CHAPTER III

RESEARCH METHODOLOGY

3.1 Chemical Materials

Methanol, chloroform and hexane used were GC-grade solvents and were purchased from Merck (Darmstadt, Germany). N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA), 1 % trimethylchlorosilane (TMCS) and Potassium hydroxide were supplied by Sigma -Aldrich (Germany).

3.2 Instruments

3.2.1 GC-MS

The gas chromatograph mass spectrometer (GC-MS) analysis was performed on an Agilent 7890A gas chromatograph (GC) directly coupled to the mass spectrometer system (MS) of an Agilent 5975C inert MSD with triple-axis detector. Column Model, BP20 (WAX) Length 30 m, Diameter 0.25 mm, Stationary phase Polyethylene glycol, Film thickness 0.25 µm from Corporation SGE.

3.2.2 LC-TOF-MS

Liquid chromatography-time of flight-mass spectrometry (LC-TOF-MS) analysis was performed on an Agilent 6230.

3.3 Sampling

Three types of date fruits Kheseab (Tamar), Segae (Rutab) and Khalas (Tamar) from Saudi Arabia were purchased local super market. The samples were stored in chiller at 0 ^{o}C before used.

3.4 Sample Preparation

An amount of 15 g of each type of date (Kheseab (Tamar), Segae (Rutab) and Khalas (Tamar) were taken and mixed together using blender (Philips) for homognization after removing the seeds.

3.5 Sample Extraction

Methanol, hexane and chloroform were used for the extraction solvents of the sample mixture of the three types of dates fruit. The usage of variety of solvents allows selecting the one with greatest ability to extract biochemical compounds in date fruits. The extraction was implemented by weighing 2 g of homogeneous sample mixture for 13 beakers and each was drenched into 20 mL of solvent for 2 h at a room temperature. The three mixture design used for extraction is shown in the **Fig. 5**, while the mixtures used were prepared as shown in **Table 6** and mixture solvents polarity is shown in **Table 7**.

The solution was then filtered using Whatman filter paper No 1 before subjecting to rotary evaporator at 40 °C for concentration of the sample for compounds identification using GC-MS and LC-TOF-MS.

Figure 5: The design of three mixtures for extraction

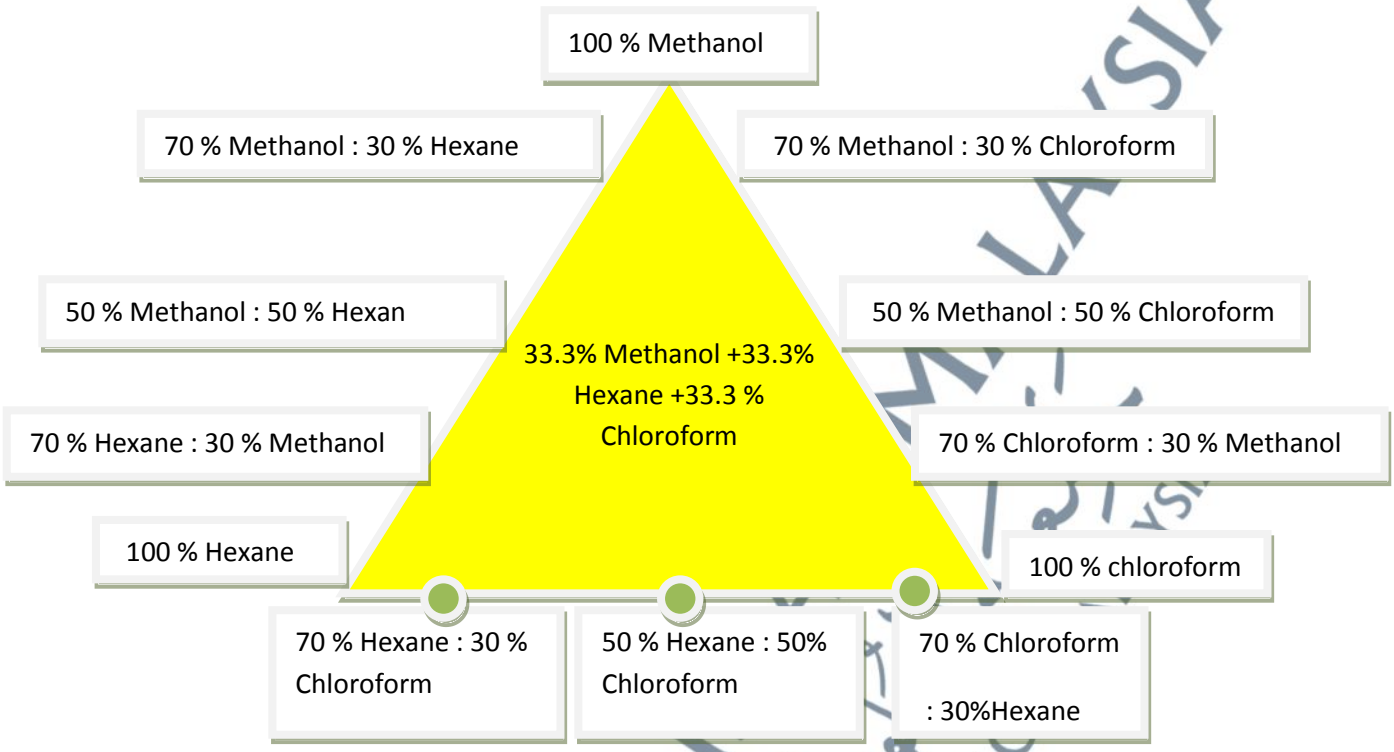

Table 6: Preparation of mixtures for designs

Design	Methanol	Chloroform	n-Haxane
1	100 % (20 mL)	0 % (0 mL)	0 % (0 mL)
2	0 % (0 mL)	100 % (20 mL)	0 % (0 mL)
3	0 % (0 mL)	0 % (0 mL)	100 % (20 mL)
4	70 % (14 mL)	30 % (6 mL)	0 % (0 mL)
5	0 % (0 mL)	70 % (14 mL)	30% (6 mL)
6	70 % (14 mL)	0 % (0 mL)	30 % (6 mL)
7	33.33 % (6.6 mL)	33.33 % (6.6 mL)	33.33 % (6.6 mL)
8	30 % (6 mL)	70 % (14 mL)	0 % (0 mL)
9	0 % (0 mL)	30 % (6 mL)	70 % (14 mL)
10	30 % (6 mL)	0 % (0 mL)	70 % (14 mL)
11	50 % (10 mL)	50 % (10 mL)	0 % (0 mL)
12	0 % (0 mL)	50 % (10 mL)	50 % (10 mL)
13	50 % (10 mL)	0 % (0 mL)	50 % (10 mL)

Table 7: Mixture solvents polarity

Design	Methanol p (0.762)	Chloroform P (0.259)	n-Haxane p (0.009)	Total polarity
1	100 % (0.762)	0 % (0)	0 % (0)	0.762
2	0 % (0)	100 % (0.259)	0 % (0)	0.259
3	0 % (0)	0 % (0)	100 % (0.009)	0.009
4	70 % (0.334)	30 % (0.077)	0 % (0)	0.411
5	0 % (0)	70 % (0.181)	30% (0)	0.181
6	70 % (0.334)	0 % (0)	30 % (0)	0.334
7	33.33 % (0.253)	33.33 % (0.086)	33.33 % (0)	0.339
8	30 % (0.228)	70 % (0.181)	0 % (0)	0.409
9	0 % (0)	30 % (0.077)	70 % (0)	0.077
10	30 % (0.228)	0 % (0)	70 % (0)	0.228
11	50 % (0.381)	50 % (0.129)	0 % (0)	0.51
12	0 % (0)	50 % (0.129)	50 % (0)	0.129
13	50 % (0.381)	0 % (0 ml)	50 % (0)	0.381

3.6 GC-MS

3.6.1 Derivatization of the Sample Extracts

500 µL of the sample extract using each design was placed in 2 mL of vial, after that 80 µL of BSTFA + 20 µL of TMCS was added to the extract. The vials were then put in oven and heated for 1 h at 65 ^{o}C for derivatization.

3.6.2 GC-MS Analysis

The gas chromatography–mass spectrometry (GS-MS) analysis was performed according to the method described by Hema et al.(2010). The derivatized sample extracts (20 mg) were dissolved into 2 mL of hexane and 20 µL of 2 N potassium

hydroxide in methanol (112 g of KOH dissolved in 1 L methanol). Then, the mixture were vortexed for 1 min, centrifuged at 10,000 rpm for 10 min and the supernatant (1 ml) were transferred to amber vial to be analyzed using GC-MS. The compounds detection was performed using an electron ionization system functioning at ionization energy of 70 eV.

The pure helium gas was the carrier at a constant flow rate of 1 mL / min. The injection volume was 0.5 µL, with a split ratio of 10:1 at injector temperature of 250 °C, where for the ion source was 280 °C. The oven temperature was set at 110 °C for 2 min and then increased to 200 °C with a ratio of 10 °C / min. Then, it was increased to 280 °C for 9 min with a ratio of 5 °C / min. Mass spectra were taken at 70 eV a scan interval of 0.5 S and fragments from 45 to 450 Da. The total running time was 65 min. The identification and interpretation of GC-MS results were conducted using the database of National Institute of Standards and Technology (NIST) by comparing fragmentation pattern of common metabolites, taking the compounds with high probability more than 70 %. The list of compounds in each extracts were tabulated in table for each design.

3.7 LC-TOF-MS Analysis

The extract from the best mixture where the highest compounds number were obtained from GC-MS analysis was verified with LC–TOF-MS technique for comparison

The liquid chromatography analysis was performed according to the method described by Shahriar et al. (2013). Degasser, column heater and autosampler; based

on manufacturer recommendations, the pumps were programmed to deliver an increasing gradient of methanol against an aqueous mobile phase of 5 Mm ammonium formate over the following time course: 5 % methanol from 0 to 0.5 min, increasing to 30 % methanol at 1.5 min, 60 % methanol at 4.5 min, to 95 % methanol at 6.5 min, and a reset to 5 % methanol as acquisition stopped at 10 min with a 3 min post-run.

Extraction was performed using 3.0 - 100 mm column. The autosampler injected 4 mL of sample per run, with automated needle washes in between. The column flow rate was kept at 0.6 L / min with the heater at a constant of 508 ºC. The mass analyzer was an Agilent 6230 TOF-MS operated in positive ion scan mode with mass scanning from 50 to 600 m/z. The instrument acquired data using the following parameters: drying gas temperature, 190 ºC; drying gas flow, 8.0 L / min; nebulizer 4 bar Focus Not active, Scan Begin 50 m/z, Scan End 600 m/z, Set Capillary 4000 V, Set End Plate Offset -500 V and Set Collision Cell RF 250.0 V.

3.8 Data Analysis with PCA

PCA was performed with the aid of "THE UNSCRAMBLER®X" software (CAMO software version 10.1) on the multivariate data from GC-MS results.