

CHAPTER 5

DISCUSSIONS

5.1 Identification of Chemical Compounds in *Rhodomyrtus tomentosa* extracts

5.1.1 Thin layer Chromatography (TLC)

Researches have shown that traditional natural plants rich in bioactive compounds such as antioxidant, flavonoids and phenolic compounds able to inhibit the development of atherosclerosis in animal models (Kim *et al.*, 2003; Chen *et al.*, 2003). Crude extracts of *R. tomentosa* were obtained by dissolving plant material into different solvents and heated up at 40°C in water bath. This way of extraction generally appears to increase the yield of extractives, possibly due to rupture of the cell structure and to the better solvent access. The use moderate heat normally during extraction in this extraction method may be helpful as heat can be used to purify or concentrate chemical compounds and increase extraction rate or create chemical reactions. Besides that, the solubility of compound in a solvent with increasing temperature facilitates penetration of the solvent into cellular structures of the plant material. However, increased temperature beyond acceptable limit might be disadvantage as the chemical compounds might be denatured or lost. In this extraction method, types of solvent used are crucial in order to get the desired extraction compound and also to maximize the extraction of plant's chemicals compounds. Polarity influences the outcome of compounds extracted. Non-polar solvent will extract non polar compound while polar solvent will extract polar solvent. In this present study the use of water, methanol, chlorofoam and petroleum ether is essential with the objective to obtain wider range of compounds from the *R. tomentosa* fruits.

Initial test to detect bioactive compounds using TLC analyses of different *R. tomentosa* extracts have qualitatively detected the presence of terpenoids, phenolic compounds and alkaloids. Different kinds of solvents were used as they can extract

different compounds at different polarity. Water, methanol, chloroform and petroleum ether extracts of *R. tomentosa* yielded quite a number of coloured bands when sprayed with reagents. It is known that the nature of the solvent determines the type of chemicals it is likely to be extracted. The best explanation to this result is because high polarity solvent such as water and methanol tend to extract more polar chemical compounds such as sugars, amino acid and glycosides meanwhile non polar solvents such as chloroform and petroleum ether may extract non polar compounds such as plant fats, waxes and volatile oils. Differences in number of band indicated that the chemical properties of plant extracts are influenced by extraction solvent. The ratio of plant extract to extraction solvent and temperature can also influence the quality of band intensity colour.

TLC was performed to identify the number of different phytochemical within extracts. TLC was performed as described in Section 3.3.1. Solvent system investigated were Toulene-ether (70:30), Toluene-EtoAc (60:40), and Chloroform-methanol (50:50). Analysis of crude water extract resulted in separation of eighteen bands (Table 4.1.1). Four bands were seen under visible light, the colour spotted ranging from yellowish green, orange and red. The red, orange and yellowish spots under visible light correspond to phenol when sprayed with aniseldehyde reagent. As some of the phenols were invisible, the TLC plate was further sprayed with anisaldehyde reagent. All together twelve bands representing phenol were detected. Terpenoid appeared to be colourless in visible light but changed to purple and brown colour when sprayed with vanillin reagent. Terpenoid is a type of essential oils that responsible for characteristic scent, odour or smell and are generally lipid soluble and are located in the cytoplasm of plant cell. Four bands of terpenoids were detected in water extract of *R. tomentosa*. The R_f value of terpenoids in water extract were between 0.183 to 0.377. Alkaloids are generally contain one or more nitrogen atoms and as part

of a cyclic system. Most alkaloids have been regarded as compound with intermediate polarity therefore can be extracted using water. Only two band of alkaloid were detected after sprayed with dragendorff reagent. R_f value of alkaloid in water extract were at 0.166 and 0.172.

Analysis of crude methanol extract detected four bands of green, yellow and orange under visible light (Table 4.1.2). Methanol extract yielded eleven bands of terpenoids with higher bands seen in Toulene-ether (70:30) and Toluene-EtoAc (60:40 solvent system. Seven bands corresponding to phenolic compounds were detected when sprayed with anisaldehyde reagent that gave blue and purple colour. Four orange bands were observed in methanol crude extract after sprayed with dragendorff and they represent alkaloid. The R_f value of alkaloid in methanol extract were between 0.178 to 0.880. The two visible green bands in Toulene-ether (70:30) and Chloroform-methanol (50:50) were identified as chlorophyll.

The phytochemical screening of crude chloroform extract revealed the presence of five bands of purplish, green and yellow bands under visible light (Table 4.1.3). The presence of terpenoids was detected in all solvent system as it is the main constituents of *R. tomentosa* fruits. The bands related to terpenoids were higher in number compared the band representing to phenolic. A total four bands representing phenol were detected in chloroform extract. In chromatogram of the chloroform extract, the blue bands representing phenol were in the range R_f 0.266 to 0.446. The phytochemical detection of alkaloids gave positive reactions in chloroform extract. Seven bands of alkaloids were detected in chloroform extract with R_f value of 0.243 to 0.788. The visibly green bands indicate the presence of chlorophyll. Chlorophyll is usually hydrophobic compound. The R_f values for chlorophyll bands were 0.502 and 0.613.

A bright yellow colour band was found in visible light and few turned into purple bands when sprayed with vanillin reagent corresponds to terpenoids (Table 4.1.4). Most of the terpenoids were detected in Toluene-ether (70:30) solvent system. There was less alkaloid detected in petroleum ether crude extract. Only one band of brown and changed to bright yellow when sprayed with dragendorff reagent at R_f value 0.525 in Toluene-EtoAc (60:40). Four band of purple and blue after react with anisaldehyde reagent corresponds to phenol compound were detected. The R_f value for phenol was 0.447 to 0.741. The chlorophyll was visible as red fluorescing zones in the chromatograms of the petroleum extract. In the chromatogram of the petroleum ether extract were found in the range of 0.153 to 0.249.

From this TLC analyses *R. tomentosa* extracts of water, methanol, chloroform and petroleum ether of varying polarity contain phytochemicals such as terpenoids, alkaloid and phenolic compound. Plants or fruits with deep coloured are rich and good source of essential compounds of phenolics such as flavonoids and anthocyanins (Cieslik *et al.*, 2006; Sass-Kiss *et al.*, 2005; Trappey *et al.*, 2005). TLC analyses of all solvents of different polarity have detected the presence of alkaloids, terpenoids and phenolic compounds. Many functions of alkaloid related to positive and beneficial effects towards cardiovascular systems have been highlighted in journals. For instances, alkaloid of boldine extracted from *Peumus boldus* was known to have the ability to decrease oxidation of low density lipoprotein LDL (Santanam *et al.*, 2004) while berberine, an alkaloid derived from berberis plant have direct effect that lowers the cholesterol level in blood that can prevent atherosclerosis (Cheng *et al.*, 2011). Over the past several years, terpenoids have been intensively studied for its cardiovascular protection effects. Terpenoids have been shown to possess antioxidative abilities especially against lipid peroxidation (Grassmann *et al.*, 2002). Interestingly, intake of terpenoids rich supplement was able to inhibit the copper-induced oxidation of linolenic

acid or the human low density lipoprotein (LDL) (Grassman *et al.*, 2001) which is the main cause of atherosclerosis. Therefore, it can be concluded that *R. tomentosa* extracts have a various different composition of chemical compounds. Such compounds may serve the purpose of combating atherosclerosis.

5.1.2 Analysis of Chemical Constituents by High Chromatography Liquid Chromatography (HPLC)

According to the result from HPLC analyses, quercetin, tannic and gallic acid were definitely identified as the type of phytochemicals found in *R. tomentosa* extract by its elution with respective standard. Standard and compounds have been identified according to their retention time and UV-vis spectra (λ_{max}). Retention times of gallic acid, quercetin and tannic acid of *R. tomentosa* extract correspond to retention time of standards of gallic acid, quercetin and tannic acid (table 4.2.1 and table 4.2.2). The chromatograms were processed at 280 or 360 nm. In this current study, C₁₈ solid phase was used to detect chemical compounds in *R. tomentosa* extract.

The presence of quercetin, gallic acid and tannic were associated with purple-skinned *R. tomentosa* fruits. Phytochemicals such as quercetin, gallic acid and tannic acid found in HPLC analyses may be increased as the fruit ripened (Araceli *et al.*, 2011). The presence of quercetin, gallic acid and tannic acid in *R. tomentosa* extract indicated that the sample was capable to have high antioxidant activity. Thus, the antioxidant activity was due to the existence of the high content of flavonoids and phenolic inside the crude extract of *R. tomentosa*.

As a potent dietary polyphenol, quercetin is known as anti-inflammatory, anti-proliferative and anti-oxidative effects (Boots *et al.*, 2008; Hirpara *et al.*, 2009 and Bischoff, 2008). Tannic acid, a naturally occurring plant polyphenol and was said to be beneficial as it can give positive changes in plasma lipid profiles as it can lower total cholesterol and high density cholesterol (Yugarani *et al.*, 1992). Tannic acid has been

shown to possess antioxidant (Lopes et al., 1999) which is useful in fight against atherosclerosis and lipid oxidation. Relatively, much of its antioxidant actions are due to hydrophobic “core” and hydrophilic “shell” (Isenburg *et al.*, 2006).

5.1.3 Analysis of Chemical Constituents by Gas Chromatography Mass Spectrophotometry (GCMS)

In GCMS study, direct infusion mass spectrometry analysis was used to determine the phytochemical profile in water extract from the fruit of *R. tomentosa*. We found that water extract of *R. tomentosa* exhibited similar spectrum as depicted in figure 4.2.13, 4.2.14, 4.2.15, 4.2.16, 4.2.17, 4.2.17, 4.2.18 and 4.2.19. Data (pseudomolecular ion, collision energy and main fragment ions observed in MS²) for compounds detected in all extracts along with their corresponding reported values for comparison are summarized in table 4.2.3. The molecular weight and fragment ions of compound no 1, 2, 3, 10, 11, 16, 21 were in agreement with those reported in the literature, in which the fragmentation pattern of each compounds have been well described. The remaining fifteen compounds at m/z 151.0608, 165.0402, 170.0173, 171.1024, 187.0975, 193.0352, 195.0510, 201.1130, 207.0510, 209.0300, 225.1128, 243.1234, 267.0716, 343.2117, 345.2272, 313.2376, 361.0767, 383.1156 were not identified and required further investigation. All together, malic acid, gallic acid, caffeic acid, dihydrocaffeic acid, quinic acid, brevifolin carboxylic acid, octadecenoid acid and galloyl glucose were elucidated from this current study.

R. tomentosa belonging to the Myrtaceae family, and the content of malic acid has been detected in other myrtle fruit known as, *Myrtus communis* (Martin *et al.*, 1999). The content of malic acid in *R. tomentosa* was elucidated with mass charge of 133.0139. One of the best documented clinical researches showed that malic acid in pomegranate juice was able to lower LDL and cholesterol at the same time increase HDL level in lipid profile parameter (Aviram *et al.*, 2000). Gallic acids are plant

polyphenols that widely distributed in plant kingdom. The presence of gallic acid was detected in *R. tomentosa* fruit as it had mass charge of 169.0142. Several studies have indicated the important use of gallic acid as antioxidant (Maggi-Capeyron *et al.*, 2001) and an important anti-inflammatory and slowing down atherosclerosis process (Chantal *et al.*, 2005). *R. tomentosa* fruits also contain naturally occurring phenolic compound known as caffeic acid. Several reports are available on the beneficial of caffeic acid like antioxidant (Kono, *et al.*, 1997), free radical scavenging (Gulcin, 2006), antimutagenic (Yamada and Tomita, 1996), anticarcinogenic (Chen *et al.*, 1996), lipooxygenase inhibitor (Nardini *et al.*, 1995) and chelator of metal ions (Psotova *et al.*, 2003). The water extract of *R. tomentosa* was found to have quinic acid. The antioxidant properties of quinic acid based on DPPH assay was proven by (Chung *et al.*, 2005) and also antiperoxidative (Luis *et al.*, 2002). Fatty acids are known to be produced by plant systems. Octadecenoic acid is one of the fatty acids found in *R. tomentosa*. Octadecenoic acids extracted from plant gained much attention in research because of their strong anti-fungal activity (Masui *et al.*, 1989). Galloyl glucose found in *R. tomentosa* fruit is a type of phenolic acid with gallic acid ester, and the galloyl groups are attached to a glucose unit by ester bonds (Cammann *et al.*, 1989). The free radical scavenging abilities of galloyl compound such as galloyl glucose was mentioned by Wolfe and Liu, 2008. Another remarkable property of galloyl glucose is inhibition of COX-2, which is the main element in inflammatory processes (Hou *et al.*, 2007) and also to alleviate lipid and fat level (Ikeda *et al.*, 2005).

5.2 Antioxidant Activity of *Rhodomyrtus tomentosa* extracts

5.2.1 DPPH radical scavenging Assay

The percentage of inhibition of DPPH radical scavenging activity is summarized in figure 4.3.1. The analyzed range of DPPH scavenging activity is between 0 μ g/ml and 200 μ g/ml. For the control ascorbic acid was used as standard assay because of its high antioxidant properties. Four different *R. tomentosa* extracts, methanol, water, petroleum ether and chloroform were used. DPPH radical is widely used because it is the stable organic nitrogen free radical with a dark purple colour when reduced to its non radical form by antioxidants becomes colourless. DPPH has a maximum absorption around 515nm and 518nm. And because the nature of its stable properties, it is useful to evaluate antioxidant activity (Sanchez-Moreno, 2002). Antioxidant activity does have direct effect on DPPH radical scavenging activity and any decrease in absorbance can be evaluated at 517nm (Sultanova *et al.*, 2001). The importance of DPPH assay in evaluating the free radical scavenging effectiveness of many antioxidants has been highlighted by (Ozcelik *et al.*, 2003). The purple colour of DPPH reagent was decolourised into light purple to yellow when the odd electron paired off in the presence of a free radical scavenger. The degree of reduction in absorbance measurement is indicative of antioxidant power of extract. The more antioxidants contain in a sample, the more reduction of the DPPH will occur. It is said that a DPPH is scavenged by antioxidant through donation of hydrogen atom to form a stable DPPH radical molecule (Shimada *et al.*, 1992).

The different kind of *R. tomentosa* extracts quenched DPPH at different scavenging activity. Based on the graph in Figure 4.3.1, we can suggest that the best extraction is in methanol extract, followed by water extract, chloroform and the least best form of extract is in petroleum ether. At concentration of 200 μ g/ml the scavenging effect of all extracts increased to 62.13% (methanol), 59.17% (water), 34.19%

(chlorofoam) and 20.29% (petroleum ether). DPPH assay proved that *R. tomentosa* extracts of methanol and water had higher scavenging activities than *R. tomentosa* extracts of chloroform and petroleum ether. From the figure 4.3.1 also shows that the scavenging activity of all extracts on DPPH radical were all relatively lower than that of ascorbic acid at the same concentration.

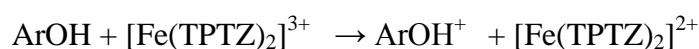
IC₅₀ value is defined as the concentration of test compound required to achieve 50% maximal inhibition. The IC₅₀ values calculated from the graphs of percentage of DPPH radicals versus concentration of crude extracts are summarised in the figure 4.3.1. Ascorbic acid (vitamin C) was used in as positive control for antioxidant activity in this study because of its strong antioxidant properties. The DPPH assay shows that ascorbic acid which act as a standard had the lowest IC₅₀ all samples which was 0.51µg/ml. Among the extract, methanol extract had the lowest IC₅₀ value at 107µg/ml. This means 107µg/ml of methanol extract of *R. tomentosa* is needed to achieve IC₅₀ value. The IC₅₀ of water extract was at 154µg/ml. Meanwhile the IC₅₀ of chloroform extract and petroleum ether extract cannot be determined because the scavenging percentage value is too low to achieve IC₅₀ value in the graph. The lower the IC₅₀ value the higher the antioxidant activity. Methanol and water extracts managed to achieve IC₅₀ value due to higher antioxidant contents. Lower antioxidant activity was observed in chloroform and petroleum ether extracts.

The antioxidant activity in plant extract is generally known by the presence of phytochemicals. The higher antioxidant activity indicated higher amount of flavonoids, phenolics, alkaloids, terpenoids and other type of phytochemicals (Maltas *et al.*, 2011) hence the higher phytochemicals in methanol extract and water extract in figure 4.3.1 explains its exhibition of better scavenging activity. This is true according to Prashant *et al.*, 2011 who have made a review on phytochemical screening and extraction stated that methanol (most polar solvent) has had higher variation of active

phytochemical components followed by water (polar solvent) and the non polar solvent with least components extracted. Therefore, the results in DPPH assay have shown that different solvent extractions gave different antioxidant scavenging activity due to chemical composition of phytochemicals in the content of the extract.

5.2.2 Ferric Reducing Antioxidant Power (FRAP) Assay

Ferric reducing antioxidant power (FRAP) assay was used to determine the free radical scavenging activity and the reducing potentials of the antioxidant constituents of *R. tomentosa* extract. This method was developed by Benzie and Strine (1996). During the reaction, an antioxidant such as polyphenol (ArOH) is able to donate a single electron, the Fe(III)-TPTZ is reduced to the blue Fe(II)-TPTZ complex which then detected at 700nm.



Antioxidant compound such as polyphenol which act as reducing agent exert their effect by donating hydrogen atom to ferric complex and thus breaking Fe(III)-TPZ radical chain reaction. After the reaction, colour changes of test solutions were seen from light yellow to green or dark blue colour solution depending on the reducing power of each antioxidant samples. FRAP assay has been commented as might not be the best form of assay test either in vivo or food system due to its low pH which may prevent electron transfer. More over the use of ferric (Fe^{3+}) has its own disadvantage as the antioxidant activity is too dependent on its ability to reduce iron (Ou *et al.*, 2002.)

The ferric reducing antioxidant power FRAP of *R.tomentosa* extracts are summerised in Figure 4.3.2. Similar to the results obtained for the DPPH free radical

scavenging activity, *R. tomentosa* extracts showed strong antioxidant activity. From the graph the absorbance of each extracts were increased with increasing concentration. Increased absorbance indicated the increasing in antioxidant activity. Butylated hydroxyanisole (BHA) was used as standard control. Based on the figure 4.3.2, the *R. tomentosa* extracts indicated antioxidant activity however its' potential activity were lowered than BHA. Methanol extract of *R. tomentosa* shows highest reducing capacity with absorbance of 0.162nm at concentration of 500µg/ml. Meanwhile chloroform extract of *R. tomentosa* show second highest absorbance at 0.129nm at concentration of 500µg/ml. In the presence of chelating agent such as antioxidant, the complex Fe(III)-TPTZ is reduced, resulting in a decrease in red colour of complex. Milos *et al.*, (2000) has claimed that high reducing activity related to high phenolic and antioxidant contents. Therefore, reducing capacity of *R. tomentosa* extracts is an indicator of its potential antioxidant activity.

Water extract of *R. tomentosa* initially had the higher absorbance reading than that of methanol extract with absorbance of 0.067nm. At concentration of 125µg/ml, the extract started off with absorbance reading of 0.089nm which is slightly higher than methanol absorbance then at concentration 250µg/ml and onwards, the reduction activity of methanol extract and chloroform apparently had exceeded the reduction activity of water extract. Finally at concentration of 500µg/ml, the water extract had the third highest absorbance which settled at 0.106nm.

The result from figure 4.3.2 showed that chloroform extract had the second highest absorbance reading at 500µg/ml. The absorbance of chloroform extract at 125µg/ml was at 0.08nm but still lowers than methanol and water extract. Then as the concentration of extract increases the reduction activity had became higher than that in water extract. At the range of 375µg/ml to 500µg/ml, the reduction activity was even greater than in water extract. The final absorbance at 500µg/ml was 0.129nm.

The higher antioxidant activity of the methanol extract as compared to chloroform, water and petroleum ether can be attributed by the presence of phytochemicals (Prashant *et al.*, 2011). It means polar solvent such as methanol was found to be easier to penetrate the cellular membrane of plant and have the intracellular components be extracted out (Wang, 2010). Chloroform was the second highest inhibition of extract (at 500µg/ml) followed by water extract. The reason why water extract had lower antioxidant activity compared to chloroform extract was could be due to the presence of active plant's polyphenol oxidase enzyme which can degrade polyphenols in water whereas in non-aqueous such as methanol and chloroform, the enzyme remains inactive (Prashant *et al.*, 2011).

5.2.3 Metal chelating ability assay

Metal chelating assay was measured according to the method by Cheung *et al.*, 2002. From the figure 4.3.3, all extracts had chelating ability, but the ability was weak. The chelating ability is dose related which means the higher the concentration of samples the higher the chelating ability. In human body or animal, ferrous ion Fe^{2+} helps the formation of ROS, the ability to form ROS can be valuable for antioxidant property. Iron is an important element for normal physiological function. But excess dose of iron can be harmful at cellular levels. According to Fenton reaction, reduced metal such as iron may form unstable reactive hydroxyl radicals which lead to oxidative stress. Since ferrous ions are good pro-oxidant (Yamaguchi *et al.*, 1988) therefore any oxidative stress disease can be prevented by removal of ferrous ions in physiological system. In nature iron ions can be form either as Fe^{2+} or Fe^{3+} but Fe^{2+} are the most powerful pro-oxidant (Gulcin *et al.*, 2004). Therefore by removing or inhibiting formation of Fe^{2+} , it can provide protection against oxidation because iron is the most important oxidation pro-oxidant due its highly reactive state.



In this assay *R. tomentosa* extracts were tested for its ability to compete with ferrozine for ferrous ion. The chelating ability is measured via absorbance with the formation of Fe^{2+} -ferrozine complex. Lower absorbance indicates higher metal chelating activity. The ferrozine only forms with free Fe^{2+} . A decrease in the amount of Fe^{2+} -ferrozine complex formed after reaction corresponds to antioxidant properties indicated by red chromophore. Therefore a measurement of colour reduction corresponds to metal chelating activity (Elmastas *et al.*, 2006).

The analyzed ranged of metal chelating assay was between 0 $\mu\text{g/ml}$ to 500 $\mu\text{g/ml}$. EDTA was selected as standard because of its strong metal chelator. The result obtained show that methanol extract of *R. tomentosa* had the highest chelating percentage. The maximum chelating ability was 36% at concentration of 100 $\mu\text{g/ml}$. In this assay, antioxidant in methanol *R. tomentosa* extract interfered with formation of ferrous Fe^{2+} and ferrozine complex. Therefore lower absorbance indicates higher antioxidant activity. Water extract demonstrated the second highest chelating with 17.6% at 100 $\mu\text{g/ml}$. This was followed by chloroform and petroleum ether with 14% and 12%. For better comparison of chelating abilities on ferrous ions, the results were also expressed as EC_{50} , which is the effective concentration at which the ions were chelated by 50%. EC_{50} of EDTA was 39 $\mu\text{g/ml}$. However, all *R. tomentosa* extracts did not have EC_{50} values because at 50% the chelating values were lower than 50%. The chelating ability of all extracts was relatively low. Few studies have proved between chelating ability with the structure of phenolic compounds, number and location of hydroxyl groups (Santos *et al.*, 2004). The figure 4.3.3 showed the EDTA had significantly higher chelating ability than the *R. tomentosa* extracts. A study by

Andjelkovic *et al.*, 2006 found out the reason why EDTA had higher chelating ability than plant extract is due to the phenolic compound itself where the phenolic contents in plants might not been able to chelate iron as good as the EDTA.

The term “antioxidant” refers to nutrients or substance found in certain foods which can slow down or prevent oxidative damage in our body. Antioxidants main function is to neutralize free radicals which are the cause of premature aging, heart disease, cancer. Our body cannot produce enough antioxidants for maintaining optimal cellular and body function. However inadequacy of endogenous antioxidants can be supplied by antioxidants derived from diet and foods. It needs to be pointed out that although many studies have shown strong antioxidant activities of various plants and fruits however no such studies were done specifically on *R. tomentosa* fruits. Therefore current antioxidant studies gave first hand knowledge on antioxidant contents in *R. tomentosa* fruits. Flavanoids and phenolic compounds detected in water and methanol extract play an important role in antioxidant activity tests of DPPH, FRAP and metal chelating assay.

Data obtained from all three antioxidant assays revealed that *R. tomentosa* extract especially water and methanol had the highest antioxidant scavenging effects. DPPH and metal chelating assay was best presented with IC₅₀ value. In DPPH assay, methanol and water extract had the best IC₅₀ at 107µg/ml and 154µg/ml respectively. Lower IC₅₀ means better DPPH radical scavenging activity. In this study, methanol extract was stronger radical scavenging than water extract for DPPH radical. By theory, the number of reduced DPPH molecules is equal to the number of their available hydroxyl groups (Sanchez *et al.*, 1998) therefore methanol extract had more reduced DPPH molecules than the water extract. However, DPPH assay alone is not enough to give information about antioxidant contents in particular samples as one assay system is not necessarily an effective antioxidant in another system (Miliauskas *et*

al., 2004). Further test involved FRAP assay. Data obtained from FRAP assay had slightly different result compared to DPPH assay. Methanol extract had the highest reducing capability at 500µg/ml, chloroform extract as the second highest reducing capability and water extract as third highest reducing capability. Initially at concentration of 125µg/ml, water extract and methanol extract were almost the same in term on reducing capability. However at the concentration of 500µg/ml, chloroform extract had overtook water extract as the second highest reducing capability. These findings indicated that with as the concentration of extracts increases, the methanol and chloroform extracts also had their reducing capability increase. Antioxidants compounds in *R. tomentosa* extracts cause the reduction of Fe³⁺-ferricyanide complex to the ferrous form which indicated by Perl's Prussian blue complex. Therefore, Fe²⁺ can be monitored by measuring the formation of blue solvent at 700nm. The result of this assay indicated the presence of reducing agents such as antioxidants and phenolic compounds that play a significant role as physiological role and dietary antioxidants against oxidative damage.

Iron and copper are transitional metals known as pro-oxidants that accelerates lipid oxidation. Human body contains these transitional metals. These metals are essential for adequate functioning of various biochemical processes in our body. The intake of transitional metals must be at normal level. Too little and too much of transitional metal in our body can be harmful, Deficiency of copper may be serious and lethal, as shown in Menkes' Disease but too much can also be harmful to liver and brain as in Wilson's Disease (DiDonato and Sarkar, 1997). Therefore maintaining proper iron and copper homeostasis is vital for well-functioning of organs and biological systems. When in ionic state, iron accelerates lipid oxidation by breaking down hydrogen and lipid peroxides. This reaction is called "Fenton Reaction". Metal chelating assay provides information on how well *R. tomentosa* extracts react to iron in

term of chelating ability. The effective of *R. tomentosa* extracts may give protection against oxidative damage by removing ferrous ion (Fe^{2+}) that may participate in hydroxyl radical as described in Fenton Reaction. Physiologically minimizing ferrous ion (Fe^{2+}) protects against oxidative damage by inhibiting production of reactive oxygen species and lipid peroxidation. The iron (II)-chelating ability of *R. tomentosa* was determined by measuring the formation of iron-ferrozine complex.

All extracts exhibited lower chelating activities than standard control. Only methanol extract showed strongest chelating ability. Methanol IC_{50} value was $39\mu\text{g/ml}$. Again much of the chelating capacity in *R. tomentosa* can be pointed to phytochemicals such as phenolic compounds.

From all three antioxidant assays we can make conclusion that there are no single antioxidant assay that can give the best result because different assay might give different kind of antioxidant capabilities. This because in theory, there's no single specific group of phytochemical or specific individual compound can be accredited for the one single total antioxidant activity. The antioxidant capacity is considered when all antioxidants act synergistically or additively which contribute to the antioxidant activities.

Much of the antioxidant scavenging activities depends on the concentration and type of antioxidant compounds and also the polarity of solvents. These factors have been pointed out by some researchers that polarity of particular solvent does change its efficacy to extract a specific group of antioxidant compounds and influences the antioxidant properties of the extracts (Zhou and Yu, 2004).

5.3 Determination of Flavonoid and Phenolic Contents

5.3.1 Total Flavonoid Contents (TFC) in *Rhodomytus tomentosa* crude extract

Polyphenols such as flavonoids in fruits and vegetables have been reported to be beneficial in preventing the occurrence of cardiovascular disease (Huxley and Neil, 2003; Joshipura *et al.*, 2001). The nature of radical scavenging activity of flavonoids relies on the molecular structure and the configuration of hydroxyl groups (Van Acker *et al.*, 1996). Rajalakshmi and Narasimhan, 1996; Ratty and Das, 1988 explained that the increase number of hydroxyl groups on the B-rings at C-30, the higher scavenging activity of flavonoids. In cardiovascular disease, flavonoids have been proved to to protect against free-radical damage and low density lipoprotein oxidation, platelet aggregation, and endothelium- dependent vasodilatation of arteries (Cao *et al.*, 1997; Heinonen *et al.*, 1998). Some even have suggested the role of flavonoids in biological effects such as inhibition of key enzymes, mitochondrial respiration, protection against coronary heart disease and anti-inflammatory, anti-tumor and anti-microbial (Harborne and Williams, 2000).

Quercetin (3,3,4,5,7-pentahydroxyflavone) was chosen as standard in total flavonoids contents simply because it is one of the most abundant flavonoid in human diet and a very potent antioxidant (H-atom donor) in which it is able to quench ROO^\cdot and O^{2-} (Larson, 1988). And when compared to kaempferol, quercetin had a higher antioxidant activity (Ratty and Das, 1988). Several studies have found that quercetin reduces blood pressure and significantly improved endothelial function in animal models (Duerte *et al.*, 2001; and Ajay and Mustafa, 2005).

So far no other reports have focused on total flavonoid content in *R. tomentosa* fruits. Flavonoid contents were found in smaller amount than total phenol contents. The amounts of total flavonoids were varied from 0.395mg/ml to 1.828mg/ml. The variations of total flavonoids content were expected for *R. tomentosa*

extracts, due to the presence of different types of phenols and flavonoids. Flavonoid contents of water extract was the highest (1.828 ± 0.018 mg/ml) followed by methanol extract (1.602 ± 0.003 mg/ml). In chloroform extract the total flavonoid was lower at 1.499 ± 0.003 mg/ml. Petroleum ether extract contained much less flavonoid contents at 0.395 ± 0.010 mg/ml.

From the results relating antioxidant assays and total flavonoid content is concerned, the highest flavonoid content was confined to water extract compared to methanol extract. This has been supported by a literature data that flavonoids such as flavan-3-ols catechin and gallic catechin are more stable in water due to intermolecular hydrogen bonding (Zainol *et al.*, 2009) which exactly supports the reason of higher flavonoid measurement in water extract than that in methanol extract. On contrary to total flavonoid content result, the antioxidant assays gave different result where methanol extract was the highest antioxidant capacity. This could likely be due to the fact that apart from flavonoids and flavonols, other antioxidant compound such as phenolic, terpenoids and alkaloids compounds present in methanol extract in antioxidant assays (DPPH, FRAP and Metal chelating) that contribute to the overall higher antioxidant activity. The total flavonoid measurement in the current experiment was deemed accurate because the use of $AlCl_3$ which has its own advantage over other method of using 2,4-dinitrophenylhydrazine because $AlCl_3$ is said to be specific for total flavonoid that comprises flavanols and flavones whereas 2,4-dinitrophenylhydrazine only for flavanol (Edrini *et al.*, 2002).

Intake of large amount of flavonoids inversely associated with cardiovascular disease. On animal studies, flavonoids could reduce blood cholesterol concentration (Hodgson, 2008). Meanwhile the effect of flavonoids intake in human consumption and atherosclerosis has been recorded. Debette *et al.*, (2008) proved that less development of aortic plaque with consumption of flavonoid rich tea. There was

even a study on decreased of carotid atherosclerosis in middle-aged Finnish men when was associated high intake of flavonoids (Mursu *et al.*, 2007).

5.3.2 Total Phenolic Contents (TPC) in *Rhodomyrtus tomentosa* crude extracts

From the result, the amount of total phenolic content varied in different analysed extracts. The variation can be expected due to presence of different amount of phenols dissolved in extracts. Among the four samples of extracts, the *R. tomentosa* water extract demonstrated the highest phenolic content 66.515 ± 0.009 mg/g dry mass. Methanol extract of *R. tomentosa* had the second highest phenolic content with 40.000 ± 0.003 mg/g dry mass. This followed by *R. tomentosa* chloroform extract with 13.985 ± 0.006 mg/g dry mass. Petroleum ether extract of *R. tomentosa* contained the lowest total phenolic content compared to other extracts. However there is an explanation to this different results because different phenolic compounds might have been extracted by different solvents, and have different response in Folin- Ciocalteu method (Heinonen *et al.*, 1998; Kahkonen *et al.*, 1999).

R. tomentosa extracts contain numerous phenolic compounds. Different kind of phenolic compounds subsequently result in complex changes of antioxidant activity. Proven study of correlation between antioxidant activity and total phenolic content has been reported by Deepa *et al.*, 2007. The degree of antioxidant activity depends on the contents of phenolic compounds in plant (Robards *et al.*, 1999). Plants that have flavonoids as phenolic content usually have higher antioxidant activity. This because flavonoids structurally have more hydroxyl groups and orthosubstitution with electron-donating alkyl or methoxy groups which can stabilize the free radicals and then increase its antioxidant potential (Rice-Evans *et al.*, 1995). Taking *R. tomentosa* fruits is beneficial as it contains high in phenol compounds. Supplements at least 1g daily of fruits or vegetables riched with polyphenolic compounds may have inhibitory effects on

mutagenesis and carcinogenesis in humans (Tanaka *et al.*, 1998). In some diseases, iron overload is positively correlated CVD risk, by consumed phenolic compounds may have inversely the risk iron overload by lowering iron status in the body (Samman *et al.*, 2001). Samples extracted in water had higher contents of phenolic compounds than samples extracted in methanol, chloroform and petroleum ether. This implies that water extract was more powerful solvent at extracting phenolic compounds.

The present data (table 4.4.1) of total phenolic content on *R. tomentosa* extract confirmed the relation of total phenolic and antioxidant assays. Water extract in comparison with the methanol extract had been more efficient at determining the contents of phenolics. Water extract allows the phenolic compounds to bond with the oxygen molecules in the water. The main reason was because of the ability of water extract to form the hydrogen bonds with the phenolic compounds (Carey, 2003). Besides that, water was also able to form hydrogen bonds with phenolic compounds that may not have numerous hydroxyl groups, and therefore, water extract was better form of solvent at extracting more phenolic compounds than methanol extract (Carey, 2003). Another factor that might contribute to better higher phenolic content in water extract was that the water itself can extract sugar more than methanol does, therefore any phenolic compounds that chemically attach with sugar may be easily extracted with water (Normala and Suhaimi, 2011).

The relation between phenolic content and antioxidant activity was that the higher the phenolic content the higher the antioxidant activity. Phenolic contents made up from hydroxyl groups are noted for their antioxidant activity (Sanchez-Moreno *et al.*, 1999) by free radical scavenging. Some literature data have found the relation of total phenolic content and its significant correlated with antioxidant activity such as DPPH scavenging activity (Velioglu *et al.*, 1998 and Beta *et al.*, 2005). Hence, it could be

inferred that the antioxidant activity were mainly due to phenolic presence (gallic acid, tannic acid).

While considering relationship of phenolic content and antioxidant activity, the antioxidant assays such as DPPH, FRAP and Metal chelating assay somehow showed methanol extract had much better antioxidant activity than that of water extract. It is best to conclude that the higher antioxidant activity in methanol extract may be attributed to other non-phenolic phytochemicals such as flavonoids.

5.4 Toxicity of *Rhodomyrtus tomentosa*

5.4.1 Brine Shrimp lethality Assay

The aim of this assay was to evaluate the toxicity effect of *R. tomentosa* extracts on brine shrimp *Artemia salina*. Crude water extract of *R. tomentosa* was the safest of all extracts in terms of toxicity effect which gave LC₅₀ at 616.083µg/ml giving LC₅₀ values greater than 100µg/ml. This means that 616.083 µg/ml of water extract is needed to kill the 50% population of the brine shrimp. When the LC₅₀ value is higher it means that the toxicity of the crude extract is lower. Methanol extract showed intermediate toxic extract with LC₅₀ value at 316.228µg/ml. Extracts of petroleum ether was the most toxic of all extracts with LC₅₀ 31.623µg/ml. In this study, brine shrimp lethality assay measures the ability of the extracts to kill shrimp larvae. A study by (Hlywka *et al.*, 1997) has proved that there is a correlation between number of dead shrimps and concentration of extract. So far no research has been reported to determine the toxicity values of extract from *R. tomentosa* by using brine shrimp lethality assay (BSLA). From this experiment it can be concluded that *R. tomentosa* extracts of various solvents have indicated varied levels of toxicity.

5.5 Animal Study

5.5.1 Water extract as Treatments

In the present study, oral administration of the aqueous water extract of *R. tomentosa* was given along with cholesterol diet 1%. This mode of extract administration is similar as described by Tijburg *et al.*, (1997). However different method was applied by Christina *et al.*, (2007) where extract was given and mixed with solid diet with the reason of active components are better absorbed when in the solid diet. Extract given orally using force feeding needle is more suitable as the exact amount of extract can be administered accordingly and it eliminates wastage. The main reason water extract was chosen in this animal study because from the result water extract possessed the highest margin of safety and comparatively less toxic than other form of extracts. The brine shrimp bioassay resulted in LC₅₀ 616.083µg/ml. was not considered toxic than other type of extracts tested. The data is supported with the study by Hart and Humpf, 2000 where there are link between toxicological level of the plant extract and the mortality of brine shrimp. Brine shrimp larvae are fast growing and highly developing creature, so any extracts that kill them disrupts with cell growth and proliferation. In *in vitro* studies, some anticancer agent provides a positive result in brine shrimp assay with cancer as a model of cells that grow out of control (Mohammed *et al.*, 2006). In human and larger animals, we do not know how the extract effect on the human cells, tissue and organs. By applying the same reasonable concept we can make conclusion that the extract will have the same effect on physiological human and larger animal models. Besides that, there were comparatively higher concentrations of phenolic and flavonoid content in total phenolic content (TPC) and total flavonoid content (TFC) in water extract than in other form of solvent extracts.

5.5.2 Preliminary evaluation of Maximum Tolerated Dose (MTD) of *Rhodomyrtus tomentosa* on rabbits

The subchronic evaluation of rabbits that received water extract at oral doses of 50, 100 and 500mg/kg/day did not manifest any clinical sign of toxicity as the rabbit gained weight throughout the course of experiment. None of the doses tested in water extract could produce mortality in rabbit during the observation period of 1 month duration. Hence it is safe to say that water extract did not give any toxicological effect in long term duration. Meanwhile methanol extract of *R. tomentosa* produces mortality in male and female rabbits. Mortality was seen in male rabbits at 500mg/kg/day while female rabbits started to die at concentration 100mg/ml respectively. The cause of mortality was due to methanol toxicity where the methanol is rapidly metabolized in the liver to form formate, a by-product that responsible to acute toxic effect of methanol (NTP-CERHR, 2004). No statistical difference of weight in water extract because not much weight difference between control and treated rabbit the while there were mortalities of rabbits in methanol extract. The current experiment showed that water extract is safe to be consumed by rabbit even at higher dose concentration of 500mg/kg/day. This allows further in vivo test to be done in *R. tomentosa* water extract.

5.5.3 Animal Organ parameter

Result from the experiment showed that liver was the most susceptible and most damaged by the supplementation of cholesterol diet (figure 4.8.2). It was observed that morphological structure of liver in cholesterol group was so severe than that in simvastatin group and tomentosa group possibly due to hepatocytes swelling. Previous study by Ines *et al.*, (2005) on rats showed similar result, the liver weight in rats fed increased with cholesterol rich diet compared with to rats fed with control diet. This is because liver is the site of cholesterol synthesis in physiological system. Heart and kidney did not show any significant effect in weight and external morphological feature

among different groups as the heart and kidney organs were less susceptible in term of pathological damage.

5.5.4 Body Weight

The experimental model of non genetic-hypercholesterolemia rabbits has been widely accepted in studies of atherosclerosis, different levels of dietary cholesterol fed for different periods of time to generate the wanted experimental disease. The rabbit's abdominal aorta is a perfect model for atherosclerotic which can imitate human lesions if correct food and dietary conditions are given to the rabbit (Orlandi *et al.*, 2000; Weinberg and Cremers, 2003). Using experimental animals such as rabbit is crucial as it allows to consider factors such as diet, length of experiment, pathogenesis of disease, interaction of dietary and animal model, the dosage and the duration of supplementation which later translate to better and reliable results. This study showed that supplement of high cholesterol diet had significantly increase ($p < 0.05$) of body weight in cholesterol group as compared to normal group. The weight gain in rabbits on the cholesterol diet supplemented with *R. tomentosa* 50mg/kg/day was smaller than those in cholesterol group. The continue increase of weight gain shown along the treatment indicated that the rabbits were in healthy condition. This marked a good and suitable parameter for experiment on the rabbits as the animal models without any interruption or errors that might affect the outcome of this experiment.

5.5.5 Lipid Profile test

The study of lipid profiles in hypercholesterolemic rabbits was done based on comparative significant and non-significant level of TG, total cholesterol, HDL and LDL among four different groups (normal group, cholesterol group, *R. tomentosa* group and simvastatin group) since there are no specific reference range of blood lipid profiles level for rabbit's hypercholesterolemic condition. Unlike rabbit, humans have specific range of blood lipid profile which can indicate if patients suffer from various blood and lipid disorders. Reference range for healthy man are as follows: (TG < 1.7mmol/L, total cholesterol < 5.2 mmol/L, HDL > 1.1mmol/L and LDL < 2.59).

5.5.5.1 Total Cholesterol (TC)

The dietary fat intake and cholesterol rich food showed significantly positive correlation with the serum total cholesterol. In this study, the supplementation of high cholesterol diet had increased the total cholesterol (TC) level significantly in the cholesterol group. This showed that 1% of cholesterol added to rabbit's food was sufficient enough to give hypercholesterolemic effect as compared to TC level in the normal group. This may support the previous study documented that high intake of cholesterol in diet can induce high level of serum cholesterol in rabbit because it can absorb cholesterol efficiently (Chin *et al.*, 1990). The elevation of TC was related to the high content of cholesterol in the rabbit pellet food. Physiologically high TC level is due to excessive loads of cholesterols by the liver beyond acceptable level. These cholesterols are supposed to be metabolized in the liver however the excess cholesterol then being returned in the circulating blood.

Supplementation of *R. tomentosa* extract was found to give significant favourable result. It is shown that *R. tomentosa* extract gave significant lower TC level when compared to cholesterol group. As the rabbits were in hypercholesterolemic state

in week 10, *R. tomentosa* extract managed to reduce down the level significantly ($P < 0.05$) down to 32% when compared to cholesterol group, while the simvastatin treatment group able to reduce to 36% reduction of TC level significantly. This showed that *R. tomentosa* extract has positive effect in lowering the TC level in hypercholesterolemic state. The reductions of atherosclerosis plaque seen in simvastatin and *R. tomentosa* group were primarily due to reduction in serum cholesterol. Lowering the serum total cholesterol is the primary objective of any treatment in CVD in attenuating hypercholesterimic condition. Any 10% reduction on total serum cholesterol concentration can reduce the mortality rate to 15% (Gould *et al.*, 1998). Administrations of 5mg/kg/day of simvastatin, a HMG-coA reductase inhibitor were thought to reduce total cholesterol by several ways. Intake of sufficient dose somehow managed to increase LDL receptor proteins in the liver tissue (Reihner *et al.*, 1990; Kume *et al.*, 1989; Miyazaki *et al.*, 1989). At the same time there will be an increased of LDL receptor mRNA as the receptor function is to eliminate LDL in the blood which further translates as lower total cholesterol level. Total cholesterol level related to the level of VLDL which secreted from the liver (Shiomi and Ito, 1994). The main component for VLDL production is cholesteryl ester (CE) (Musanti *et al.*, 1996; Kohen *et al.*, 1995; Carr *et al.*, 1995). HMG-co reductase inhibitor inhibits production of cholesterol biosynthesis of cholesteryl ester. As such inhibition of CE leads to lower concentration of VLDL.

5.5.5.2 Low Density Lipoprotein (LDL)

The cholesterol group had significant higher LDL level as compared to normal group, *R. tomentosa* group and simvastatin group. The higher LDL level can be attributed to the down regulation of LDL receptor by cholesterol in the liver (Mustad *et al.*, 1997). As it possessed the highest LDL level among all groups, it is postulated that

high cholesterol diet intake can induce elevation level of TC, LDL and lower down HDL level as well.

As in the LDL level, Both *R. tomentosa* group and simvastatin group gave reduction down to 29% and 38% respectively compared to cholesterol group at week 10. Much of the reduction in the LDL can be attributed to *R. tomentosa* extract which rich in phytochemicals. Phytochemicals such as flavonoid is a strong chelators of free metal ions (Morel *et al.*, 1994). The content of antioxidants in *R. tomentosa* extract rich in flavonoids act by attacking free radical species by preventing them from interacting with LDL. Antioxidants in *R. tomentosa* extract might protect LDL against copper mediated modification by decreasing binding of Cu^{2+} to LDL. The decreased Cu^{2+} to LDL occurred when antioxidants react with specific amino acids residue on apolipoprotein B which normally bonds with Cu^{2+} (Retsky *et al.*, 1993). Competition between antioxidants and Cu^{2+} over apolipoprotein B leads a more decreasing affinity of apolipoprotein B to Cu^{2+} ions. In 2003, studies by Weggemans and Trautwein reported the connection between flavonoids and LDL level. In their studies, flavonoids intake did decrease LDL level by through removal of cholesterol from peripheral tissue liver for catabolism and extraction. Flavonoid intake can increase the LDL receptor in liver to remove LDL by binding to apolipoprotein B. This because flavonoids in *R. tomentosa* such are hydrophilic compounds, flavonoids are likely to bind to apolipoprotein B due to protein binding properties (Haslam, 1981). Therefore phytochemicals rich *R. tomentosa* extract can be taken as supplement to prevent elevation of LDL as this lipoprotein can easily cause health complication (e.g. CVD). Besides that antioxidants in *R. tomentosa* were thought to alter 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA) and cholesterol 7 α -hydroxylase (CYP 7A1). By altering these enzymes *R. tomentosa* antioxidants were able to lower down total cholesterol and LDL level.

Simvastatin is regarded as the most potent statin against hydroxyl free radicals (Chaudiere and Ferrari, 1999). A research on simvastatin drug interaction has managed to prove the beneficial of this drug as antioxidants potential in the blood. Simvastatin administration resulted in decrease in cell oxygen production, binding to LDL surface phospholipids and also facilitate the antioxidant action on specific simvastatin metabolites (Aviram *et al.*, 1998). The great reduction shown in the current experiment similarly proven as in previous clinical studies in lowering down LDL level (Nobuhiro *et al.*, 2006; Roberto *et al.*, 2005). These data suggested that simvastatin reduced the cholesterol content in the liver by inhibiting hepatic HMGCoA reductase, increased the concentration of LDL receptor proteins, and thus lowering the concentration of LDL-cholesterol.

5.5.5.3 Triacylglycerides (TG)

There was a significant increase ($p < 0.05$) of TG level as the effect of high cholesterol diet within groups and between groups at week 5. The increase of TG with supplementation of cholesterol diet was in agreement of previous result by Mohamedain *et al.*, 2000, TG level in rabbits increased more than 3 times after being fed with cholesterol for 1 month. At week 5, the result showed that cholesterol diet increase up to 225% elevation of TG in cholesterol group and 13% increase in *R. tomentosa* group comparison with the normal group rabbit. In this experiment *R. tomentosa* extracts did not have any effect on the TG level. Instead the level of TG increased at week 10. This result contradicts to beliefs that antioxidants generally in plants like *R. tomentosa* supposed to have a positive effect at reducing TG level. Drug therapy using simvastatin was highly beneficial to the TG levels, as seen in the lipid profile test. Simvastatin managed to significantly ($p < 0.05$) lower down the TG level by 22% compared to cholesterol group at week 10. Hernandez *et al.*, (2003) discovered that

there was a reduction of plasma TG level in hypercholesterolemic induced rabbit after treated with simvastatin.

5.5.5.4 High Density Lipoprotein (HDL)

The level of HDL in the body indicates the risk factor for the developing of atherosclerosis. The lower the HDL level, the higher the risk of having atherosclerosis meanwhile increase in HDL may slow down the risk of atherosclerosis. Physiologically HDL conveys cholesterol from the peripheral tissue to the liver for catabolism to be converted into bile salt (Assmann *et al.*, 1996). This physiological pathway involving HDL as cholesterol carrier is important in reducing the cholesterol level in the blood.

In this study, treatment of *R. tomentosa* extract of 50mg/kg.day did significantly increase the HDL level in rabbits' blood compared to cholesterol group at week 10. The HDL level in cholesterol group was significantly low at week 10. This low level of HDL is considered bad when the subject is in hypercholesterolemic state where the presence of low HDL may accelerate the occurrence of atherosclerosis. The simvastatin group was also had higher HDL level compared to cholesterol group at week 10 This is in agreement from the proven clinical studies on this drug where it is able to moderately increased HDL (Nobuhiro *et al.*, 2006).

5.5.6 Lipid Peroxidation TBARs-MDA

MDA is the most widely employed assay used to determine lipid peroxidation. Malondialdehyde (MDA) forms an adduct with thiobarbituric acid and produces TBA-MDA complex (figure 5.1) which can be measured by spectrophotometry.

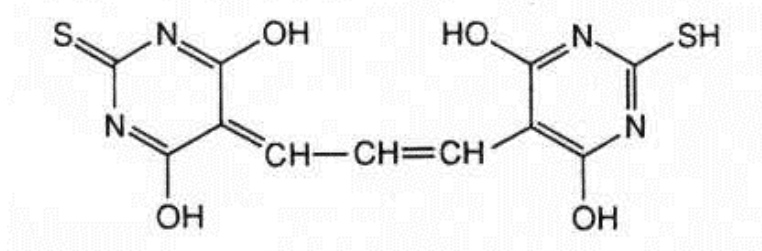


Figure: 5.1 TBA-MDA complex

The current study showed that the TBAR-MDA level in cholesterol group at week 5 were significantly elevated ($p < 0.05$) compared to other groups. And at week 10, the cholesterol groups still significantly ($p < 0.05$) higher than all groups. The increased level of TBARS-MDA level seen in cholesterol group mainly due to free expulsion into the circulation of tissue lipid peroxides caused by pathological changes (Selvam and Anuradha, 1990). *R. tomentosa* and simvastatin groups were found to have protective effect against lipid peroxidation as the level of TBAR-MDA was low in hypercholesterolemic rabbits. The supplementation of 50mg/kg/day of *R. tomentosa* extract to rabbits fed with hypercholesterolemic diet managed to suppress the TBARS-MDA almost the same as the normal group at week 10. The level of lipid peroxidation marked by the TBARs-MDA complex significantly reduced ($p < 0.05$) compared to cholesterol group at week 10. Studies by El-Demerdesch *et al.*, (2003) and Yousef *et al.*, (2004) confirmed the beneficial effect of flavonoid in lowering lipid peroxidation levels. The result obtained from this study suggested that the capability of *R. tomentosa* extract

rich in phytochemicals such as flavonoid might be capable to lowering or slowing down oxidative stress linked to lipid peroxidation. This result was proven true as the result from TBARS-MDA was found to be in line with the decreasing total cholesterol and LDL level from lipid profile test. Oxygen radicals are the causative factor to produce endothelial cell injury (Warren and Ward, 1986) which is the signal of early development of atherosclerosis. Therefore inhibiting oxidative modification of LDL by *R. tomentosa* extract rich in phytochemicals is considered to be an important therapeutic step.

5.5.7 Assessment of Aortic Lesion

Without doubt atherosclerosis is a highly prevalent disease, and is currently the major cause of morbidity and mortality in developed and developing countries. In Malaysia alone heart diseases made up 16% of principal causes of death in government hospital in Malaysia in 2009 (Ministry of Health, Malaysia, 2009). Meanwhile many diseases evolved from atherosclerosis including coronary artery disease (CAD) and myocardial infarction (MI), including hyperlipidemia, hypertension, diabetes mellitus and many more. The pathogenesis of atherosclerosis involves multi-factor processes of vascular injury, inflammation, degeneration, and thrombosis and inflammatory.

Atherosclerosis is a complex disease, with various events in early lesion development such as intimal accumulation of plasma lipoproteins, increased expression of adhesion molecules on the endothelium in the vessel wall, and monocyte margination, migration across the endothelium and accumulation in the intima, where they accumulate large intracellular deposits of lipoprotein-derived cholesterol and 'fatty streak', seen in more advanced atherosclerosis.

This study was set out to investigate if *R. tomentosa* extract rich in bioactive compounds could lower the serum cholesterol concentration in the cholesterol-fed hypercholesterolaemic rabbits and also prevent the formation of atherosclerotic plaque. This study was more on preventive approach than a treatment therefore hypercholesterolemia was induced along the course of study. Hypercholesterolemia is a major factor result in atherogenesis and in one study, diet-induced maternal hypercholesterolemia promoted fatty streak formation in New Zealand white rabbits (Napoli *et al.*, 2000).

In agreement with the findings of high serum total cholesterol and LDL in lipid profile tests, all cholesterol, simvastatin and *R. tomentosa* groups had marked

development of atherosclerotic plaque but the degrees of severity were different between groups. *R. tomentosa* extract has managed to inhibit development of atherosclerosis plaque as seen in the Sudan IV staining. The reduction in the atherosclerotic plaques could be related to the lipid lowering activity of *R. tomentosa*. *R. tomentosa* extract consists of rich antioxidant, flavonoid and phenolic compounds and other unknown beneficial bioactive compounds which very helpful in inhibition of atherosclerosis plaque. Several phytochemical compounds detected by HPLC analysis (quercetin, tannic acid, gallic acid), Thin Layer Chromatography (terpenoids alkaloids), along with the antioxidant capacity of *R. tomentosa* as seen in DPPH, Metal Chelating assay and FRAP also play role by slowing down the formation of atherosclerotic plaque. The bioactive compounds in *R. tomentosa* were believed to be acting by inhibiting absorption and endogenous synthesis of cholesterol and also increase its secretion. Certain bioactive compounds such as polyphenolics have been proved to provide inhibition of cholesterol either by absorption (Raederstorff *et al.*, 2003), endogenous synthesis (Abe *et al.*, 2000; Johnston *et al.*, 2005) and by excretion (Valsa *et al.*, 1998).

5.5.8 Assessment of Hematoxyline and Eosin Histology

5.5.8.1 Hematoxyline and Eosin Histology Examination of Aortas

Besides doing the macroscopic studies, there were also studies on histopathological examinations on the aortas. Aorta is part of cardiovascular system to carry the blood between the heart and the tissues. This oblique vessels located at posterior arch descends in the thoracic cavity where it is branching to the body wall and to the viscera. The best cell structure and morphology of normal healthy aorta can be seen in normal group. Anatomically there are three main layers in the aorta separated by layers of tissues or tunics that form the wall of typical blood vessels. The inner most layer is called tunica intima, composed of single layer of flattened, simple squamous endothelial cells. The intermediate layer is called tunica media, made up of smooth muscle cells. Between tunica intima and tunica media, there is a thin band elastic layer called internal elastic laminae however in the picture the intima was almost undistinguished from the medial layer. The outmost layer is tunica adventitia, composed manly of fibro-elastic connective tissue arranged longitudinally. The smooth muscle cells in the media are spindle-shaped and well-arranged.

There was no presence of foam cells in aortas of rabbits fed with normal diet. In normal group, there was no aortic lesion or accumulation of cholesterol deposits. Plaque free region of the aortas wall of tunica intima can be seen. This was because the rabbits in normal group were given only normal diet that does not contain cholesterol. It was observed that there were prominent in foam cells thickness in the cholesterol group compared to simvastatin group and *R. tomentosa* group.

Higher dietary intake of cholesterol effect can be seen in cholesterol group. Rabbits were given 1% cholesterol diet. The wall of aorta was surrounded with thick cholesterol plaques. Histology observation of the figure 4.11.3 and 4.11.4 show

thickening of the intimal layer and accumulation of lipid. The smooth muscle cells in the media were disordered, the intima was irregularly thickened and multilaminated, the internal elastic lamina was also thickened. Fatty streak developed at the intimal layer and progresses into plaques. Overall the plaques in the picture were considered as well developed atheromatous plaques with fibrous cap encircling the top of the plaque. Fibrous cap composed of proliferating smooth muscle cells, macrophages, lymphocytes, foam cells and extracellular matrix. In cholesterol group, the lesions were characterized by increased population of macrophage-derived foam cells that formed due to increased uptake of oxidized LDL by smooth muscle cells and macrophages. The foam cells are differentiated with other lipid cells by its nucleus which resides at the centre of the cell and stringy substances occupy the cytoplasm. This is clearly seen at the magnification of X 20. The formation of foam cells signals the early phase in the development of atherosclerosis (Keaney *et al.*, 1999). The intimal elastic layer in cholesterol group was not clearly seen between intima and media layer as the smooth muscle were infiltrated with macrophages and inflammatory cells.

The aortic lesions that were present in *R. tomentosa* group and simvastatin group were visibly thinner than those in cholesterol group. The lesion that developed in simvastatin group and *R. tomentosa* were categorized in Type III as the lesions consists some small cores but no visible calcification. Histopathological examination of simvastatin group and *R. tomentosa* group revealed less presence of foam cells. Less cholesterol deposits were seen in the aorta of the *R. tomentosa* group and simvastatin group with very little deposits observed in the intima tunica and medica tunica region and no cholesterol deposit seen in the adventitia tunica region.

Treatment with simvastatin 5mg/kg/day efficiently attenuate development of atherosclerotic plaque by reducing the plaque lipid content and alleviate macrophage infiltration. Morphological assessment revealed simvastatin group had significantly

inhibited vascular inflammation with considerably less macrophage accumulation (figure 4.11.7). The mechanism of macrophages infiltrating the lesion has been reported by Ross, 1993 and Bobryshev, 2005; where the monocytes infiltrate into subendothelial layer of the intima media and then differentiate into macrophages and dendritic cells. The intensity of inflammatory cells infiltration in simvastatin group showed a correlation with cytokines and tissue factor. Much of the better prognosis of simvastatin group (figure 4.11.8) was attributed from the adhesion molecules, cytokines and tissue factor expression in the lesion (Sukhova *et al.*, 2002). Another study by Dunzendorfer *et al.*, 1997 has recognized statins as a useful drug in combating of inflammation by inhibiting the neutrophils chemotaxis and decrease pro-inflammatory cytokines thus decreasing infiltration of inflammatory cells in tissues and organs. Another factor that leads to the migration of inflammatory cells may be due to the enhanced cell expression of some cell adhesion molecules, including ICAM-1 and E-selectin (Leeuwenberg *et al.*, 1992; Blankenberg *et al.*, 2003; Quehenberger 2005). Some studies had suggested the role of platelets in atherosclerosis by secretion of MPVs microvesicular platelets formed during the platelet activation process (Kahn *et al.*, 1999 and VanWijk *et al.*, 2003). High concentration of MPVs stimulates platelets adhesion and eventually enhancing inflammatory cells such as leukocytes or T-cells adhesion via increase in the expression of the ICAM molecules (Arita *et al.*, 1999).

Foam cells still can be seen in simvastatin groups but in less severe than the cholesterol groups (figure 4.11.8). Decrease depositions of cholesterol in simvastatin group were represented by lesser lipid laden foam cells which originally derived from circulating total cholesterol. The accumulations of atherosclerotic plaques are related to the amount of serum total cholesterol (Smith 1990). This was clearly seen when cholesterol found within the arterial wall was proportional to the serum cholesterol level in the lipid profile test.

5.5.8.2 Hematoxyline and Eosin Histology Examination of Kidney

The kidney contributes to the body's biochemical homeostasis by eliminating metabolic waste products, regulating fluid and electrolyte balance and influencing acid-base balance. The kidney also produces the following hormones, prostaglandins, erythropoietin, 1,25-dihydroxycholecalciferol and rennin. Kidney section of normal group in figure shows normal architecture of cell morphology of glomerulus and parenchyma cells. The parenchyma was divided into cortex and medulla. The cortex comprised renal corpuscles, proximal and distal convoluted tubules. Each glomerulus consists of capillaries wall layered with epithelial cells and surrounded with Bowman's space (arrow). All capillary walls contribute to the filtration barrier. The capillaries were supported with mesengial cells that become more prominent in some diseases.

Atherosclerosis can also lead to renal dysfunction (Moorhead *et al.*, 1982) and glomerular lipid deposition (Amatruda *et al.*, 1974). Besides that, oxidised LDL cholesterol and free oxygen radical may have direct effect on renal parenchyma and vasculature (Kasiske *et al.*, 1990) and also interstitial injury and glomerular disease (Magil, 1999). Previous study also proved that an association of renal vasculature impairment linked to high cholesterolemia in atherosclerosis disease both in-vitro (Stulak *et al.*, 2001) and in-vivo (Feldstein *et al.*, 1999).

Histopathology examination of cholesterol group kidney revealed some abnormality when compared with normal group kidney. Glomerular defects were noted in figure 4.10.16 with swollen and finely vacuolated cytoplasm. This provides the direct evidence that hypercholesterolemia induces glomerular injury. Other histological changes observed in cholesterol group were when the kidney was atropic and the mesengial cells were contained with lipid fill in the glomeruli. Heavy infiltrations of inflammatory cells were seen scattered all over kidney tissues. An increased level of oxidised LDL in the blood was one of the factor that encourage the infiltration of

macrophages into glomeruli figure 4.11.12 observed in cholesterol group. The same histological observation in cholesterol is coherent with the finding by Mune *et al.*, 2002 where kidneys from rat animal model were infiltrated with high oxidised LDL. This because any oxidised LDL can act as macrophages chemo-attractants associated with intracellular signalling for atherogenic lipoprotein in glomerular injury (Kamanna *et al.*, 1999). Low density lipoprotein (LDL) also a kind of atherogenic lipoprotein that can change pathophysiology in glomeruli and interstitial damage that result in reduction of renal function (Keane *et al.*, 1988; Matoba *et al.*, 1997; Kodama *et al.*, 1997).

There were no obvious differences between *R. tomentosa* group and simvastatin group in glomerular morphology and also infiltration of inflammatory cells (figure 4.11.13, 4.11.14, 4.11.15 and 4.11.16). In *R. tomentosa* group, the infiltrations of inflammatory cells were reduced compared to cholesterol group. This may be related to its antioxidant function and inhibition of LDL oxidation. *R. tomentosa* extract that rich in antioxidants act by scavenging the free radicals that cause LDL peroxidation in renal blood and at the same time the antioxidants in *R. tomentosa* enhance endothelium wall condition.

The beneficial effects of simvastatin were clearly seen not only on its lipid lowering properties, but also on renal morphology of hypercholesterolemic rabbit. Any statins treatments did improve renal vasculature. This approach has been studied in hypercholesterolemic patients. In the current study, hypercholesterolemia or high cholesterol in the blood was exerted in cholesterol group with 1% high cholesterol diet. Oxidised LDL in hypercholesterolemic blood did cause inflammation and endothelial injury to tissues and blood vessels represented by deranged and loss of radial arrangement of kidney tissues (figure 4.11.11 and 4.11.12). Hypercholesterolemia was able to impair endothelium-dependent vasodilatation and decrease nitric oxide (NO) production through scavenging by superoxide anion production (Wolfrum *et al.*, 2003).

With simvastatin treatment of 5mg/day/kg in simvastatin group, the better assessment of cell arrangement and improved blood vessels provided by the endothelial barrier. The better endothelium and kidney tissues arrangement were seen in simvastatin group (figure 4.11.15 and 4.11.16) compared to cholesterol group. In the endothelium, simvastatin increases levels of eNOS and enhance (NO) bioavailability by stabilizing the endothelial nitric oxide synthase mRNA (Mital *et al.*, 2000). Another study managed to prove the link between simvastatin and migration of C18 neutrophil (Lefer *et al.*, 1999). These data suggest that HMG-co reductase inhibitor such as simvastatin may maintain endothelial function and microvascular homeostasis in inflammatory conditions in renal.

5.5.8.3 Hematoxyline and Eosin Histology Examination of Liver

Figure 4.11.17 and 4.11.18 show pictomicrograph of a normal group. Healthy liver in normal group had sparse connective tissue which composed of uniform parenchymal cells called the hepatocytes. The hepatocytes looked well arranged in hexagonal shaped with central lightly stained nucleus and the cytoplasm were evenly distributed without vacuolation. Histologic analysis in figure 4.11.18 presented with no fat accumulation nor obvious inflammatory infiltration.

The hematoxylin and eosin-stained sections from figure 4.11.19 shows the condition of liver of cholesterol group. Evidence of hepatocytes scattered randomly around portal and central vein and cellular necrosis were seen. Severe fatty changes were observed with vacuolation of liver cells. Fatty change refers to any abnormal accumulation of triglycerides within parenchymal cells. Fatty change was most often seen in the liver, since this is the major organ involved in fat metabolism. Early fatty change was seen by light microscopy as small fat vacuoles in the cytoplasm

around nucleus (arrow) but it did not displace centrally located nucleus. In later stage, the vacuoles coalesce to create cleared spaces that displace the nucleus to the cell periphery. The vacuolation of cells was often due to accumulation of lipid droplets as a result of a disturbance to endoplasmic reticulum and ribosomal function (Caldwell *et al.*, 2006; Caldwell *et al.*, 2007). The liver in cholesterol group had large population of inflammatory cells. A combination of fatty changes and inflammation eventually progresses into steatohepatitis or retention of lipid. Figure 4.11.20 shows degeneration of liver cells in cholesterol group near to the portal tract with dense inflammatory cell infiltration which can lead to progression erosion of liver. A significant number of inflammatory cells can be seen along central vein (CV) and portal track. Hepatic lobule filled with blood was observed. There was also calcification and necrosis around central vein and portal track (figure 4.11.19) of blood vessels. This calcification was caused by calcium built up surrounding the vessel walls. Normally calcification can lead to loss of liver architecture and in some serious condition it may progress to destruction of liver tissues.

Figure 4.11.21 represents *R. tomentosa* group with having inflammatory cells scattered all over liver. A few fat vacuoles were spotted. There were less slightly inflammatory cell infiltrations. In figure 4.11.22 shows the dense lymphocytic of inflammatory cells was only confined to portal tracts and there was no erosion of hepatic architecture. *R. tomentosa* treatment led the improvement of fatty liver. The incidence of fatty changes was lower. Only a few fat vacuoles were seen at magnification of 20 X.

Microscopic examination of tissue sections from simvastatin group revealed similar histologic condition as in cholesterol group and *R. tomentosa* group. Fatty changes represented by fat vacuoles were also present. There has been study related fatty liver disease and heart disease risk (Brea *et al.*, 2005). One remarkable

study even managed to prove between oxidative stress of fat injury in hepatocellular to oxidative stress as seen in atherosclerosis (Zatloukal *et al.*, 2004). Oxidative stress contributes to the damage of the endoplasmic reticulum as a result of fat accumulation and loss of cytoskeleton function, further impede the fat droplet interaction with VLDL synthesis (Albano *et al.*, 2005; Puri *et al.*, 2008).

5.5.9 The Evaluation of Atherosclerotic Area Macroscopically

Rabbit aorta is a suitable atherosclerotic model because it can mimic the human lesion (Fan and Watanabe, 2003). Not only that, rabbit is also considered as phylogenitacally closer to human than rodents (Graur *et al.*, 1996). The evaluation of aorta macroscopically showed that there were no atherosclerotic plaques in normal group. This is true because the normal group rabbits only fed with normal diet. In cholesterol group (positive control) there were marked accumulations of atherosclerotic plaques $36.72\% \pm 7.653$. In this case there rabbits were given 1% cholesterol diet daily. High cholesterol diet not only causes hypercholesterolemia but also leads to the development of atheromatous plaque. From the figure 4.12.3, these plaques can be frequently seen at the proximal of aorta (aorta-celiac junction) and a few at the middle of aorta (mesenteric branch). The proximal and the middle of the aorta are the location where the aorta bends and the junction to large arteries that supply blood to abdomen region. The depositions of cholesterol at these locations are related to hemodynamics of blood and structure of the aorta's wall (Olson, 1987; Texon, 1996). As we know the sole function of heart is to supply energy required for the circulation of blood in the cardiovascular system. Most of the cholesterol deposits in the proximal due to the high pressure of heart pumping large volume of blood to the systemic organs at high pressure that creates irregular hemodynamics of blood flow. Irregular heamodynamic effects also known as "disturbed flow" such as low wall shear stress (WSS) levels, high particle residence times and excessive arterial wall strains contribute to the localization of cholesterol (DeBakey *et al.*, 1985; Nerem, 1992; Ross, 1993; Texon 1996). In naked eye, cholesterol deposits on the lumen of the aorta look like a whitish waxy substance, but after stained with Sudan IV, the waxy appearance became prominent as dark reddish deposits. These deposits can be very dangerous in human when in full advanced, as the accumulation restricts the blood flow via vessels and can lead to ischaemia. In some

extreme cases, ruptured plaques can be conveyed to artery causing embolism (Stary *et al.*, 1992).

The supplementation of *R. tomentosa* extract 50mg/kg/day together with 100mg cholesterol pellet to the rabbits for 10 weeks did cause the formation of aortic lesions, however the degree of formation was slightly lower $15.17\% \pm 8.646$. In the picture 4.12.4 of *R.tomentosa* group, the aortic lesion is not extensive as in the cholesterol group. Still, there were atherotoma at the proximal and middle of aorta. That was 21.55% decrease of lesion size compared to cholesterol group and it was rather significant. The progression of lesions in *R. tomentosa* and simvastatin group is so small compared to cholesterol group. Few small lesions in *R. tomentosa* and simvastatin group can be seen as fatty streaks. Fatty streaks composed of lipid filled foam cells (arrow) that started as multiple yellow, flat spots (fatty dots) of lipid rich and less than 1mm in diameter and progresses into elongated streaks. In pathological understanding of atherosclerosis, the formation of fatty streak signifies earliest pathogenesis of atherosclerosis however some fatty streaks may not develop into advanced atherosclerosis lesions.

The same can be said in simvastatin group (figure 4.12.5) as positive treatment group. Simvastatin group was the group that fed with cholesterol diet and at the same time was given 5mg/kg/day simvastatin. Distribution analysis of lesion size in simvastatin group had significantly fewer lesions $13.64\% \pm 2.65$. The fatty lesion appeared to be reduced in number and size. The extent of atherosclerosis in simvastatin is 21.55% lower. This indicates that simvastatin drug has managed to play its role as anti-cholesterol drug. Previous studies have shown that statins such as simvastatins can act as athero-protective function besides lipid lowering function (Rosenson and Tangney, 1998).