CHAPTER 3

MATERIALS AND METHODS

3.1 Plant Materials

Fresh fruits of *Rhodomyrtus tomentosa* were collected from Kuala Rompin, Pahang in May 2008 and between October to November 2009. It has been identified by plant taxonomist Professor Dr Ong Hean Chooi from Institute of Biological Sciences, University of Malaya, Kuala Lumpur. The ripe fruits were dried under indirect sunlight. The quality of fruits must be free from plant disease, fungus and soil sediments.

3.2 Extraction of Plant Compounds

The fruits of *Rhodomyrtus tomentosa* were cleaned and dried under indirect sunlight for one week until completely dried. The dried fruits which were cut into small pieces were then ground to powder form by using Continuous Chinese Herbs Grinding Machine and it was stored to further study.

The extraction of bioactive compounds was carried out in different solvents. Different crude extracts were made by soaking dried powder into four different solvents water, methanol, chloroform and petroleum ether at a ratio of 1:10. (10g of powder were mixed into 100ml of particular solvent). The mixtures were kept at 40°C in water bath for 5 days in a closed system sealed with parafilm and occasionally shaken and filtered with filter paper (no. 1). The solvents were concentrated by rotary evaporator under reduced pressure and at a temperature below 50°C to maintain bioactive compounds from being destroyed. The crude extracts were kept in airtight container at 20°C until further used.

3.3 Separation of chemical compounds

A) Thin layer chromatography

B) High Performance Chromatography

3.3.1 Identification of chemical compounds in *R. tomentosa*

Developing chamber was prepared by adding developing solvent system to chamber to a depth of no more than 1/2cm. TLC was performed on silica gel, 60 F_{254} aluminium backed plates. Lightly draw a pencil line approximately 1 cm from the end of the plate. The extract solution was draw up with a capillary glass tube.The glass tube vertically was hold over the plate. The glass tube then aimed over the pencil line. Touch the tip of the glass tube onto the surface of the alumina-coated plate to dispense an approximately 1mm-sized spot of the solution. The spot should be small and concentrated. TLC plate was placed in developing chamber. The pencil line with spots must be above the level of the solvent in the developing chamber. The developing solvent was allowed to migrate approximately up the length of the TLC plate. The plate then removed from the chamber and immediately marked the position of solvent line. The plate was then allowed to air dry. The spots were visible but will fade with time. Circle the spots with pencil. Spray the plate with reagent and gave a light heat. Colour spot will develop and retention factor (R_f) for each spot was calculated. R_f value is used to describe the characteristic of samples. The R_f value is determined when component of particular compound (x) cm moved from the origin divided by the distance travelled by the solvent front (y) cm.

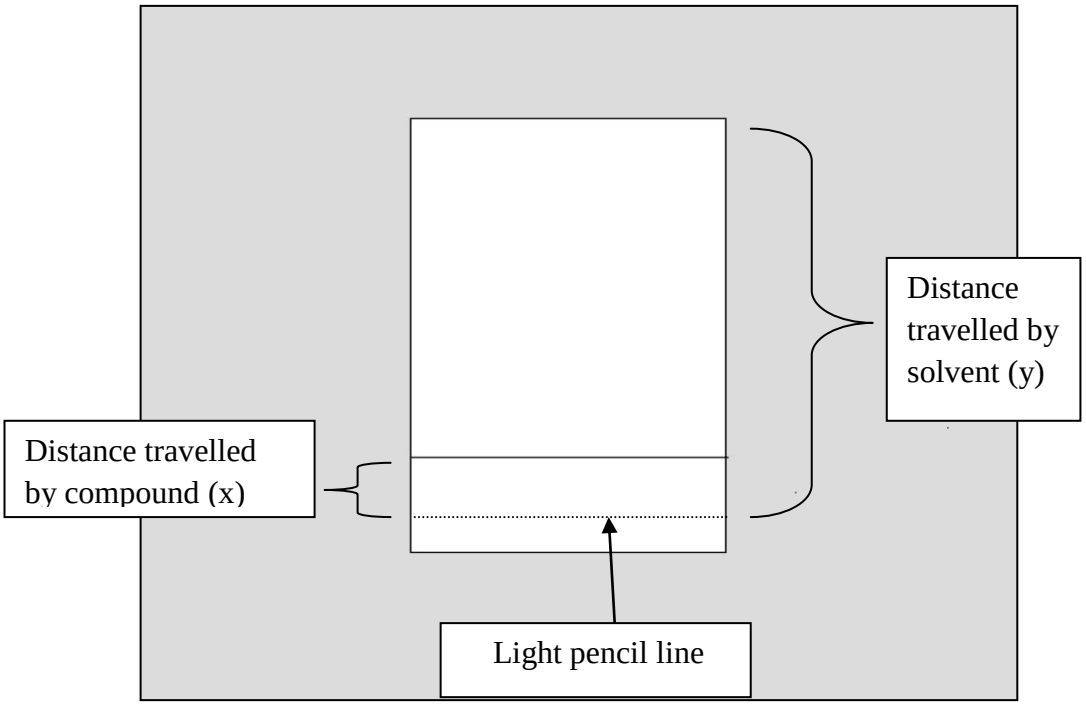


Figure 3.3: Diagram of determination of Retention factor (R_f) value on TLC plate

$$\text{Retention factor } (R_f) \text{ value} = \frac{\text{Distance travelled by compound (x) cm}}{\text{Distance travelled by solvent (y) cm}}$$

A) UV-Visible light

Under visible light, the coloured chemical compound can be seen clearly. The chemical compound that appeared is a compound that contains pigments that absorb lights. TLC chromatograms of each sample are observed under visible light. Available bands on plate were marked and R_f value were calculated. (Green colour band indicate the presence of chlorophyll in the sample while carotene gives red, orange or yellow bands).

B) Dragendorff reagent

Dragendorff reagent was used to spray the TLC chromatograms to determine the presence of alkaloid compound in the samples. Dragendorf Reagent was prepared by mixing 5ml of Solution A [1.7g basic bismuth nitrate in 100ml H_2O-HOAc (80:20)] with 5ml Solution B [40g KI in 100ml distilled water], 20g acetic acid and 70ml distilled water were added. The stock of dragendorff can stable for several weeks and can be kept in refrigerator. (Orange spot on the plate indicates the presence of alkaloid and choline in the sample, koumarin gives an orange spot). R_f value for the alkaloid compounds was calculated and recorded.

C) 10 % Vanillin reagent in H_2SO_4

After it was sprayed onto the TLC plate, it then heated at about 100ºC. If the purple colour appears, the compound is terpenoid but if the green colour appears, it shows the presence of phenol compound. Vanillin reagent was prepared by mixing 5% ethanol sulfuric acid with 1% ethanol vanillin. TLC chromatograms were dyed with Vanillin Reagent. Then they were heated in a hot plate at about 100ºC. (Terpenoid gives purple band while phenolic compound gives red or other colour spot). R_f value of each coloured spot was calculated and recorded. All the chemical compounds available in each sample were isolated from TLC plate and transferred into sample tubes.

D) Aniseldehyde

Anisaldehyde solution is a universal reagent for detecting and identifying a wide range of natural and synthetic products. Spray with a solution of freshly prepared 0.5ml p-anisaldehyde in 50ml glacial acetic acid and 1ml 97% sulphuric acid. Phenols turn violet, blue, red, grey or green when react with aniseldehyde reagent

3.4 High Performance Liquid Chromatography (HPLC) and Gas Chromatography Mass Spectrophotometry Analysis

3.4.1 HPLC analysis

3.4.1.1 Sample preparations for HPLC

Methanol and water extracts were dried using rotorary evaporator under low pressure. Thick crude extract then kept in freezer at -20°C. Water crude extract then subjected to further extraction by freeze drying using freeze dryer. Methanol crude extract was obtained by drying in an oven at 40°C for 7 days.

3.4.1.2 Instruments and HPLC analyses

HPLC analysis of *R. tomentosa* was based on method by (Hao *et al.*, 2001). *R. tomentosa* extract was diluted and filtered through 0.45µm syringe filter. Analyses were performed using HPLC reverse phase DAD on C_{18} hypesil column. Flavonoids and phenolic acids were detected at 280nm and 360nm wavelength. Mobile phase were performed by the dual pumping system, Mobile A [water-acetic acid (97:3, (v/v] and mobile phase B (methanol) with flow rate at 1.0min/ml. HPLC analysis was done using Shimadzu liquid chromatography. Standard of gallic acid, quercertin and tannic were purchased from Sigma-aldrich (Life Science) and Acros Organics (New Jersey, USA). HPLC analyses and mobile phase solvents were degassed using sonicator to get rid of air bubble of the solvent before it is pumped into the HPLC machine. Detections of flavonoids and phenolic compounds were done by comparing retention times of the peaks detected with those of standard compounds and *R. tomentosa* samples.

3.4.2 GCMS analysis

3.4.2.1 Sample preparations for GCMS

Crude water extract of *R. Tomentosa* was the only extracts used in GCMS analyses as preliminary examinations indicated that they were suitable according to the specific column used in GCMS machine. Water extract was extracted using rotorary evaporator under low pressure. Thick crude extract then kept in freezer at -20°C. Water crude extract then subjected to further extraction by freeze drying using freeze dryer. The GCMS analysis of crude water extract was carried out at the Institute of Medical Research (IMR), Kuala Lumpur.

3.4.2.2 Instruments for GCMS analyses

The direct infusion mass spectrometry technique was carried out on AB SCIEX TripleTOF 5600-1 quadrapole time-of-flight mass spectrometer (AB SCIEX, California, USA) with an electrospray ionization (ESI) source. Data acquisition and processing were performed using Analyst TF 1.5 software. Positive ionization mode was recorded in the range of m/z 100-2000. The capillary and voltage of the ESI-MS source were maintained at 350°C and 5.5 kV, respectively. All other parameters were as follows: nitrogen was used as ion source gas for nebulisation, curtain gas; 15 psi, collision gas; 10 psi, declustering potential; 80V. The scan mode was used to screen the sample profile and the product ion mode was used to determine the characteristic ions for structural information.

3.5 Antioxidant Assay

3.5.1 DPPH assay

The DPPH test was adopted from Yen and Hsieh (1998). 8mg/ml DPPH was prepared by adding 0.04g of DPPH in 5ml methanol. A stock of ascorbic acid in methanol was prepared at the concentration of 400µg/ml. Then 0.02g of ascorbic acid is dissolved in 50ml methanol. The stock solutions were kept in flask which was wrapped in aluminium foil. Different concentrations of ascorbic acid and plant extracts were prepared according to the table 3.5.1.

Table 3.5.1: Reaction of mixtures containing ascorbic acid, DPPH, and methanol as positive standard references.

Concentration of ascorbic acid (µg/ml)	**Volume of methanol(µl)**	**Volume of ascorbic acid (µl)**	**Volume of DPPH solution (µl)**
200.00	475.00	500.00	25.00
100.00	725.00	250.00	25.00
50.00	850.00	125.00	25.00
25.00	912.00	62.50	25.00
12.50	943.75	31.25	25.00
6.25	959.38	15.63	25.00
3.12	967.19	7.81	25.00
1.56	971.09	3.91	25.00
Control	975.00	-	25.00

The reaction mixtures were incubated at room temperature to react for 30 minutes. The DPPH radical was used alone without any ascorbic acid as negative control. The quenching of free radicals by ascorbic acid was measured

spectrophotometrically at 517nm. Methanol was used as blank. The degree of discoloration indicates the free radical scavenging efficiency of ascorbic acid. Analyses of water, methanol, chlorofoam and petroleum *R. tomentosa* extracts were made by preparing the same procedure but by substituting ascorbic acid with plant extracts.

The percentage of inhibition of DPPH was determined using the formula:

$$\% \text{ of Inhibition} = [(OD_{control} - OD_{sample}) / OD \text{ control}] \text{ X } 100$$

Where;

$OD_{control}$ = absorbance value of control

OD_{sample} = absorbance value of sample or crude extract

Percentage of inhibition for each concentration for the active extract was calculated by using the above formula. A graph of percentage of inhibitions against concentration was plotted so that IC_{50} value could be determined. The IC_{50} value is the concentration at which 50% of DPPH radicals is inhibited.

3.5.2 Ferric Reducing Power Assay

The method of Benzie and Strain, (1996) was used. Generally, vary amount of 5mg, 10mg, 15mg, and 20mg of each crude extract were dissolved in 1ml methanol and vortexed until it mixed completely. 1ml of crude extracts prepared was then added with 2.5ml of 0.2M Phosphate Buffer (pH 6.6) and later with 2.5ml of 1% (w/v) potassium ferricyanide. The mixtures are incubated in water bath at 50ºC for 20 minutes. Following incubation, 2.5ml of 10% trichloroacetic acid (TCA) solution was added to each mixture and then centrifuged at 1000 rpm for approximately 10 minutes. 2.5 ml of aliquot of the upper layer is transferred in test tubes and added with 2.5 ml of distilled water and 0.5 ml of 0.1% (w/v) ferric chloride solution. The colour changes were

observed. The mixtures were then transferred in cuvetts. Optical density (OD) or absorbance of the reaction mixtures was taken using spectrophotometer at 700nm. Increased absorbance of the reaction mixtures indicates greater reducing power. Butylated hydroxyanisole (BHA) was used as positive reference standard using the same methods mentioned above. All tests were carried out in triplicates and the readings were averaged out. Mean values for three independent samples are calculated for each extract. Methanol was used as blank.

3.5.3 Metal Chelating Assay

Ethylenediaminetetraacetic acid (EDTA) was used as positive reference standard in this assay. EDTA stock of 0.1g/ml was prepared by dissolving 1g of EDTA in 4 ml deionized water. The pH was adjusted while stirring with NaOH solution until most of EDTA was dissolved. Once the EDTA was dissolved, total volume of EDTA stocks was then mixed with deionized water, followed by ferrous ($FeCl_2$) and ferrozine respectively in centrifuge tubes.

The reaction mixture was shaken vigorously and left incubated in the room temperature for 10 minutes. 1ml of mixture was transferred into 3 cuvettes each. The absorbance reading was measured at 562nm. Deionized water was used as blank. All 6 samples are tested in triplicate and the readings were averaged out. Percentage of inhibition of ferrozine Fe^{2+} complex in each reading was calculated. Different concentration of mixtures was prepared in Table 3.5.3

Table 3.5.3: Reaction mixtures of deionized water, EDTA, $FeCl_2$ 2mM and ferrozine 5mM

Concentration of EDTA (mg/ml)	**Volume of deionized water (ml)**	**Volume of EDTA (ml)**	**Volume of $FeCl_2$ 2mM (ml)**	**Volume of ferrozine 5mM (ml)**
Control	3.7	-	0.100	0.200
1	3.7	0.040	0.100	0.200
2	3.7	0.080	0.100	0.200
3	3.7	0.120	0.100	0.200
4	3.7	0.160	0.100	0.200
5	3.7	0.200	0.100	0.200

i) Determination of metal chelating activity of crude extracts samples

Crude extracts were tested in different concentration of 1mg/ml, 2mg/ml, 3mg/ml, 4mg/ml and 5mg/ml and are prepared by dissolving 20mg/ml of crude extract in 1ml methanol. The crude extracts were then added with deionized water, followed by ferric chloride ($FeCl_2$) and ferrozine respectively in centrifuge tubes. The reaction mixture was shaken vigorously and left incubated in the room temperature for 10 minutes. 1 ml of mixture was transferred into 3 cuvettes each. The absorbance reading was measured at 562 nm. All 6 samples are tested in triplicate and the readings were averaged out. Percentage of inhibition of ferrozine Fe^{2+} complex of each reading was calculated.

ii) Determination of percentage of inhibition of ferrozine Fe^{2+} complex

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

% Inhibition = [(Abs control – Abs sample) / Abs control] X 100.

Where;

Abs control = absorbance reading of control

Abs sample = absorbance reading of sample

3.6 Total Phenolic Contents

The Total Phenolic Content was determined by Follin-Ciocalteau method with slightly modification (Velioglu *et al.,* 1998). 3mL water was added to each test tube, then add 50µL sample, or standard or solvent (blank). 250 µL FC reagent was added to each tube, vortex for 5 seconds. 750 µL 20% Na_2CO_3 solution was added to each tube. The mixtures were then incubated at 45^0C for 15 minutes. The absorbance was measured at 765nm using spectrophotometer against a blank after 2 hours. Total phenolic was determined using a standard curve with gallic acid (0-125mg/ml). Measurements of every sample were taken in triplicate and the results were expressed as milligram gallic acid equivalents (GAE)/g dried weight.

Numerous studies of TPC involve different kind of standards as equivalents. A review by Prior *et al.,* 2005 has expressed TPC in terms of ferulic acids equivalents, gallic acid equivalents, catechin equivalents, tannic acid equivalents, chlorogenic acid equivalents, caffeic acid equivalents, protocatechuic equivalents, and vanilic equivalents. Gallic acid is the most commonly used TPC standard for phenolic contents and expressed as mg gallic acid equivalents per (g) sample. Using this method phenolic sample was reduced from yellow (FC) to dark blue. During the reduction, phenolic groups are deprotonated leading to a phenolate anion. The chemical nature of the FC reagent thought to be a phosphomolybdate-phosphotungstate complex (Huang *et al.,* 2005). In this complex, molybdenum Mo(IV) has the characteristic yellow, after reduction to Mo(V) by phenolate ions it then becomes blue.

3.7 Total Flavonoid Content

Flavonoid contents were determined using aluminium chloride colorimetric method with slightly modification (Liu and Zhu, 2007). 1ml diluted solution containing flavonoids, 0.7ml of 5% (w/w) NaNO2 and 10 ml of 30% (v/v) ethanol were mixed for 5 min, and then 0.7ml of 10% $AlCl_3$ (w/w) was added and mixed. Six minutes later, 5ml of 1mol/l NaOH was added. Subsequently, the solution was diluted to 25ml with 30% (v/v) ethanol prior to measurement. The absorbance of the solution was measured at 510nm using spectrophotometer with methanol as a blank. Total flavonoid was determined using a standard curve with quarcetin (0mg - 2.5mg/ml).

3.8 Brine Shrimp Lethality Assay

Dried *Artemia salina* (brine shrimp) cysts were hatched in filtered artificial seawater (1 g cyst per liter) at 28°C, under conditions of strong aeration and continuous light regime (Vanhaecke *et al*., 1981). Approximately 24 hours after hatching. The phototropic nauplii were collected with a pipette and concentrated in small vial. Various concentrations of extracts ranged from 0µL/mL, 100µL/mL and 1000µL/mL were prepared in 6x4 cell culture well plates. The concentration were obtained by transferring the corresponding volume of the extracts from the stock solution to different wells and added with artificial sea water to make up the final volume of 1mL. The wells were then gently shaken to ensure that the compounds diffused adequately in the aqueous solution. Three replicates were used for each treatment and control. Each well consisted of exposing groups of 10 *Artemia* aged 24 hours to various concentrations of the extracts tested. The lethality was determined after 24 hours of exposure where the larvae were observed under stereo microscope. The numbers of survivors were counted and percentages of deaths were calculated. Larvae were considered dead if they did not exhibit any movements during observation. The data was analysed with Finney computer programme to determine LC_{50} values and 95% confidence intervals.

3.9 Animal Study

3.9.1 Maximum Tolerated Dose (MTD)

Maximal Tolerated Dose is defined as the highest dose that can exert a toxic but not life threatening in a small number of animals including consideration of body weight, survivality, gross examination, histopathology and toxicology endpoints (Foran, 1997). Sixteen rabbits were used in this experiment, and divided into two groups, four male and four female rabbits. All rabbits had free access of water and given normal diet food. Safety parameters included treatment-emergent adverse events, laboratory measurements, and changes in vital signs. All animals were examined clinically on daily basis during the experiments. *R. tomentosa* water extract and methanol was oral administered to different groups at doses of 50, 100, and 500mg/kg of body weight daily, 7 days a week for four weeks. *R. tomentosa* methanol and water extracts were diluted with water shortly before use. At each dose level, any changes on physical appearance, body weight were observed and recorded everyday.

3.9.2 Experimental Animals

This experiment was conducted at the Laboratory Animal House and Biomedical Lab of Faculty of Medicine University Malaya for 10 weeks. The location was chosen for its complete experimental facilities and convenient reason for the treatment preparation and data collection due to its near location for both laboratories. This experiment fulfilled the ethical code sanctioned by the animal ethic committee of Faculty of Medicine, University Malaya (Ethic No ISB/11/03/2009/MFM [R]). 32 adult, male *New Zealand White* rabbit were used in this 10 weeks of experiment (Zulkhairi *et al.,* 2008). The rabbits aged weeks were weight between 1.5 – 2.0kg. Rabbits were purchased from the Animal House, Faculty of Medicine, UM and kept in individual cages under room temperature, 37°C, 70 - 80% humidity with 12 hours light and dark cycle for one-week acclimatization. Animal used in concordance with procedure accepted by animal Care and Use Committee, Faculty of Medicine, UM.

3.9.2.1 Dietary composition to Experimental Animals

Table 3.9.2.1: Dietary composition and treatment for each group of animal

Experimental Group	Dietary Composition
A	Normal diet 100g/day
B	Cholesterol diet 1% 100g/day
C	Cholesterol Diet 1% 100g/day + Extract *R. tomentosa* 50mg/kg/day
D	Cholesterol Diet 1% 100g + Simvastatin 5mg/kg/day

In the beginning, all rabbits will be allowed for acclimatization to new environment for 2 weeks by having normal rabbit pallet, and water ad-libitum. The subsequent week, they were divided into 4 groups, consisting of 8 rabbits respectively. Groups labelled as group A (negative control), group B (positive control-high cholesterol diet), group C (Cholesterol Diet 1% and extract 50mg/kg/day) and group D (Simvastatin drug treatment 5mg/kg/day).

3.9.2.2 Preparation of simvastatin drug treatments

Commercial Simvastatin drug was obtained from Pharmaniaga in form of tablets. The dosage of commercial simvastatin drug is 20mg/tablet. The dosage given to the treatment of rabbit in group D was reduced to 5mg/kg. From previous studies by Hernandez-Presa *et al*., 2003 and Wiviott *et al*., 2006; 5mg/kg is the ideal dosage for rabbit as the ratio of the highest clinical dose of simvastatin in patients to the highest experimental dose of simvastatin in rabbits. By diluting the 5mg/kg of Simvastatin according to rabbit's average body weight, each subject was subjected to concentration at amount of 5ml. Treatment was given to the subject orally through force-feeding needle.

Dosage of simvastatin = [Body weight (kg) X Dose (mg)] + 5ml dH_2O

3.9.2.3 Preparation of plant extracts treatment

The dosage of *R. tomentosa* water extract used for this experiment was 50mg/kg/day. The concentration of dose was based on study made by Aylin *et al.*, (2004). In their studies, the concentration of 50mg/kg/day was potent enough to exert the desired physiological effect to the rabbits. By diluting the right amount of plant extract according to subject's average body weight, each subject was subjected to

concentration at amount of 5 ml. Treatment was given to the subject orally through force-feeding needle.

Dosage of *R. tomentosa* = [Body weight (kg) X Dose (mg)] + 5ml dH_2O

3.9.2.4 Preparation of blood samplings

Blood were taken from ear vein of non-anaesthetized rabbit at week 0, week 5 and week 10 of the experiment period. Rabbits were kept under fasting condition at least 12 hours before blood sampling to allow the relevant estimation of lipid profile levels. Blood volume was collected minimally at 7 ml using 21 G syringe. The animals were kept in rabbit restrainers, the ear, were disinfected by wiping the central vein area thoroughly with 10% alcohol swab. Then, the needle was inserted at the ¼ the length of the needle distally into the central vein, with the tips of the needle pointing toward the based of the ear. When needle is in place, blood collected into open plain tube, as it should begin to flow immediately through the needle. Repositioning the needle, or gently massaging the vein, pushing blood toward the tube, may help. Excessive suction on the syringe will cause the artery to "fade out," and blood will cease flowing. Changing the position of the ear, inserting the needle further, or withdrawing the needle partially, may aid in restoring blood flow through the needle. If blanching of the artery occurs, repositioning the ear should solve the problem. Massaging may also increase blood flow. EDTA tube for serum collection and Gel Tube for plasma collection, In order to obtain the serum, blood in the tube were left to clot for 30 minutes. Then, the tubes were centrifuged at 3000 rpm for 10minutes. Serum and plasma obtained from the centrifugation was separated into 3 appendorf tubes for duplicate and kept under -80ºC before further analysis.

3.10 Lipid Profile analysis

Blood samples were taken to the Clinical Diagnostic Labarotary, University Hospital. Lipid profiles observed are the total cholesterol, triglycerides, high-density lipoprotein (HDL) and low-density lipoprotein (LDL). Analysis was done using automatic analyser (Type 7170A, Hitachi, Tokyo, Japan) at Clinical Diagnostic Laboratory, University Hospital, University of Malaya.

3.10.1 Total Cholesterol (TC)

The total cholesterol method used on the Dimension Clinical Chemistry system is an in vitro diagnostic test intended for the quantitative determination of total cholesterol in human serum and plasma. The total cholesterol method is based on the principle first described by Stadtman (1957) and later adopted by (Rautela and Liedtke, 1978).

Lipids and lipoproteins in circulation have been strongly associated with coronary heart disease (CHD), associated lipid metabolism disorders and atherosclerosis, a cause of CHD. In figure 3.10.1 showed that cholesterol esterase (CE) catalyzes the hydrolysis of cholesterol esters to produce free cholesterol (reaction 1) which, along with pre-existing free cholesterol, is oxidised in a reaction catalyzed cholesterol oxidase (CO) to form cholest-4-ene-3-one and hydrogen peroxide (reaction 2). In the presence of horseradish peroxide (HPO), the hydrogen peroxide thus formed is used to oxidised N,N-diethylaniline-HCL/4-aminoantipyrine (DEA-HCL/AAP) to produce chromophore that absorbs at 540nm (reaction 3). The absorbance due to oxidised DEA-HCL/AAP is directly proportional to the total cholesterol concentration and is measured using polychromatic (452, 540, 700nm) end point technique.

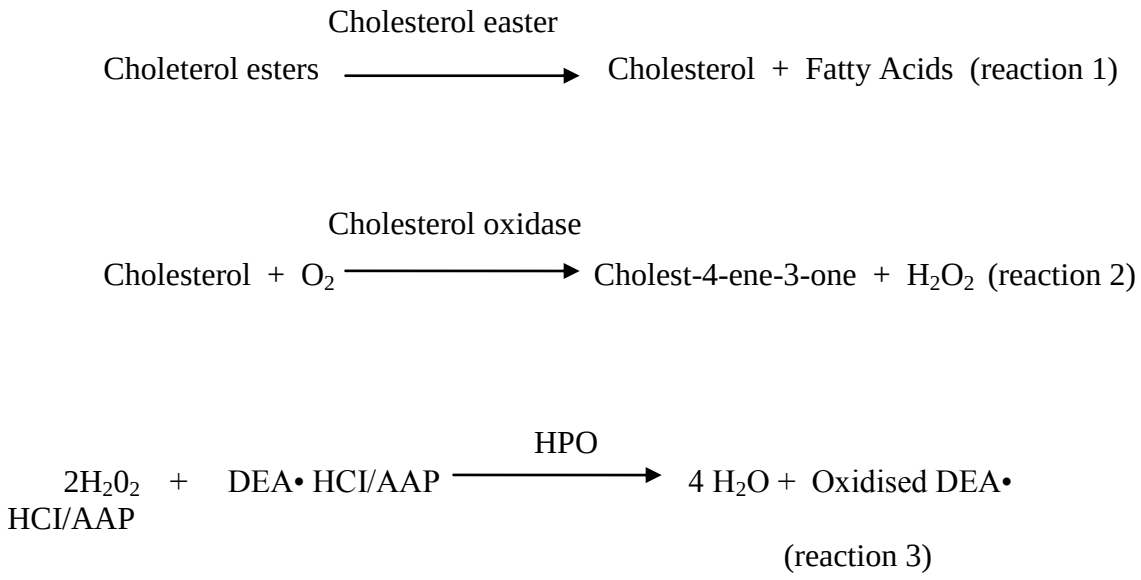


Figure 3.10.1: Pathways of chemical reactions in total cholesterol (TC) measurement

3.10.2 High Density Lipoprotein (HDL)

The AHDL method for the Dimension clinical chemistry system is an in vitro diagnostic test intended to quantitatively measure high density lipoprotein cholesterol (HDL-C) in human serum and plasma. HDL-C measurements are used as an aid in the diagnosis of lipid disorders. Plasma lipoproteins are spherical particles containing varying amounts of cholesterol, triglycerides, phospholipids and proteins. The phospholipids, free cholesterol and protein constitute the outer surface of the lipoprotein particle, while the inner core contains mostly esterified cholesterol and triglycerides. These particles serve to solubilise and transport cholesterol and triglycerides on the bloodstream.

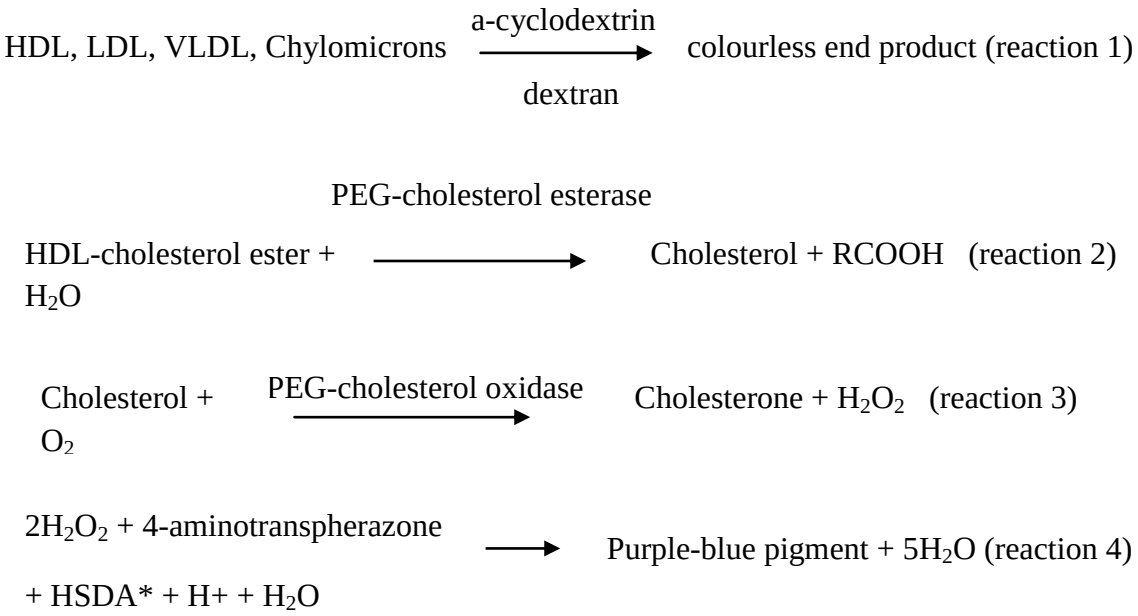
The relative proportions of protein and lipid determine the density of these lipoproteins and provide a basis on which to begin the classification. These classes are: chylomicrons, very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL).

The principle role of HDL in lipid metabolism is the uptake and transport of cholesterol from peripheral tissues to the liver through a process known as reverse cholesterol transport (a proposed cardio protective mechanism). Low HDL-C levels are associated with an increased risk of coronary heart disease and coronary artery disease. Hence, the determination of serum HDL-C is a useful tool in identifying high-risk patients. The Adult Treatment Panel of the National Cholesterol Education Program (NCEP) recommends that adult 20 years of age and over should have their total cholesterol and HDL cholesterol levels measured at least 5 years to screen for coronary heart disease risk.

The reference method for the quatification of HDL-C combines ultracentrifugation and chemical precipitation to separate HDL from other lipoproteins, followed by cholesterol measurement using the Abell-Kendall assay. This method is too

time consuming for use in routine analysis. Therefore, most laboratories utilize one of several methods for selective precipitation and removal of LDL and VLDL, followed by the enzymatic measurement of HDL-C in the supernatant fraction. Since these methods require off-line pretreatment and separation steps the assay procedures cannot fully automated. As a result, routine determination of HDL-C has suffered from long handling times and poor reproducibility.

The AHDL Cholesterol assay is a homogeneous method for directly measuring HDL-C levels without the need for off-line pretreatment or centrifugation steps. The method is in two reagent format and depends on the properties of a unique detergent as shown in figure 3.10.2. The first reagents involved a-cyclodextrin and dextran sulphate in order to stabilize LDL-C, VLDL and chylomicrons (reaction 1). The second reagent contains PEG-modified enzymes that supposedly only to react with the cholesterol present in the HDL particles (reaction 2). This selective reaction by PEG-modified enzyme only allowed HDL to be measured.



HSDA* - N- (2-hyroxy-3-sulphurophil)-3,5-dimetosiavalin

Figure 3.10.2: Pathways of chemical reactions in high density lipoprotein (HDL) measurement

3.10.3 Triglycerides (TG)

Triglycerides measurements are vital in the diagnosis and treatment of cardiovascular disease involving lipid metabolism. Besides that, its also used in liver and kidney diseases such as diabetes, nephrosis, endocrine disorders and liver obstruction.

The measurement of triglycerides involves the use of triglycerides GPO reagent (figure 3.10.3). The first step was hydrolization of sample to glycerol and fatty acids with the aid of lipase enzyme (reaction 1). The next step began with a multi-pathway involved three coupled enzymatic steps using glycerol kinase (GK) (reaction 2), glycerophosphate oxidase (GPO) (reaction 3), and horseradish peroxidise (reaction 4). Through these processes the oxidative coupling of 3,5-dichloro-2-hydroxybenzenesulfonic acid (DHBS) with 4-aminoantipyrine to form a red quinoneimine dye. The intensity of red red quinoneimine dye was calculated by colourimetric.

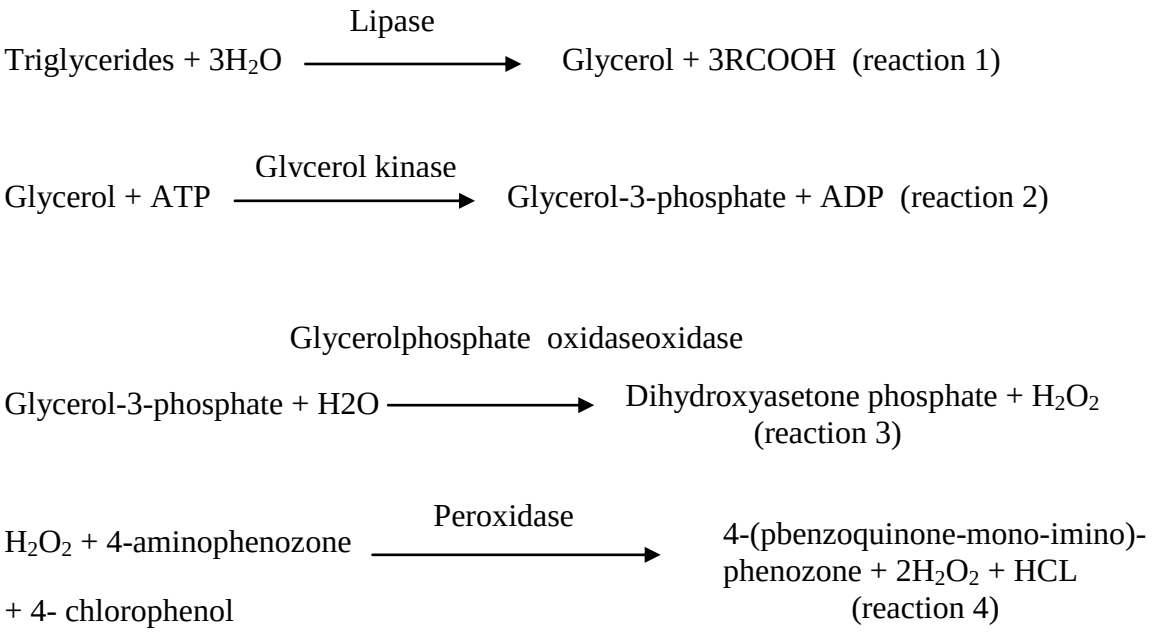


Figure 3.10.3: Pathways of chemical reactions in triglycerides (TG) measurement

3.10.4 Low density Lipoprotein (LDL)

The method of LDL measurement (figure 3.10.4) employs the special reagents that solubilize only the non-LDL lipoprotein particles through reaction of cholesterol esterase and cholesterol oxidase (reaction 1). Second reaction also involves the reaction of cholesterol esterase and cholesterol oxidase to form cholestenon and peroxide (reaction 2). Peroxide generated in then consumed by a peroxidise reaction with N,N-bis (4-sulphobutyl)-m-toluidine-disodium (DSBmT) yielding colour product (reaction 3).

$$\text{HDL, LDL VLDL, Chylomicrons} \xrightarrow[\text{Cholesterol oxidase}]{\text{Cholesterol esterase}} \text{Non colour product}$$

(reaction 1)

$$\text{LDL-C} \xrightarrow[\text{Cholesterol oxidase}]{\text{Cholesterol esterase}} \text{Cholestenon} + H_2O_2 \quad \text{(reaction 2)}$$

$$\text{H2O2} + \text{DSBmT} + \text{4-Aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Colour development}$$

(reaction 3)

Figure 3.10.4: Pathways of chemical reactions in low density lipoprotein (LDL) measurement

3.11 Lipid Peroxidation index, TBARs-Melondialdehyde (MDA) analysis

Malondialdehyde concentration was estimated using Ledwozyw *et al*, (1986) with several modifications. Standard MDA was prepared by adding together 0.01ml 1,1,3,3-tetraetoxipropane (malondialdehyde tetraetil asetal) 4.05M or MDA reagent into 1L distilled water to form 0.04mM MDA. Five different MDA standard concentrations (0.25, 0.5, 1.0, 2.0 and 4.0nmol/ml) were prepared. 0.5ml from each concentration of each standard prepared were pippeted out and inserted into 5 new test tubes. Then, 2.5ml trichloroasetic acid 1.22M (TCA 1.22M in HCl 0.5M) and 1.5ml tiobarbituric acid (0.67% TBA in 0.05M NaOH) newly prepared was added into the test tubes. Test tubes were ensured to tightly closed. Test tubes were then boiled in 100ºC water bath for 30 minutes to allow the formation MDA-TBA complex. After 30 minutes, the test tubes taken out and allow cooling at room temperature.

For the extraction of MDA-TBA complex, 4 ml n-butanol were added and vortexed vigorously for 3 minutes. Then, the mixture was centrifuged for 10 minutes at 3000 rpm (to separate between n-butanol and aqueous layers). The supernatant layer is taken out and the absorbance was read at 532 nm using spectrophotometer.

In estimation of MDA concentration in serum sample, serum was diluted 5 times by adding 0.1ml serum in 0.4ml distilled water. Then, the samples were subjected to the same method as described in the preparation of MDA standard procedure, except for the addition of TCA, the sample were kept at room temperature for 15 minutes. MDA concentration in the sample was determined using the MDA standard curve as given below;

$$\text{MDA} = \text{MDA concentration from standard curve (nmol/ml)} \times \frac{\text{Vn (4.5ml)}}{\text{Vo (0.1 ml)}}$$

V n = final volume V o = early volume

During oxidation process, malondialdehyde can be determined by TBA method. The TBA test is a colorimetric technique in which the absorbance of a red chromogen formed between TBA and malondialdehyde was measured (Rhee, 1978).

3.12 Histology analysis

3.12.1 Aorta Preparation

The full length of the aorta from the ascending to the common iliac were isolated immediately after exsanguinations and washed with ice-cold normal saline to remove debris and blood clot. Proximal of the aorta approximately 3cm was cut and fixed in 10% buffered formalin for hematoxylin and eosin histology analysis. The remaining aorta was used for Sudan IV staining. The aorta was stripped from excess adventitial and fat tissues. A longitudinal section was made on the aorta using scissor to expose the lumen. The tissue was pinned on a wooden board with the luminal surface is exposed above following immersion in 10% formalin for 24 hours. After the incubation period, the tissue was rinsed with 70% ethanol followed by immersion in Sudan IV staining for 15 min to indentify lipid containing atheromatous plaque. The tissue was rinsed with tab water to remove excess staining and the luminal surface was photographed using a single lens reflex digital camera (Canon EOS 300D, Japan). The intimal lesion was measured using computerised image analyser system and area ratio was calculated as area of lesion/area of intimal surface. The image analysis system consisted of a Macintosh lix computer (Apple) equipped with a Frame Grabber Card (Quick Capture, Data Translation), a Sony high-resolution video camera, and a Trinitron Super Mac 21 inch colour monitor.

3.12.2 Sudan IV Staining

At the end of week 10, all the rabbits' aortas were dissected free, cleaned and stained with Sudan IV. Sudan IV staining is used to stain the atherosclerotic plaques in deep red colour. Generally Sudan IV red compounds are hydrophobic azo dyes which can form hydrogen bonds with the polar head groups of membrane phospholipids. Such characteristic is useful to detect lipid accumulation in living tissues.

After the aortas were dissected free and cleaned with normal saline, they were sectioned longitudinally and stretched onto a piece of wooden board and followed by fixing with 10% buffered formalin for 24 hours. Subsequently, they were rinsed off with 70% alcohol. Then, the aortas were immersed in Sudan IV for 2-3 minutes. They were then consecutively washed in running tap water for 1 hour. The staining allows a clear depiction of the plaques due to their deep red colour.

The photographs of the aortas were made by digital camera (Nikon, Japan). The total atherosclerotic areas of the intimal surface of the aorta were measured in mm^2 using an image analysis machine. The extent of atherosclerosis was expressed as percentage of the luminal surface that is covered by atherosclerotic changes (Prasad *et al.,* 1997). The lesion area was estimated as a percentage of the intimal surface area affected by the atherosclerotic lesion using the following formula (Bocan *et al.,* 1993):

$$\text{Lesion area} = \frac{\text{lesion area in intimal surface of the aorta}}{\text{Whole area of the aorta}}$$

3.12.3 Haematoxyline and Eosin Staining

Approximately 3mm of aortas from the rabbits were fixed in 10% buffered formalin for a few days and prepared for light microscopy by dehydrating the tissue samples in an ascending series of alcohol solution for 14 hours in an automated tissue processor machine (TP1020). The series of steps are shown in table 3.12.3.

Table 3.12.3: Tissue dehydration in a tissue processor machine

Dehydration solution	Time taken
70% alcohol	1 hour
80% alcohol	1 hour
95% alcohol	1 hour
95% alcohol	1 hour
100% alcohol	1 hour
100% alcohol	1 hour
100% alcohol	1 hour
Chlorofoam 1	1 hour
Chlorofoam 2	1 hour
Chlorofoam 3	1 hour
Wax at 68° C	1 hour

3.12.4 Tissue Sectioning and Staining

For the preparation of sections in 4µm ranges, blocking using paraffin wax was carried out. Ready blocks were kept at 0°C for 3 hours and the trimming was carried out using a microtorm (Leica R M 2145) to 29 micron. Hematoxylin and eosin (H&E) were used to stain the tissue sections. The slides underwent process, colourisation and dehydration aging as shown in table 3.12.4. After perfectly dried from xylene, the slides were mounted with cover slips and a drop of DPX. The slides were then dried at room temperature for two days and tested under light microscope (Olympus CK2).

Table 3.12.4: Colourisation with Hematoxyline and Eosin (H&E)

Process	Time taken
Xylene 1	3 min
Xylene 2	3 min
70% alcohol	3 min
90% alcohol	3 min
100% alcohol	3 min
Wash under running water	3 min
Hematoxylin	3 min
Wash under running water	2 min
1% alcohol acid	5 seconds
Wash under running water	10 min
Dip in distilled water	3 min
Eosin	3 min
70% alcohol	3 min
95% alcohol	3 min
100% alcohol	3 min
Xylene 3	3 min
Xylene 4	3 min

3.13 Statistical analysis

All data were expressed as mean ± standard deviation. Statistical analysis was done by one-way ANOVA using the SPSS 17.0 computer software. One-way anova was used for statistical differences between groups and Bonfferoni post-tests were performed for multiple group comparison. In all cases statistical significance was set at $p < 0.05$.