-8-O-xylo-3-O-glu MS/MS fragmentation. The retention time for herbacetin-8-O-xylo-3-O-glu was 5.315 minute. The mass spectrum fragmentation of herbacetin-8-O-xylo-3-O-glu was displayed the following major peak at m/z: 595.1, 300.0, 271.1 and 255.1. The count per second (cps) of the tallest peak was 6.6e7 cps.

The LCMS/MS chromatogram shows a peak at retention time 5.315 minute was identified as herbacetin-8-O-xylo-3-O-glu based on ACD/Labs advanced chemometrics mass fragmentations predictive software. The chemical formula for herbacetin-8-O-xylo-3-O-glu is $C_{26}H_{28}O_{16}$. The molecular weight is 596.13 g/mol. herbacetin-8-O-xylo-3-O-glu also known as Rhodalidin.

Figure 4.47: Molecular structure of herbacetin-8-O-xylo-3-O-glu



The molecular ion of herbacetin-8-O-xylo-3-O-glu appears as large peak at m/z 595.1. The ion spectrum showed fragment ions at m/z 300.0 $[C_{15}H_8O_7]^+$ indicate the molecular ion of herbacetin-8-O-xylo-3-O-glu has lost of $C_9H_{10}O_9$ molecule. The ion spectrum also showed fragment ions at m/z 271.1 $[C_{14}H_8O_6]^+$ indicate the molecular ion lost of CO molecule. The ion was then produce fragment at m/z 255.1 $[C_{14}H_6O_4]^+$ due to lost of O.

Figure 4.48: Fragmentation of Quercetin rutinoside: (A) Full chromatograms of UKMR-1 variety in the negative mode. (B)Fragmentation of peak at retention time 5.798 min results in a MS spectrum with m/z 609.1 dominating.

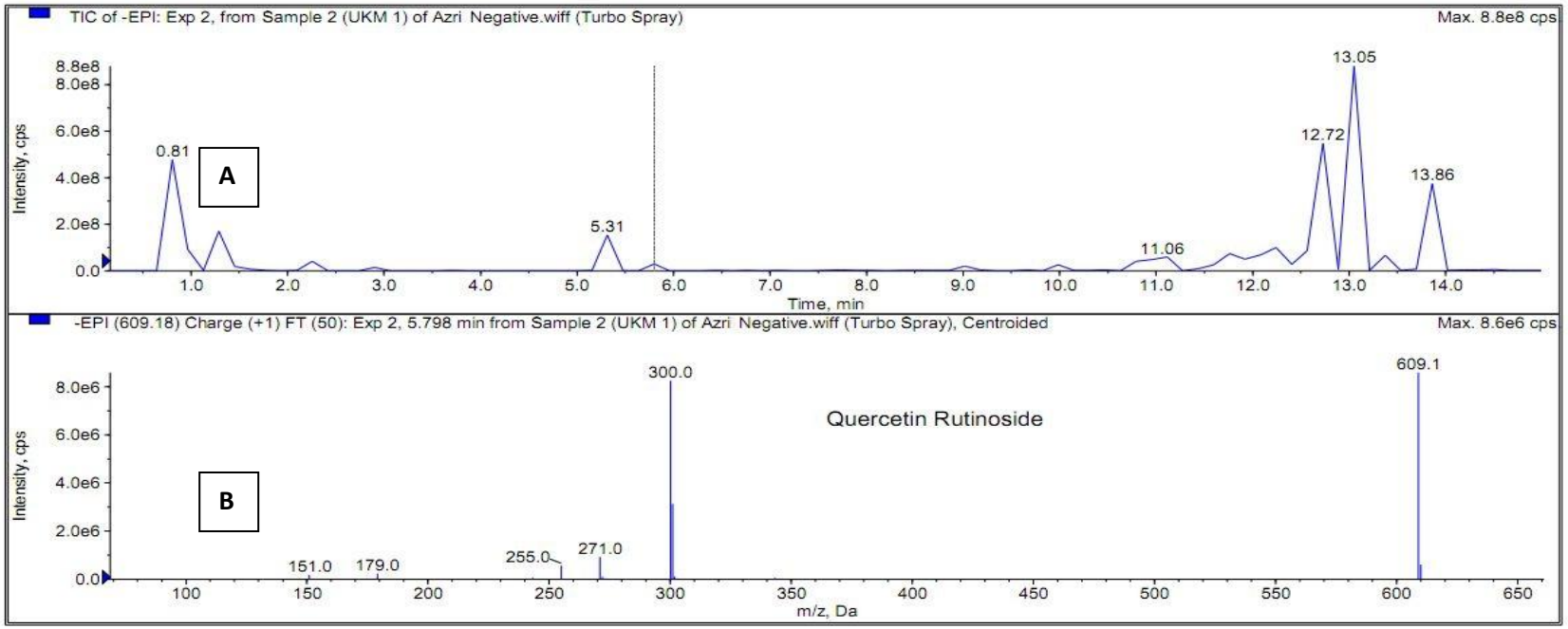
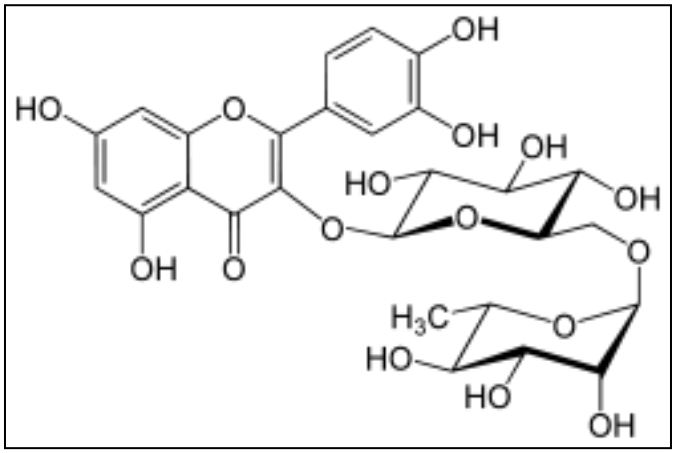


Figure above shows full scan (negative mode) of UKMR-1 variety with Quercetin rutinoside MS/MS fragmentation. The retention time for Quercetin rutinoside was 5.798 minute. The mass spectrum fragmentation of Quercetin rutinoside was displayed the following major peak at m/z: 609.1, 300.0, 271.0 and 255.0. The count per second (cps) of the tallest peak was 8.6e6 cps.

The LCMS/MS chromatogram shows a peak at retention time 5.798 minute was identified as quercetin rutinoside based on ACD/Labs advanced chemometrics mass fragmentations predictive software. The chemical formula for Quercetin rutinoside is $C_{27}H_{30}O_{16}$. The molecular weight for Gossypetin is 610 g/mol.

Figure 4.49: Molecular structure of quercetin rutinoside



The molecular ion of Quercetin rutinoside appears as large peak at m/z 609.1. The ion spectrum showed fragment ions at m/z 300.0 $[C_{15}H_8O_7]^+$ indicate the molecular ion of Quercetin rutinoside has lost of $C_{12}H_{22}O_9$ molecule. The ion spectrum also showed fragment ions at m/z 271.0 $[C_{13}H_5O_4]^+$ indicate the molecular ion lost of CO molecule. The ion was then produce fragment at m/z 255.0 $[C_{13}H_5O_3]^+$ due to lost of O.

4.9.3 LC-MS/MS Analysis of UKMR-2 Variety

LCMS/MS phytochemical analysis of *H. sabdariffa* from UKMR-2 variety detected the present of several chemical compounds mostly from flavonoids group. Four different compounds were detected from aqueous extract of UKMR-2 variety which are hibiscetine, delphinidin, herbacetin-8-O-xylo-3-O-glu and quercetin rutinoside.

Figure 4.50: Full chromatograms of UKMR-2 variety in the positive mode.

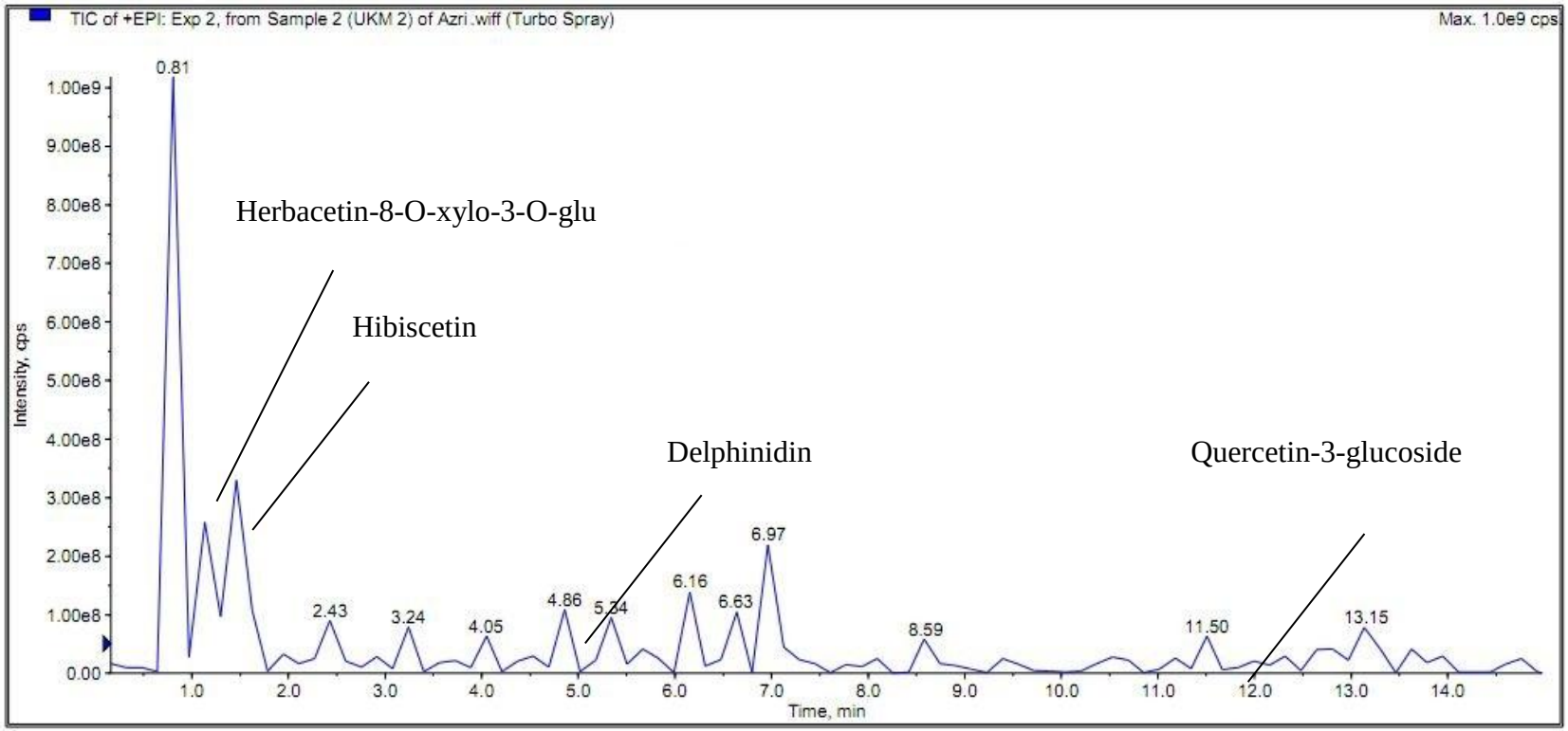


Figure 4.51: Fragmentation of hibiscetin: (A) Full chromatograms of UKMR-2 variety in the positive mode. (B) Fragmentation of peak at retention time 1.785 min results in a MS spectrum with m/z 377.0 dominating.

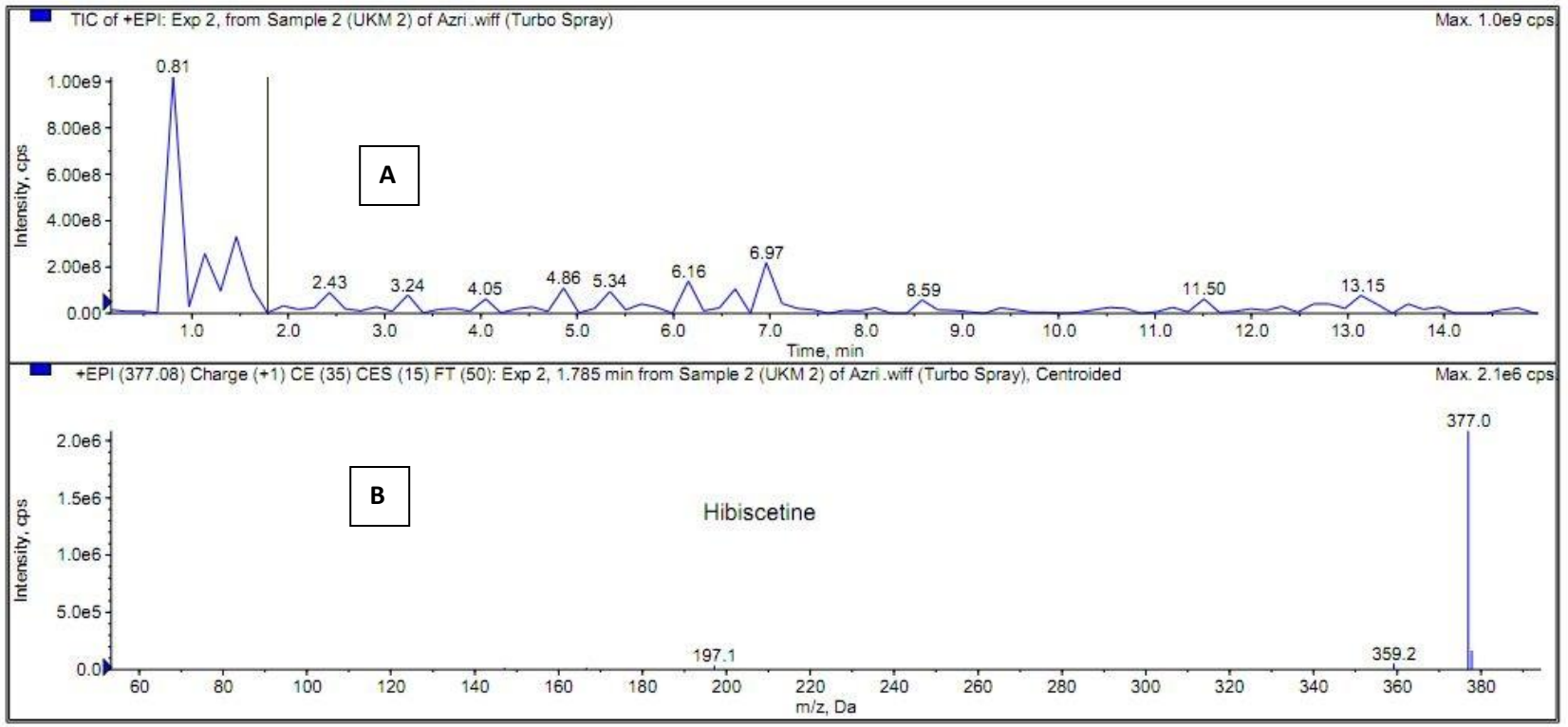


Figure above shows full scan (positive mode) of UKMR-2 variety with hibiscetin MS/MS fragmentation. The retention time for hibiscetin was 1.785 minute. The mass spectrum fragmentation of hibiscetin was displayed the following major peak at m/z: 377.0 and 197.1. The count per second (cps) of the tallest peak was 2.1e6 cps.

The mass spectrum fragmentation in Figure above was appearing as large peak at m/z 377.0. The product ion spectrum of the ion (m/z 377.0) showed fragment ions at m/z 359.0 $[C_{18}H_{14}O_8]^+$ (loss of water). Thus, by looking at the fragmentation pattern, the LCMS/MS chromatogram shows a peak at retention time 1.785 minute was identified as hibiscetin based on ACD/Labs advanced chemometrics mass fragmentations predictive software. The chemical formula for hibiscetin is $C_{18}H_{16}O_9$. The molecular weight is 376 g/mol.

Figure 4.52: Chemical structure of hibiscetin

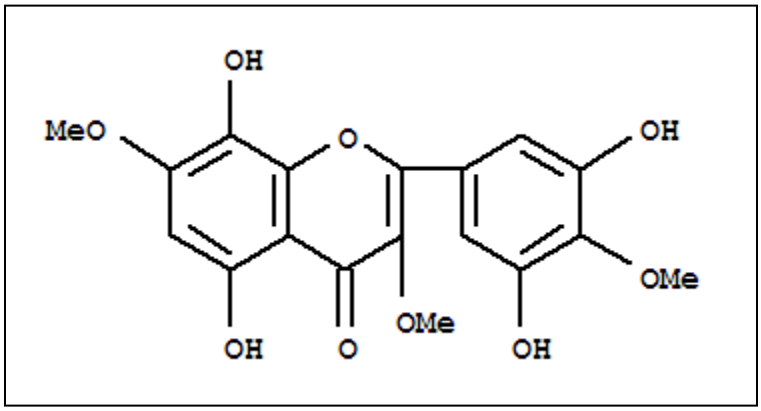


Figure 4.53: Fragmentation of delphinidin: (A) Full chromatograms of UKMR-2 variety in the positive mode. (B) Fragmentation of peak at retention time 5.505 min results in a MS spectrum with m/z 303.1dominating.

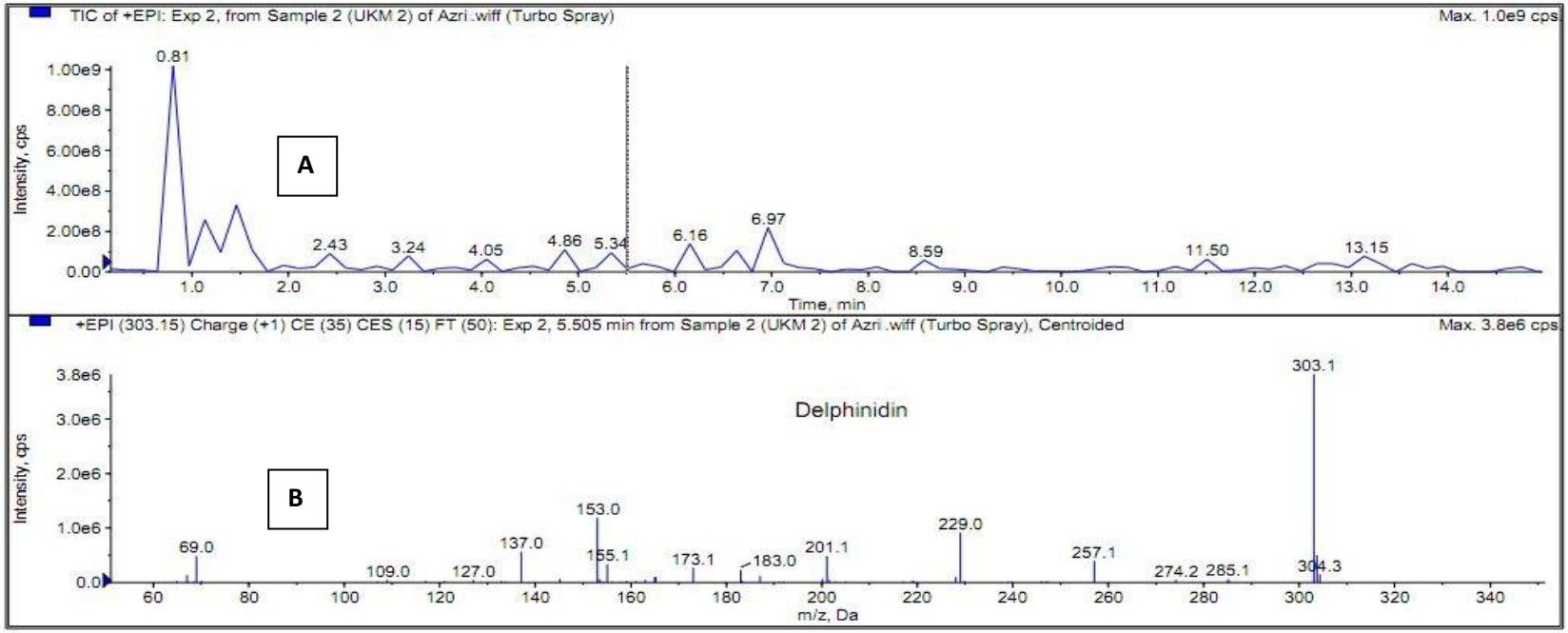
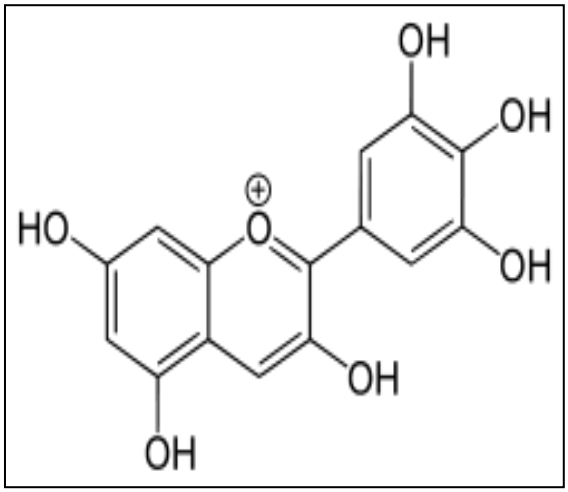


Figure above shows full scan (positive mode) of UKMR-2 variety with delphinidin MS/MS fragmentation. The retention time for Delphinidin was 5.505 minute. The mass spectrum fragmentation of delphinidin was displayed the following major peak at m/z: 303.1, 229.0, 153.0 and 69.0. The count per second (cps) of the tallest peak was 3.8e6 cps.

The LCMS/MS chromatogram shows a peak at retention time 5.505 minute was identified as Delphinidin based on ACD/Labs advanced chemometrics mass fragmentations predictive software. The chemical formula for delphinidin is $C1_5H_{11}O_7$. The molecular weight is 303.24 g/mol. Delphinidin, which belongs to the anthocyanidins is highly water-soluble and is easily degraded by hydrolysis

Figure 4.54: Molecular structure of delphinidine



The mass spectrum fragmentation of delphinidin was appearing as large peak at m/z 303.1. The product ion spectrum of the delphinidin ion (m/z 303.1) showed fragment ions at m/z 285.1 $[C_{15}H_9O_6]^+$ (loss of water), m/z 257.1 $[C_{13}H_7O_3]^+$ (loss of CO and water) and m/z 229.0 $[C_{11}H_5O]^+$ (loss of two CO and water)

Figure 4.55: Fragmentation of herbacetin-8-O-xylo-3-O-glu: (A) Full chromatograms of UKMR-2 variety in the positive mode. (B) Fragmentation of peak at retention time 1.616 min results in a MS spectrum with m/z 303.1dominating.

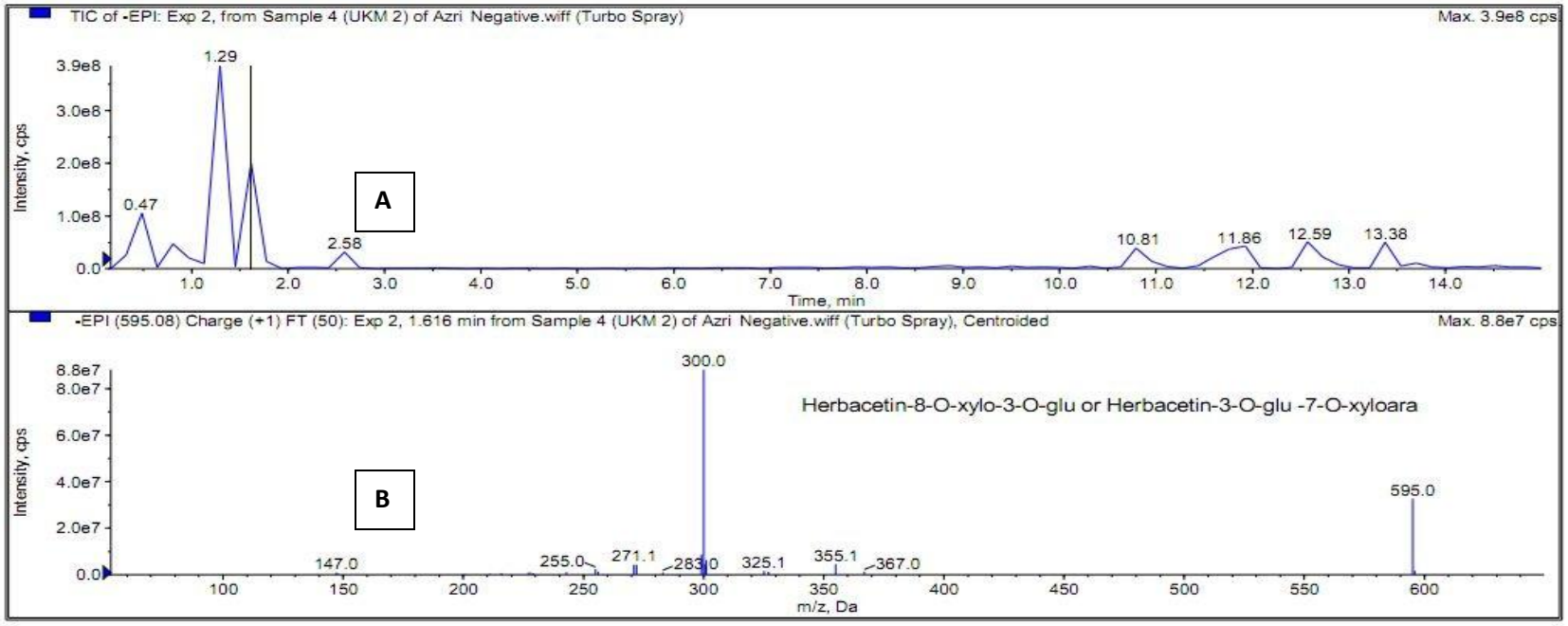
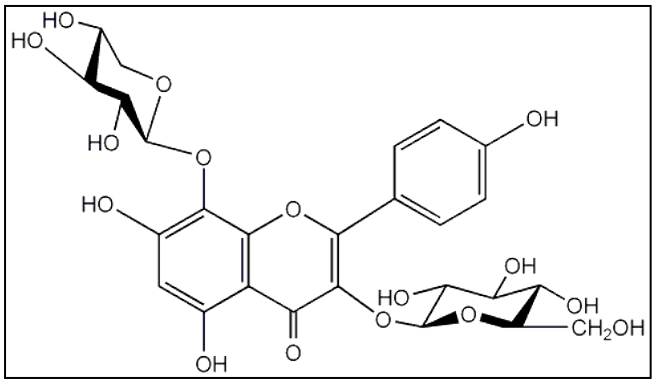


Figure above shows full scan (positive mode) of UKMR-2 variety with herbacetin-8-O-xylo-3-O-glu MS/MS fragmentation. The retention time for herbacetin-8-O-xylo-3-O-glu was 1.616 minute. The mass spectrum fragmentation of herbacetin-8-O-xylo-3-O-glu was displayed the following major peak at m/z: 595.0, m/z 355.1 and m/z 300.0. The count per second (cps) of the tallest peak was 8.8e7 cps.

The LCMS/MS chromatogram shows a peak at retention time 1.616 minute was identified as herbacetin-8-O-xylo-3-O-glu based on ACD/Labs advanced chemometrics mass fragmentations predictive software. The chemical formula for herbacetin-8-O-xylo-3-O-glu is $C_{26}H_{28}O_{16}$. The molecular weight is 596.13 g/mol. Herbacetin-8-O-xylo-3-O-glu also known as Rhodalidin.

Figure 4.56: Molecular structure of herbacetin-8-O-xylo-3-O-glu



The molecular ion of herbacetin-8-O-xylo-3-O-glu appears as large peak at m/z 595.1. The ion spectrum showed fragment ions at m/z 300.0 $[C_{15}H_8O_7]^+$ indicate the molecular ion of herbacetin-8-O-xylo-3-O-glu has lost of $C_9H_{10}O_9$ molecule. The ion spectrum also showed fragment ions at m/z 271.1 $[C_{14}H_8O_6]^+$ indicate the molecular ion lost of CO molecule. The ion was then produce fragment at m/z 255.1 $[C_{14}H_6O_4]^+$ due to lost of O.

Figure 4.57: Fragmentation of quercetin-3-glucoside: (A) Full chromatograms of UKMR-2 variety in the positive mode. (B) Fragmentation of peak at retention time 11.923 min results in a MS spectrum with m/z 463.3 dominating.

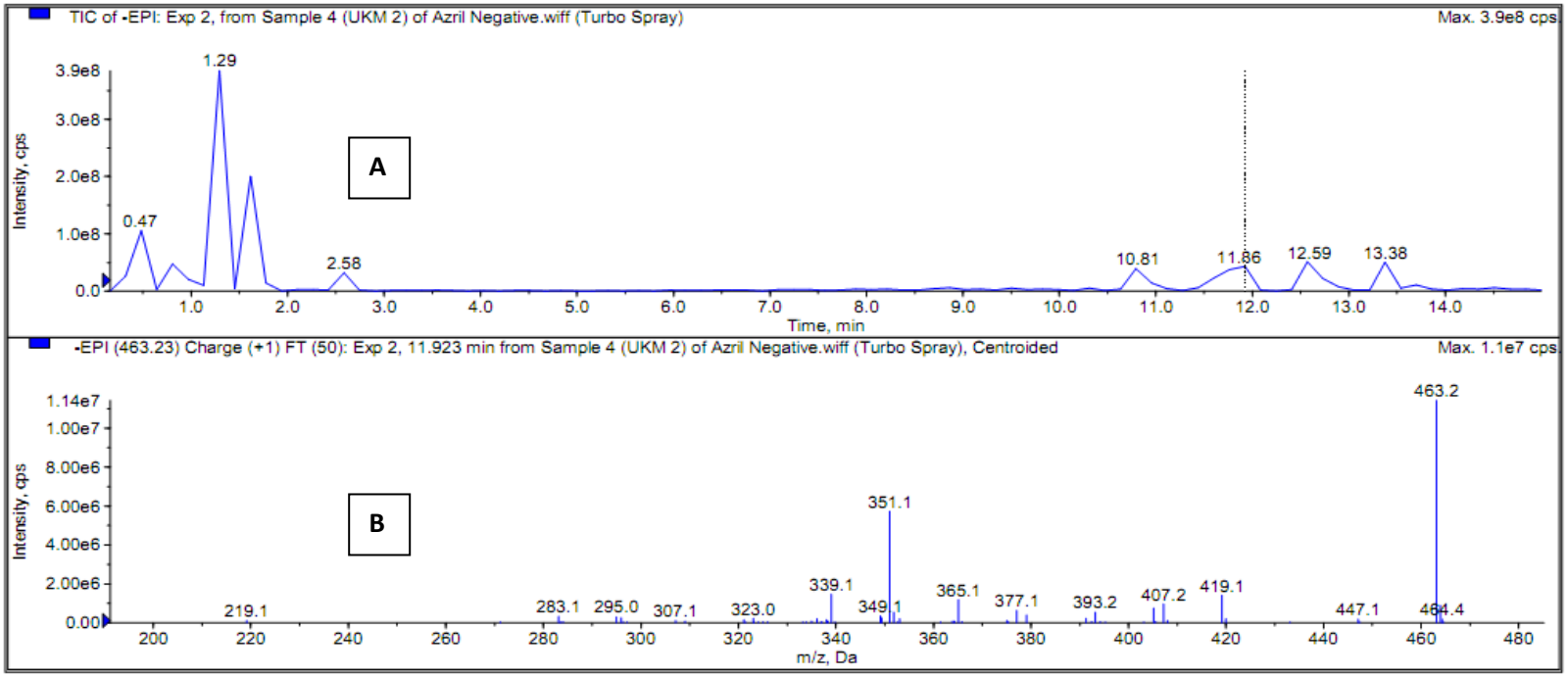
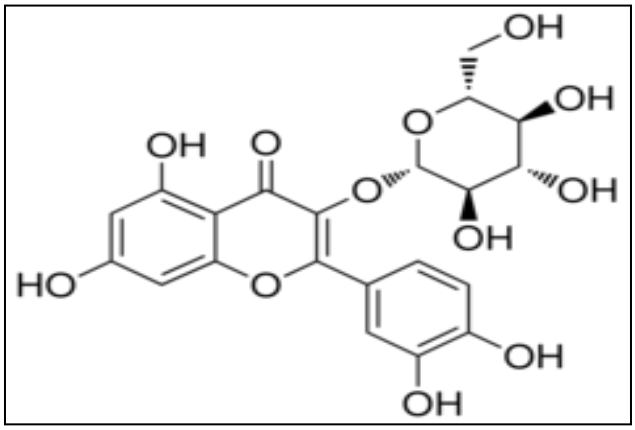


Figure above shows full scan (positive mode) of UKMR-2 variety with quercetin-3-glucoside MS/MS fragmentation. The retention time for Quercetin-3-glucoside was 11.923 minute. The mass spectrum fragmentation of quercetin-3-glucoside was displayed the following major peak at m/z: 463.2, 419.1, 365.1 and 351.1. The count per second (cps) of the tallest peak was 1.1e7 cps.

The LCMS/MS chromatogram shows a peak at retention time 11.923 minute was identified as quercetin-3-glucoside based on ACD/Labs advanced chemometrics mass fragmentations predictive software. The chemical formula for quercetin-3-glucoside is $C_{21}H_{20}O_{12}$. The molecular weight is 464.38 g/m

Figure 4.56: Chemical structure of quercetin-3-glucoside



The molecular ion of quercetin-3-glucoside appears as large peak at m/z 654.4. The ion spectrum showed fragment ions at m/z 463.4 $[C_{21}H_{19}O_{12}]^+$ indicate the molecular ion of quercetin-3-glucoside has lost of H atom. The ion spectrum also showed fragment ions at m/z 447.1 $[C_{21}H_{19}O_{11}]^+$ indicate the molecular ion lost of O atom. The ion was then produce fragment at m/z 419.1 $[C_{20}H_{19}O_{11}]^+$ due to lost of CO.

Table below summarize the chemical constituent detected in Arab, UKMR-1 and UKMR-2 variety. delphinidin and herbacetin-8-o-xylo-3-o-glu were detected in all variety extracts. In contrast, cyanidin 3-sambubioside, gossypetin-3-o-glu-7-o-xylo and kaempferol-3-o-rutinoside were only detected in Arab extract. While gossypetin was only detected in UKMR-1 extract.

Table 4.20: Chemical constituents detected in Arab, UKMR-1 and UKMR-2 extracts.

Chemical constituents	**Varieties of *H. sabdariffa***		
	Arab	**UKMR-1**	**UKMR-2**
Cyanidin 3-sambubioside	√		
Delphinidin	√	√	√
Delphinidin-3-sambubioside	√	√	
Gossypetin		√	
Gossypetin-3-o-glu-7-o-xylo	√		
Herbacetin-8-o-xylo-3-o-glu	√	√	√
Hibiscetine		√	√
Kaempferol-3-o-rutinoside	√		
Quercetin rutinoside		√	√