CHAPTER 3: MATERIALS AND METHODS

3.1 Experimental Material

Three different varieties of *H. sabdariffa* samples which are Arab, UKMR-1 and UKMR-2 variety were used in this study. *H. sabdariffa* samples were collected from Universiti Kebangsaan Malaysia (UKM). Only calyx part was used in this study. The selected calyces were ensured to be free from any diseases. The calyces were washed and chopped into small pieces for efficient drying process. The calyces were then grounded into fine powder for extraction.

3.2 Preparation of extracts

For extraction, 100 gram of *H. sabdariffa* fine powder from Arab, UKMR-1 and UKMR-2 variety were extracted with 1L of distilled water. The mixtures were placed in the environmental shaker at room (27ºC) temperature for 24 hours. After 24 hours, the mixtures were filtered using filter paper. The filtered extracts were then evaporated to a concentrated crude extracts using a rotary evaporator at 40-45ºC and converted to powder by freeze dryer. The extract powder eventually transferred into a specimen tubes, wrapped with aluminium foil and was placed in desiccators and kept at -20ºC until used.

3.3 Antioxidant Activity Assay

In this study, the antioxidant activities of each crude extracts from the selected variety of *H. sabdariffa* were evaluated using three different assays:

a) 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) Free Radical Scavenging Activity Assay

b) Reducing Power Assay

c) Metal Chelating Assay

3.3.1 DPPH Free Radical Scavenging Activity Assay

3.3.1.1 Ascorbic Acid as a Positive References Standard

The potential antioxidant activity of *H. sabdariffa* extracts was determined on the basis of scavenging activity on the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. Ascorbic acid was used as the positive reference standard in the DPPH free radical scavenging assay. The reaction was initiated by the addition of 500µl DPPH radical solution to ascorbic acid that was dissolved in methanol at various concentrations. Reaction mixtures of ascorbic acid; DPPH and methanol were prepared according to Table 3.1.

Table 3.1: Reaction mixtures containing ascorbic acid, DPPH and methanol

Concentration of Ascorbic Acid (µg/ml)	Volume of Methanol (µl)	Volume of DPPH (µl)
1000.00	3000	500
500.00	3500	500
250.00	3750	500
125.00	3875	500
62.50	3937.5	500
0.00	4000	500

The reaction mixtures were incubated at room temperature in a dark room for 30 minutes to allow reactions. The absorbance was measured using spectrophotometer at 517 nm with methanol as a blank. The DPPH without ascorbic acid served as negative control. Lower the absorbance of the reaction mixtures indicates higher free radical scavenging activity.

3.3.1.2 DPPH radical scavenging activity of crude extracts

H. sabdariffa extracts at different concentrations were tested for their ability to scavenge DPPH radical resulting in action of decolorizes the purplish color of DPPH solution. The similar procedure for positive reference standard test was repeated for the *H. sabdariffa* extracts. All tests were run in triplicates and the readings were averaged.

3.3.1.3 Determination of Percentage of Inhibition

H. sabdariffa extracts capability to scavenge the DPPH radical was expressed as the percentage of inhibition. The percentage of inhibition of *H. sabdariffa* extracts was calculated according to the following equation:

$$\left[\% \text{ of inhibition} = \frac{OD_{control} - OD_{sample}}{OD_{control}} X\ 100 \right]$$

Where:

$OD_{control}$ = absorbance of the control

OD_{sample} = absorbance in the presence of the samples of crude extract

The control only contains methanol and DPPH without ascorbic acid/ extract

3.3.2 Reducing Power Assay

3.3.2.1 Reducing Power of Standard BHA

The reducing power assay was done to evaluate the ability of *H. sabdariffa* extracts to reduce Ferricyanide complex/ Fe^{3+} to Ferrous/ Fe^{2+}. This assay was done according to the method described by Oyaizu (1986) with slightly modification. Butylated hydroxyanisole (BHA) was used as positive reference standard. The reducing power of BHA of different weights (1mg, 0.5mg, 0.25mg, 0.125mg and 0.0625mg) was dissolved in 1.0 methanol and vortexes until it mix completely. 1.0 ml of BHA extracts prepared was then added to 2.5 ml of 2.0 M phosphate buffer (pH 6.6) and later with 2.5 ml of 1% (w/v) potassium ferricyanide (sigma) in centrifuge tubes. The mixtures were incubated in water bath at 50ºC for 20 minutes.

Following incubations, 2.5 ml of 10% trichloroacetic acid (TCA) solution was added to the mixture and the mixtures then centrifuged at 1000rpm for approximately 10 minutes. A 2.5 ml aliquot of the upper layer from the centrifuged solution was transferred into test tubes and added with 2.5 ml of distilled water and 0.5 ml of 0.1% (w/v) ferric chloride solution. The mixtures were then transferred in cuvettes to measure the absorbance of the reaction mixtures. Optical density (OD) or absorbance of the reaction mixtures was taken using spectrophotometer at 700 nm after 30 minutes incubation. Increased absorbance of the reaction mixtures indicates greater reducing of BHA.

3.3.2.2 Reducing Power of Crude Extracts

A serial dilution of the extract was performed (1mg/ml, 0.5mg/ml, 0.25mg/ml, 0.125mg/ml, and 0.0625mg/ml) to test for their reducing power activity. The similar procedure as the above positive reference standard test were repeated. All tests were carried out in triplicates and the readings were averaged. Mean values and standard deviation were calculated.

Table 3.2: Reaction mixtures containing crude extracts, phosphate buffer, potassium ferricyanide and ferric chloride

Concentration of crude extracts (µg/ml)	Phosphate Buffer 0.2M (mg/ml)	Potassium Ferricyanide (mg/ml)	Ferric Chloride (mg/ml)
1000.00	2.5	2.5	2.5
500.00	2.5	2.5	2.5
250.00	2.5	2.5	2.5
125.00	2.5	2.5	2.5
62.50	2.5	2.5	2.5
0.00	2.5	2.5	2.5

3.3.3 Metal Chelating Assay

The purpose of metal chelating assay is to evaluate the ability of each extracts to chelate ferrous ion and preventing the formation of ferrozine-Fe^{2+} complex. The chelating of ferrous ions by plant extracts was determined by the method described by Dinis *et al*. (1994)

3.3.3.1 Metal Chelating for EDTA Positive Reference Standard

EDTA (ethylenediaminetetraacetic acid, sigma) was used as positive reference standard in this assay. EDTA stock of 1 mg/ml was prepared by dissolving 0.01 g of EDTA in 4 ml deionized water. The pH was adjusted while stirring with NaOH solution until most of EDTA dissolved. Once the EDTA dissolved, total volume of the solution was added with deionized water until 10 ml. 1 ml of various concentrations of EDTA at 1mg/ml, 0.5mg/ml, 0.25mg/ml, 0.125mg/ml, 0.0625mg/ml and control (without EDTA) were added to a solution of 2.0 mM $FeCl_2$ (0.05 ml). The reaction was initiated by the addition of 5.0 mM ferrozine (0.2 ml).

Table 3.3: Reaction mixtures containing EDTA, $FeCl_2$ and ferrozine

Concentration of EDTA (µg/ml)	**Volume of 2.0 mM $FeCl_2$ (µl)**	**Volume of 5.0 mM ferrozine (µl)**
1000.00	50	20
500.00	50	20
250.00	50	20
125.00	50	20
62.50	50	20
0.00	50	20

The mixture was shaken vigorously and left standing at room temperature for 10 minutes. The optical density (OD) or absorbance of resulting solution was then measured at 562 nm. Deionized water was used as blank. All tests were carried out in triplicates and the readings were averaged. Percentage of inhibition of ferrozine-Fe^{2+} complex each reading was calculated.

3.3.3.2 Metal Chelating for Crude Extracts

Crude extracts at concentrations of 1mg/ml, 0.5mg/ml, 0.25mg/ml, 0.125mg/ml, 0.0625mg/ml and control (without crude extract) were tested for metal chelating activity. Crude extract stock of 1mg/ml was prepared by dissolving 0.01 g of crude extract in 10 ml methanol. The similar procedure as the above positive reference standard test above were repeated. All tests were carried out in triplicates and the readings were averaged. Percentage of inhibition of ferrozine-Fe^{2+} complex each reading was calculated.

3.3.3.3 Determination of Percentage of Inhibition

The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the formula given below:

$$\left[\% \text{ of inhibition} = \frac{OD_{control} - OD_{sample}}{OD_{control}} X\ 100 \right]$$

Where:

$OD_{control}$ = absorbance of the control

OD_{sample} = absorbance in the presence of the samples of crude extract

The control only contains $FeCl_2$ and ferrozine without EDTA/crude extract

3.4 Toxicity Test

The toxicity effect of three different variety of *Hibiscus sabdariffa* namely UKMR-1, UKMR-2 and Arab was studied using two different tests:

a) Brine Shrimp Lethality Assay

b) Oral Toxicity Test: 14 Days Repeated Oral Administration

3.4.1 Brine Shrimp Lethality Assay

Brine shrimp lethality bioassay was carried out to investigate the cytotoxicity of extracts of *Hibiscus sabdariffa*. It is the simplest way to test the toxic effect of plant extract. The procedure determines lethal concentrations of active compounds in brine medium. The activities of a broad range of active compounds are manifested as toxicity to the shrimp.

3.4.1.1 Sample Preparation

The toxicity of plant extract was test on *Artemia salina* at concentration of 1000µg/ml, 500µg/ml, 250µg/ml, 100µg/ml, 50µg/ml, 25µg/ml, 10µg/ml. Samples were prepared by dissolving 50 mg of plant extract in 5 ml of water and then the samples solution were diluted to make the final concentration become 1000µg/ml, 500µg/ml, 250µg/ml, 100µg/ml, 50µg/ml, 25µg/ml, 10µg/ml. Each concentration of plant extract was test triplicate.

3.4.1.2 Hatching the Shrimp

Brine shrimps (*Artemia salina*) were hatched using brine shrimp eggs in a conical shaped vessel (1L), filled with sterile artificial seawater (prepared using sea salt 38 g/L and adjusted to pH 8.5 using 1N NaOH) under constant aeration for 48 hours. After hatching, active nauplii free from egg shells were collected from brighter portion of the hatching chamber and used for the assay.

3.4.1.3 Test Procedure

Ten of *Artemia salina* were exposed to the plant extract solution at respective concentration for 24 hours. After 24 hours, the lethality of the *Artemia salina* was observed to determine the toxicity of plant extract. Experiments were conducted along with control group. The percentage lethality was determined by comparing the mean surviving larvae of the treated group and control group. The data then analyzed with Finney Statistical Program to determine the LC_{50} dose of the plant extract.

3.4.2 Oral toxicity Test

The oral toxicity study is based on Organization for Economic Co-operation and Development (OECD) Guide Line for the Testing of Chemicals, Repeated Dose 28/14-day Oral Toxicity Study in Rodents, updated version of Guideline 407. The toxicity of three different variety of *Hibiscus sabdariffa i.e.* Arab, UKMR-1 and UKMR-2 were study in male Sprague Dawley rat through repeated oral administration for fourteen days at dose of 500 mg/kg body weight/day and 1000 mg/kg body weight/day. Each group was fed with the diet as shown in Table 3.4. The rats were observed everyday for abnormal clinical signs. On day 14, the animal were anesthetized and killed. The blood from the animals was subjected to hematology and clinical biochemistry analysis. Histological analysis was conducted on the kidney and liver from all treatment groups.

3.4.2.1 Animals and Maintenance

Adult Sprague-Dawley rats, 5 week-old, were purchased from the Animal House, University of Malaya. For each variety of *H. sabdariffa* extract, the rats were allocated to eight groups of 6 male and female rats (two control group and six treatment group) and were housed individually in cage in a controlled environment (temperature 25°C ± 1°C and artificial lighting was sequenced at 12-h light/dark cycles). Commercial feed and tap water was provided *ad libitum.* The animals were kept at their cages for about 7 days for acclimatization prior the experimentation.

3.4.2.2 Administration of Doses

H. sabdariffa (UKMR-1, UKMR-2 and Arab variety) extracts were administered by oral gavages to male SD rats. The treatment groups were treated with the extracts at 250, 500 and 1000 mg/kg body weight/day for 14 days. Food and drinking water were provided *ad libitum* except for overnight fasting prior to necropsy.

Table 3.4: The diet for specific group of experimental animal in 14 days of oral toxicity study

Group	Diet
Group 1 (Control Group)	Normal rat chow
Group 2 (Treatment Group)	Normal rat chow + 1000 mg/kg body weight/day of UKMR-1/ UKMR-2 / Arab variety extracts.
Group 3 (Treatment Group)	Normal rat chow + 500 mg/kg body weight/day of UKMR-1/ UKMR-2 / Arab variety extracts.
Group 4 (Treatment Group)	Normal rat chow + 250 mg/kg body weight/day of UKMR-1/ UKMR-2 / Arab variety extracts.

3.4.2.3 Clinical Observation

The experimental animals were observed twice daily for abnormal clinical sign. All animals were observed for the following parameters: fur appearance, change in skin, fur and eye color, mucous membranes, bizarre movement, convulsions, and lacrimation. Body weights of the experimental animals were also recorded on day 0, 7 and 14.

3.4.2.4 Clinical Biochemistry Analysis

In this 14-day repeated oral administration toxicity study, the clinical biochemistry analyses of blood serum from the experimental animals were conducted. Blood samples were drawn from posterior vena cava using a syringe needle under ether anesthesia. The animals had been 16 hours overnight fasted (water was not restricted) prior to necropsy and blood collecting. The blood samples were collected into vacutainer tube containing cloth activator and inert separating gel and were centrifuged at 3000 rpm for 10 min to get the serum.

Bloods serum were analyses for liver function test to determine Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), G-Glutamyl transferase, Total protein (TP), Albumin (ALB), Total bilirubin (TB), Conjugated bilirubin and Globulin levels using autoanalyzer. In addition, blood serum also subjected to renal function test to measure the level of chloride (Cl), sodium (Na), carbon dioxide (CO_2), anion gap, urea, creatinine, and potassium (P) using an ion autoanalyser.

3.4.2.5 Histopathology

Immediately after collecting the blood samples, all experimental animals were sacrificed and a thorough necropsy was performed on all animals' organs. Liver and kidney from the experimental animal were removed and vascular perfusion was performed for tissue fixation using isotonic saline followed by 10% buffered formalin solution. The organs tissues samples from the control group and the treated group were embedded in paraffin and subjected to hematoxillin-eosin staining. The pathological observations of all tissues were performed on gross and microscopic bases. The tissues samples were examined for lesions.

3.5 Hypercholesterolemic Assay

Six New Zealand White rabbits aged 8 weeks old, weighing between 1.8 – 2.0 kg were assigned to 5 groups. Each group was fed with the diet as shown in Table 4.1. Water was given *ad libitum* and the rabbits were housed in cages under a 12 h-light and 12 h-dark cycle along 8 weeks of study. Blood samples from middle artery of the ear were collected before (0) and after 4^{th} and 8^{th} week of the experiment on the respective experimental diets for measurements of Triglycerides (TG), Total Cholesterol (TC), High-density Lipoprotein (HDL) and Low-density Lipoprotein (LDL) activity. Blood sample was collected in lithium heparin tube. Complete blood clot was ensuring to take place before centrifugation. Blood serum was physically separated from cells as soon as possible with a maximum limit of two hours from the time of collection. Specimens were ensured to be free of particulate matter. Cholesterol in serum is stable for 5 -7 days at 4ºC, three month at -20ºC, and many years at -70ºC.

Table 3.5: Group of experimental animals with specific diet

Group	Diet
Group 1 (Normal Control)	100g/day normal lab rabbit chow
Group 2 (Cholesterol Control)	100g/day rabbit chow with 1% cholesterol diet
Group 3 (Treatment)	100g/day rabbit chow with 1% cholesterol diet + 250mg/ kg body weight / day of crude extract of Arab variety.
Group 4 (Treatment)	100g/day rabbit chow with 1% cholesterol diet + 250mg/ kg body weight / day of crude extract of UKMR-1 variety.
Group 5 (Treatment)	100g/day rabbit chow with 1% cholesterol diet + 250mg/ kg body weight / day of crude extract of UKMR-2 variety.

3.5.1 Total Cholesterol Measurement

The total cholesterol measurement method is based on the principle first described by Stadtman (1957) and later adapted by other workers including Tietz (2006). All reagents are contained in an analytical test pack and the test is performed on Siemens Dimension Vista Automatic Clinical Analyzer, which mixes the sample and reagents and performs the necessary absorbance measurements and calculations.

The cholesterol esters are hydrolyzed to produce free cholesterol by cholesterol esterase (CE). Free cholesterol produced in this reaction along with preexisting cholesterol in the serum is then oxidized in a reaction catalyzed by cholesterol oxidase (CO) to form cholest-4-ene-3-one and hydrogen peroxide. In the presence of horseradish peroxidase (HPO), the hydrogen peroxide formed then is used to oxidize N, N-diethylaniline-HCL/4-aminoantipyrine (DEA-HCL/AAP), to produce a chromophore that absorbs at 540 nm. The absorbance due to oxidized DEA-HCL/AAP is directly proportional to the total cholesterol concentration in the serum and is measured using a polychromatic (540, 452, 700 nm) end point technique.

$$\text{Cholesterol esters} \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol + Fatty Acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholest-4-ene-3-one} + H_2O_2$$

$$H_2O_2 + \text{DEA-HCL/AAP} \xrightarrow{\text{Horseradish peroxidase}} 4H_2O + \text{oxidized DEA-HCL/AAP}$$

3.5.2 Triglycerides Measurement

The triglycerides measurement method is based on an enzymatic procedure in which combinations of enzymes are employed for the measurement of serum triglycerides. The sample is incubated with lipoprotein lipase (LPL) enzyme reagent that hydrolyzed triglycerides into free glycerol and fatty acid. Glycerol kinase (GK) catalyzes the phosphorylation of glycerol by adenosine-5-triphosphate (ATP) to glycerol-3-phosphate. Glycerol-3-phosphate-oxidase (GPO) oxidizes glycerol-3-phosphate to dihydroxyacetone phosphate and hydrogen peroxide (H_2O_2). The catalytic action of peroxidase (POD) forms quinoneimine from H_2O_2, aminoantipyrine and 4-chlorophenol. The change in absorbance due to the formation of quinoneimine is directly proportional to the total amount of glycerol and its precursors in the sample and is measured using a bichromatic (510, 700 nm) endpoint technique.

$$\text{Triglycerides} \xrightarrow{\text{Lipoprotein lipase}} \text{Glycerol + Fatty acids}$$

$$\text{Glycerol + ATP} \xrightarrow{\text{Glycerol kinase}} \text{Glycerol-3-phosphate + ADP}$$

$$\text{Glycerol-3-phosphate + } O_2 \xrightarrow{\text{Glycerol-3-phosphate-oxidase}} \text{Dihydroxyacetone phosphate} + H_2O_2$$

$$2H_2O_2 + \text{Aminoantipyrine} + \text{4-Chlorophenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine + HCL + } 4H_20$$

3.5.3 High Density Lipoprotein (HDL) Cholesterol Measurement

The high density lipoprotein (HDL) assay measures serum HDL cholesterol levels directly without the need for sample pretreatment or specialized centrifugation steps, using a two reagent format. In the first reaction, chylomicrons, VLDL and LDL form water soluble complexes with dextran sulfate in the presence of magnesium sulfate. These complexes are resistant to the polyethylene glycol (PEG)-modified cholesterol esterase (CE) and cholesterol oxidase (CO) that react with HDL cholesterol. In the second reaction, in the presence of oxygen, the HDL cholesterol is oxidized to 4-cholestenone and hydrogen peroxide. The generated hydrogen peroxide then reacts with 4-aminoantipyrine (4-AAP) and N-(2-hydroxy-3, 5-dimethoxyaniline (HSDA) in the presence of peroxidase to form a colored dye that is measured using a bichromatic (600/700 nm) technique. The color intensity of the dye is directly proportional to the serum HDL-C concentration.

$$\text{HDL, LDL, VLDL' Chylomicrons Chylomicrons} \xrightarrow[MgSO_4]{\text{Dextran } SO_4} \text{Non-reactive LDL, VLDL, + HDL Cholesterol esters}$$

$$\text{HDL Cholesterol esters} + H_2O \xrightarrow{\text{PEG-CE}} \text{HDL Cholesterol, RCOOH}$$

$$\text{HDL Cholesterol} + O_2 \xrightarrow{\text{PEG-CO}} \text{Cholestenone} + H_2O_2$$

$$H_2O_2 + \text{4-AAP} + \text{HSDA} + H^+ + H_2O \xrightarrow{\text{Peroxidase}} \text{Color development} + H_2O$$

3.5.4 Atherosclerotic Plagues Analysis

3.5.4.1 Sample Abstraction

The experimental animals were sacrificed after 8 weeks of study. Dissection was done to the ventral side of the rabbit, followed by abstraction of aortas from experimental animals for assessment of atherosclerotic plagues. The aorta between its origin and bifurcation into the iliac arteries was dissected out. Several steps were done to prepare the aorta sample from each group of experimental animal before the total area of aorta covered by atherosclerotic plague can be measured. The abstraction of the aortas was done as soon as possible to prevent the organs from damage

3.5.4.2 Fixation and Staining of Organ Specimens

The aorta was rinse with 0.9% normal saline to eliminate excessive blood fluids. The deposited fat around outer surface of aorta was cleaned and removed. Then the aortic strips were opened longitudinally and immersed in 10% buffered formalin solution for 24 hour and then rinsed briefly in 70% alcohol. The tissue was then immersed in Herxheimer's solution that contained Sudan IV (5 gm), ethyl alcohol 70% (500 ml) and acetone (500 ml) at room temperature for 15 min and washed in running water for 1 hour. The total atherosclerotic areas of the intimal surface of the aorta were measured using ImageJ Software. The extent of atherosclerosis was expressed as a percentage of the luminal surface that is covered by atherosclerotic changes to the total area of aortic strip.

3.6 Phytochemical Analysis

3.6.1 Thin-layer Chromatography (TLC)

TLC is a simple and quick procedure to separate and determine how many components are in a mixture. Separation experiment was performed on silica-based TLC plate that was product of Merck (Darmstadt, Germany). Before sample application, the TLC plates (100 × 100 mm) were cut to a working size of 30 × 100 mm. A sample starting line was placed 5 mm from the plate bottom edge, allowing a maximum eluent front migration distance of 95 mm. The sample was applied to the TLC plate as a small spot of a solution.

Solvent mixture of acetone and methanol (3:7) was used as mobile phase. After the solvent has evaporated, the TLC was propped vertically in a closed container. The plant sample spot on the thin layer plate must be positioned above the level of the solvent in the container. If it is below the level of the solvent, the spot will be washed off the plate into the developing solvent. When the solvent front has nearly reached nearly the top of the adsorbent, the thin layer plate was removed from the container.The bands developed on the TLC plate were check under visible and UV-light and the R_f value for each bands were calculated. The TLC plate then was sprayed with dragendorf, anisaldehyde, vanillin-H_2SO_4 and iodine to determine the compounds.

Calculation of R_f value of each compound using the following equation:

$$R_f = \frac{\text{Distance traveled by compound (x)}}{\text{Distance traveled by solvent front (y)}}$$

3.6.2 LC-MS/MS Analysis

3.6.2.1 Sample Preparation

The dried and powdered calyxes of Arab, UKMR-1 and UKMR-2 *H. sabdariffa* variety were extracted with distilled water. After 24 hours, the extracts were filtered. The filtered extracts were evaporated to a concentrated crude extracts under reduced pressure using a rotary evaporator at 40-45^{o}C and then converted to powder by freeze drying. 0.02g powdered sample extracts of each *H. sabdariffa* variety were re-dissolved with methanol and filtered with 0.2uM nylon filter prior to analysis.

3.6.2.2 LCMS/MS conditions

A Phenomenex Aqua C18 column (50mm x 2.0mm x 5uM) was used for the separation. The mobile phase consisted of solvent A, H_2O and solvent B, acetonitrile containing 0.1% formic acid and 5mM ammonium formate (v/v). The mobile phase (delivered at 1.0 mL/min) was a linear gradient elution system of solvent A and solvent B, and the elution system was programmed as follows: 10%–90% B for 0 – 8.0 min. After holding the composition of 90% solvent B for the next 3 min, the column was returned to its starting conditions until 4.0 min for column balance.

3.7 Statistical analysis

The experimental results are expressed as the means ± SD. Data were assessed using analysis of variance (ANOVA). Bonferroni's multiple tests were used to determine the difference among groups. A P-value less than 0.05 ($P < 0.05$) was considered statistically significant.