CHAPTER 5: DISCUSSIONS

5.1 Preparation of Plant extracts

Three different varieties of *H. sabdariffa* calyx (Arab, UKMR-1 and UKMR-2) were used in this study. *H. sabdariffa* calyxes were obtained from Department of Biology, Faculty of Science and Technology, UKM. There are several things to take into account regarding the moment at which plant materials were harvested, especially time the calyxes of *H. sabdariffa* were collected since the levels of plant metabolites vary throughout the day. For example, is known that the levels of several primary metabolites, e.g. malic acid and sugars, fluctuate following a daily cycle (Queiroz, 1974). This is also true for secondary metabolites (Urbanczyk-Wochniak *et al*., 2005). Thus, only matured calyxes of *H. sabdariffa* were processed as plant materials in this study.

Harvested *H. sabdariffa* calyxes immediately processed to ensure its phytochemicals preserved. Harvesting and processing plant materials were conducted very rapidly and were done with special care. This is because rapid metabolite changes, such as those due to enzymatic degradation or oxidation, could occur during the process and may affect the results considerably (Kim and Verpoorte, 2009). The Arab, UKMR-1 and UKMR-2 calyxes were dry at room temperature to prevent the bioactive compounds from degrade. Water content in the calyxes of UKMR-2 is higher compared to UKMR-1 and Arab calyxes. About 90.28 % of UKMR-2 sample weight is made up of water. While percentage of water content in UKMR-1 and Arab samples are 89.55 % and 85.63 % respectively.

Plant materials consist of a wide variety of compounds at very different levels and with very different polarities. Because of that, solvent characteristics, ratio solvent and sample, duration of extraction and temperature are several aspects to be considered for extraction (Kim and Verpoorte, 2009). In this study, distilled water was used as solvent of extraction because wide range of solute can dissolve in water. Furthermore, water does not react with phytochemicals in *H. sabdariffa* thus can prevent the alteration of the chemical structure. *H. sabdariffa* calyxes were extracted for 24 hours at room temperature to maximize the extraction. Nearly 5.82g of crude extract was obtained from 100g of dried sample of Arab variety. While 5.41g and 4.30g of crude extract were obtained from each 100g of UKMR-2 and UKMR-1 dried sample.

5.2 Analysis of Antioxidant Activity

Antioxidant capacity is widely used as a parameter for medicinal bioactive components. In this study, antioxidant activities of each extracts of *H. sabdariffa* variety were evaluated using three different assays namely DPPH free radical scavenging activity assay, reducing power assay and metal chelating assay. Each assay evaluate antioxidant activities in different way because antioxidant compounds do not all act in the same way. Antioxidants can deactivate radicals by two major mechanisms, hydrogen atom transfer (HAT) and single electron transfer (SET). The end result is the same, regardless of mechanism, but kinetics and potential for side reactions differ (Prior *et al*., 2005).

Ishige *et al*. (2001) reported that both oxidants and antioxidants have different chemical and physical characteristics. Individual antioxidants may, in some cases, act by multiple mechanisms in a single system or by a different single mechanism depending on the reaction system. Furthermore, antioxidants may respond in a different manner to different radical or oxidant sources. Thus, because of multiple reaction characteristics and mechanisms as well as different phase localizations are usually involved, no single assay will accurately reflect all of the radical sources or all antioxidants in a mixed or complex system.

5.2.1 DPPH free radical scavenging activity assay

2,2-Diphenyl-1-picryl-hydrazyl (DPPH) is a stable free radical which has an unpaired valence electron at one atom of nitrogen bridge (Eklund *et al*., 2005). It is widely used to test the ability of compounds to act as free radical scavengers or accept hydrogen radical to become a stable molecule. The DPPH method can be used for solid or liquid samples and is not specific to any particular antioxidant component, but applies to the overall antioxidant capacity of the sample (Miller *et al.*, 2000). DPPH produces a violet or purple solution in organic solvent such as methanol. The purple chromogen is decolorized in the presence of antioxidant molecules, giving rise to the color ranged from yellow to uncolored solution. The resulting of the decolorization is stoichiometric with respect to the number of electrons captured (Lee & Halliwell, 2001). Thus the radical scavenging activity can be determined from the reduction of optical absorbance reading at 517 nm.

In this study, calyx extracts of *H. sabdariffa* from Arab, UKMR-1 and UKMR-2 variety exhibit DPPH radical scavenging activity. Table 4.1 shows the extract from Arab variety possesses better scavenging activity when compared to UKMR-1 and UKMR-2 variety. However, only extract of *H. sabdariffa* from Arab and UKR-2 variety shows good radical scavenging activity with IC_{50} = 462.0 µg/ml and 640.0 µg/ml respectively, while extracts from UKMR-1variety exhibit poor DPPH radical scavenging activity when compared to ascorbic acid. The extracts from Arab and UKMR-2 variety scavenge approximately 92% and 82% of DPPH radicals at concentration of 1000 µg/ml. This is quite good enough since at same concentration, ascorbic acid scavenges nearly 98 % of DPPH radical, thus, from statistical analysis, the DPPH radical scavenging activity extracts of *H. sabdariffa* from Arab and UKMR-2 variety are not statistically different from ascorbic acid.

5.2.2 Reducing power assay

Reducing power is generally associated with the presence of reductones which exert antioxidant action by breaking the free radical chain. Reductones can also act as primary and secondary antioxidants because they can reduce the oxidized intermediates of lipid peroxidation processes. The reducing power assay measures the electron-donating ability of antioxidants compound in each *H. sabdariffa* variety extracts to reduce ferricyanide complex/ Fe^{3+} to Ferrous/ Fe^{2+}. In this assay, the yellow color of the test solution changes to various shades of green and blue depending on the reducing power of antioxidant samples and this reaction can be measured as the increase in the absorbance at 700 nm. Increased absorbance of the reaction mixture indicates greater reduction capability.

The calyx extracts of *H. sabdariffa* from all variety studied exhibit reducing activity toward ferricyanide complex/ Fe^{3+}. The extracts of *H. sabdariffa* calyx from Arab variety exhibit reducing capability slightly higher compared to the extract from UKMR-2 variety and the extract from UKMR-1 variety exhibit the lowest reducing capability. However, the extracts of *H. sabdariffa* calyx from all variety showed poor reducing capability when compared with the standard, BHA (Figure 4.3). The extracts from all variety showed very low reducing capability even at the highest concentration, 1000 µg/ml. Statistical analysis on the reducing power of extracts of *H. sabdariffa* calyx from all variety showed that the reducing powers of Arab, UKMR-1 and UKMR-2 extract were significantly lower than the standard.

5.2.3 Metal chelating assay

The antioxidant activities of each *H. sabdariffa* variety extracts were also analyzed by the chelating ability of the extracts against Fe^{2+}. Metal chelating activity is claimed as one of antioxidant mechanisms, since it reduces the concentration of the catalyzing transition metal in lipid peroxidation. Among the transition metals, iron is known as the most important lipid oxidation pro-oxidant due to its high reactivity ions (Halliwell & Gutteridge, 1984). The ferrous state of iron accelerates lipid oxidation by breaking down hydrogen and lipid peroxides to reactive free radicals via the Fenton reaction ($Fe^{2+} + H_2O_2$ - $Fe^{3+} + OH^{\bullet} + OH^{\bullet}$). Ferrozine can quantitatively form complexes with Fe2+. In the presence of chelating agents, the Ferrozine-Fe^{2+} complex formation is disrupted, resulting in a decrease in the red color of the complex. Therefore, measurement of color reduction allows estimating the metal chelating activity of the coexisting chelator (Yamaguchi *et al*, 2000). Lower absorbance indicates higher metal chelating activity.

The study on metal chelating activities of extract of all *H. sabdariffa* variety found the extracts exhibit metal chelating activity. The extract from Arab variety shows higher metal chelating activity compared to its new variety. However only extract from Arab variety possess good metal chelating activity with IC_{50} 370 µg/ml when compared to the standard, while the extract from UKMR-1 and UKMR-2 shows very poor metal chelating activity. Both extract only shows approximately 18% of inhibition against Fe^{2+} at the highest concentration of extract i.e. 1000 µg/ml. Both new varieties have very significant different of metal chelating capability compared to their parent Arab variety.

As seen in this study, the aqueous extracts of *H. sabdariffa* calyx from different varieties exhibited varying degrees of antioxidant activity. *H. sabdariffa* from Arab variety shows better activity in antioxidant assay compared to UKMR-1 and UKMR-2. These results are consistent with the fact that the calyx of *H. sabdariffa* is a composite of several antioxidants, such as protocatechuic acid (PCA) (Tseng *et al.,* 1996; Liu *et al.,* 2002), anthocyanins (Ali *et al.,* 2003; Wang *et al.,* 2000), ascorbic acid (Prenesti *et al.,* 2005) and b-carotene (Wong *et al.,* 2002). Other previous work also proved that the calyx of *H. sabdariffa* rich in vitamin C (141 mg/100 g), anthocyanins (2.52 mg/100 g), b-carotene (1.88 mg/100 g), lycopene (164 µg/100 g)(Wong *et al.,* 2002).

5.3 Toxicity test

5.3.1 Brine Shrimp Lethality Bioassay

Bioactive compounds are almost always toxic in high doses. Thus, *in vivo* lethality in a simple zoologic organism can be used as a convenient monitor for screening in the discovery and monitoring of bioactive natural products. The study by Mclaughlin *et. al* (1998) reported a positive correlation between brine shrimp toxicity and human nasopharyngeal carcinoma cytotoxicity thus used the brine shrimp test as prescreen for antitumor assays.

Brine shrimp lethality activity of the calyx extracts of *H. sabdariffa* from all variety were shown in Tables 4.7. The data demonstrate that the extracts of all studied variety exerted a toxic effect on *A. salina* at highest dose i.e. 1000 µg/ml. Among three extracts of *H. sabdariffa* variety, the most active extract was the extract from Arab variety with LC_{50} = 146.88 µg/ml. The extract from UKMR-1 and UKMR-2 variety needed higher dose to cause lethality to brine shrimp or less active compared to their parent variety with LC_{50} = 503.58 µg/ml and LC_{50} = 325.56 µg/ml respectively. Crude extracts resulting in LC_{50} values less than 250 µg/ml were considered significantly active and had the potential for further investigation (Rieser *et al.*, 1996).

5.3.2 Oral Toxicity Study

The toxicity assessments on extracts of all *H. sabdariffa* variety in this study were also done on mammalian animal. The methodology of this study was based on Organization for Economic Co-operation and Development (OECD) Guide Line for the Testing of Chemicals, Repeated Dose 28/14-day Oral Toxicity Study in Rodents, updated version of Guideline 407. Compared to brine shrimp lethality assay, the oral toxicity study on mammalian animal offered several advantages. A part from the number of the experimental animals survived at the end of the study and the concentration of tested compound that cause lethality to the half of the experimental animal (LC_{50}), this oral toxicity study also analyze on how the tested compound can give effect to experimental animals' neuro- behavioral, organ functions, and biochemical activities in the body. Thus, the toxicology mechanism of tested compound or chemical are more understood because the analyzed data are covered from wide range of parameters.

5.3.2.1 Clinical observation

The present study has demonstrated that no mortality was found in all groups of rats attributed to any effect after sub-chronic administration of a very high dose (1000 mg/kg body weight), as well as treatment with a slightly lower dose (500 mg/kg body weight) of the extracts from different variety of *H. sabdariffa* for 14 days. There was also no significant dose – related changes observed in the neuro-behavioral of the experimental animals as judged by clinical signs of toxicity during the experimental period. Gross pathological findings and histopathological examination also indicated that there was no evidence of toxicity caused by the extracts on all rats tested at any dose given.

Researchers from Nigeria have recently studied the effect of sub-chronic administration of aqueous extracts of *H. sabdariffa* calyx on rats (Orisakwe *et al.*, 2004). Rats were given high dose of an aqueous extract of *H. sabdariffa* calyx which were 1150, 2300 and 4600 mg/kg/day in the drinking water for up to 12 weeks. At the end of the treatment period, there was a steady decrease in body weight of rats. However, Sub-chronic oral administration of calyx extracts of *H. sabdariffa* from Arab, UKMR-1 and UKMR-2 variety at a dose of 1000 mg/kg body weight and 500 mg/kg body weight for 14 days produced no significant different in body weight of all rats when compared to the control group. Treatment with these extracts regimen did not affect the mean body weight of the experimental animals in all groups indicate that the extracts may not produce any adverse or toxic effect since previous study has demonstrated that the administration of toxic substances to rats in certain period of time can give significant loss in body weight (Teo *et al*., 2002).

5.3.2.2 Clinical biochemistry analysis

i. Liver function test

It is well known that the liver is important organ in the detoxification processes. Thus, any abnormality in the liver function or liver activity has been widely used as a sign of toxicity effect. In this study, several biochemical compounds in the blood serum that plays vital roles in liver function were measured as parameters to determine the liver activity. Significant fluctuation or changes of any of these biochemical compounds may indicate toxicity sign to the rats tested.

Oral administration of calyx extracts of Arab variety at dose 500 mg/ kg body weight for 14 days give no abnormal changes on serum biochemical analysis results as shown in Table 4.7. There were no significant differences changes in liver function activity parameters between the rats treated daily with the extracts of Arab variety at dose 500 mg/kg body weight and control groups. Only marginal changes on the mean alanine aminotransferase and aspartate aminotransferase level were observed in treated rat. Alanine aminotransferase level was slightly higher (130.00 IU/L) in the Arab-treated group compared to the control group (127.00 IU/L). By contrast, aspartate aminotransferase level was marginally less (434.75 IU/L) in the Arab-treated group (control = 461.00 IU/L). As the differences were very small, these are unlikely to be important and statistically insignificant compared to normal control. Others parameters (Total protein, Albumin, Globulin, Total bilirubin, Conjugated bilirubin, Alkaline phosphatase and G-Glutamyl transferase) concentration level in serum biochemical analysis were falls within normal ranges and also gives insignificants different changes compared to control group.

Nearly same results were demonstrated by group of rats administered with Arab variety extract at high dose (1000 mg/kg body weight) for 14 days (Table 4.7). Each

parameter measured to determine the activity of liver in serum biochemistry analysis didn't shows any drastic changes compared to normal control. Total protein, Albumin, Globulin, Total bilirubin, Conjugated bilirubin, and G-Glutamyl transferase mean level gives results in minimal changes when the animal treated with the extract of Arab variety. Slightly increment on Alanine aminotransferase (137.00 IU/L, normal = 127.00 IU/L) and alkaline phosphatase (228.75 IU/L, normal = 211.75 IU/L) mean level was detected in this group. While deflation on aspartate aminotransferase mean level (448.50 IU/L, normal = 461.00 IU/L) also was observed. However these changes didn't give any significant different ($p < 0.05$) and can be considered as normal since the mean level of alanine aminotransferase, alkaline phosphatase, and aspartate aminotransferase still within normal ranges when compared to normal control group.

Daily administration of extracts from UKMR-1 variety at dose 500 mg/ kg body weight for 14 days also give results in several changes on the parameters measured (Table 4.7). Although there were several fluctuations on the mean levels of parameters measured, but overall finding on serum biochemistry analysis for the rats treated with UKMR-1 variety extract found that the liver activity was normal. Alanine aminotransferase (109.25 IU/L, normal = 127.00 IU/L) and Aspartate aminotransferase (403.25 IU/L, normal = 461.00 IU/L) mean levels were detected lower in blood serum of the animals tested compared to normal group. But the reductions of these two enzymes were statistically insignificant and considered still within the reference values when compared no normal group.

An interesting observation in the study of toxicity effect on oral administration of extracts of UKMR-1 variety at higher dose (1000 mg/ kg body weight) for 14 days was that treatment with this extract significantly decreased the Aspartate aminotransferase (143.50 IU/L, normal = 461.00 IU/L) and alkaline phosphatase (88.00 IU/L, normal = 211.75 IU/L) mean level in blood serum. The mean level of total protein (76.50 g/L, normal = 65.50 g/L) also found markedly increases. Despite this, there were no others significant alterations in liver function parameters including albumin, globulin, total bilirubin, conjugated bilirubin and liver enzymes (e.g. Alanine aminotransferase and G-Glutamyl Transferase).

The result of the sub-acute toxicity test suggest daily oral administration for 14 days of extract of UKMR-2 at dose of 500 mg/ kg body weight caused mean concentration levels of certain liver enzymes in treated rats to fluctuate. The slightly elevation of alkaline phosphatase (228.75 IU/L, normal = 211.75 IU/L) was detected in blood serum of rats. On the other hand, Alanine aminotransferase (109.25 IU/L, normal = 127.00 IU/L) and Aspartate aminotransferase (403.25 IU/L, normal = 461.00 IU/L) concentration were found to be marginally lower than normal control. However, statistical analysis of these fluctuation of liver enzymes eventually results in insignificant different when compared to normal control. It was also noted that others parameters measured in serum biochemistry analysis to determine the activity of liver were not significantly affected by treatment with this extract.

Daily oral intake of this new variety of *H. sabdariffa* at dose 1000 mg/ kg body weight for same period of time demonstrated no toxicology evidence on liver activity (Table 4.7). Several changes on concentration of biochemical compounds and enzymes in blood serum resulted from the treatment were statistically analyze and indicated that no dose-related toxicity effect was observed and all of biochemistry parameters fell within the reference

values, thus considered to be normal. The concentrations of alanine aminotransferase (138.50 IU/L, normal = 127.00 IU/L) and alkaline phosphatase (241.00 IU/L, normal = 211.75 IU/L) were found to be slightly higher. In contrast, aspartate aminotransferase (416.00 IU/L, normal = 461.00 IU/L) level exhibit decreasing at the end of 14-days of treatment.

According to research that has been conducted by Liu *et al*. in 2006, administration of *H. sabdariffa* extract with various doses (1–5%) for 9 weeks significantly decreased the elevation in plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT). In this study, the reduction in plasma aspartate aminotransferase (AST) was also recorded in rats treated with 1000 and 500 mg/ kg body weight of Arab, UKMR-1 and UKMR-2 variety. Alanine aminotransferase (ALT) level in rats treated with 500 and 1000 mg/kg body weight of UKMR-1 and UKMR-2 variety extract were also shows reduction. However these reductions in plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) is still in normal reference ranges, except in rats treated with high dose of UKMR-1 variety.

The anthocyanins of H. sabdariffa were also shown to have a protective effect against tert-butylhydroperoxide-induced hepatic toxicity in rats (Wang *et al.*, 2000). The anthocyanins were able to quench the free radicals of 1, 1-diphenyl-2-picrylhydrazyl and this antioxidant effect was also demonstrated by the ability of the anthocyanins to reduce the cytotoxicity induced by tert-butylhydroperoxide in rat primary hepatocytes and to attenuate hepatotoxicity in rats (Wang *et al.*, 2000). Administration of the anthocyanins isolated from the plant (100 or 200 mg/kg/day for 5 days) significantly reduced the activities of the serum enzymes indicative of liver damage, ameliorated histological lesions and reduced oxidative liver damage. Similar dosages of H. sabdariffa anthocyanins were

effective in significantly mitigating the pathotoxicity induced by paracetamol in mice (Ali *et al*., 2003). It has also been reported that anthocyanins protect against DNA damage induced by tert-butyl-hydroperoxide in rat smooth muscle and hepatoma cells (Lazze *et al*., 2003).

ii. Renal Function Test

The result from blood serum analysis to determine the activity of kidney of the rats after treated with Arab variety at dose 500 mg/kg body weight revealed that none of the parameters assessed deviate from the reference values range. Minimal increasing were exhibited in sodium (137.50 mmol/L, normal = 136.83 mmol/L) and carbon dioxide (19.58 mmol/L, normal = 17.67 mmol/L) levels. The different in concentration of other parameters were found statistically not significant from the normal control. A normal activity of kidney also can be seen in rats administered orally with Arab variety extract at same period of time but at higher dose (1000 mg/kg body weight). The fluctuation in concentration of Sodium, Potassium, Chloride, Carbon dioxide, Anion gap, Urea and Creatinine were observed but statistically not significant compared to normal control, thus, the kidney was assumed function in normal condition.

Daily oral administrations of extract of UKMR-1 variety at dose 500 mg/ kg body weight induce the significant increasing in mean concentration of sodium (142.00 mmol/L, normal = 136.83 mmol/L) in the treated rats. Though so, others parameters were remain at normal level. However, results from group of rats treated with 1000 mg/ kg body weight of same extract demonstrate that the sodium level (138.50 mmol/L, normal = 136.83 mmol/L) was fell in reference value range, thus give evidence that the increasing in mean concentration of sodium in rats treated with 500 mg/ kg body of UKMR-1 extract were not dose dependent. Meanwhile, concentration of creatinine (53.50 ± 0.71 µmol/L, normal = 42.50 ± 6.11 µmol/L) in rats treated with 1000 mg/ kg body weight of UKMR-1 extract also elevated above normal range. Nevertheless, others parameters measured in blood serum analysis for renal function test shows no significant abnormal in concentration changes, therefore indicate that the kidney function normally.

The results in biochemical analysis also proved that administration of UKMR-2 variety extract at low dose cause no adverse effects to kidney activities of rats. The kidney were found to be normal as results from serum blood analysis for kidney function found all parameters measured were fell in normal ranges. The only obvious change observed was in reduction of carbon dioxide level (14.84 ± 1.80 mmol/L normal = 17.67 ± 4.99 mmol/L). However, this reduction was still considered very small and normal. Daily administrations of the extract at dose 1000 mg/kg also cause no toxicity effect related to the treatment on the rats. Creatinine level was detected slightly 7.00 µmol/L higher than control (49.50 ± 6.36 µmol/L, normal = 42.50 ± 6.11 µmol/L). The changes in others parameter of serum biochemistry levels were found to be very small and statistically insignificant. Thus, the data from this study suggest that repeated oral administration of UKMR-2 extract for 14 days at dose of 500 and 1000 mg/kg didn't give any adverse effect to kidney activities.

The study by Kirdpon *et al.* (1994) reported the changes in urine composition that follow the consumption of *H. sabdariffa* extract at different concentrations and for various periods of time in six normal Thai subjects. The study indicated that consumption of *H. sabdariffa* extract resulted in significant decreases in the urinary concentrations of creatinine, uric acid, sodium, and potassium. Interestingly, the study was found that the low dose of *H. sabdariffa* (16 g/day) caused a more significant decrease in salt output in the urine than a high dose (24 g/day). However, the results in this study shows treatment with three different variety of *H. sabdariffa* cause the creatinine concentration elevated especially in rats treated 1000 mg/kg of extract. Rats treated with 1000 mg/kg of UKMR-1 extract was exhibited highest and significant elevation in creatinine concentration (53.50 ± 0.71 µmol/L, normal = 42.50 ± 6.11 µmol/L) followed by Arab extract (52.50 ± 12.02 µmol/L) and UKMR-2 extract (49.50 ± 6.36 µmol/L). On the other hand urea, sodium

(except in rats treated with 500 mg/kg UKMR-1 extract), and potassium levels were found to be falls within normal range values. The contrary finding on concentrations of creatinine, uric acid, sodium, and potassium that follow the consumption of *H. sabdariffa* extract at different concentrations in this study with works done by Kirdpon *et al.*, in 1994 may be resulted from several factors including different concentration of dose and period of study. Furthermore, plant samples cultivated from different area produce different concentration of bioactive compound that eventually give different result on certain test.

5.3.2.3 Histological analysis

Liver and kidney of rats from Control, Arab, UKMR-1 and UKMR-2 groups were also subjected to histological process. The liver and kidney were undergo normal histological process for Hematoxylin and Eosin (H & E) staining. Histological analysis on liver and kidney of rats from Control, Arab, UKMR-1 and UKMR-2 groups shows no occurrence of toxicity signs such as cell morphological changes, cell degeneration, necrosis or inflammation related to administration of *H. sabdariffa* extracts. In fact, from previous studies, *H. sabdariffa* extract was proved had therapeutic effect on hepatic fibrosis induced by carbon tetrachloride (CCl4) exposure in rats (Liu *et al*., 2006). These *H. sabdariffa* extract preventive effects may be mediated by inhibition of hepatic stellate cell activation (Tseng *et al*., 1996, Wang *et al.*, 2000 and Liu *et al*., 2002). As there were no gross or microscopic pathological changes observed on rat's liver and kidney, the results suggest that oral treatment with the extracts of Arab, UKMR-1, and UKMR-2 variety at dose 500 and 1000 mg/kg body weight for 14 days in rats had no significant effect on liver and kidney function.

5.4 Hypercholesterolemic Analysis

5.4.1 Body Weight

The body weight of the experimental animals increased over the time period in all groups (normal control, cholesterol control, Arab, UKMR-1 and UKMR-2) due to the growth of animals. However, the 60 days period of induction of hypercholesterolemia through feeding 100g/day rabbit chow containing 1% cholesterol to male New Zealand White rabbits in cholesterol control group cause the body weight significantly increase compared to normal group. This conspicuous increasing in body weight of animals in cholesterol control group was contributed by cholesterol diet supplied everyday to the animals. This conclusion was support by gross examination on animal where lots of fats were found in animals' body especially in heart, liver and kidney. This condition was not found in normal group.

In contrast, feeding the animals with 100g/day rabbit chow containing 1% cholesterol diet and supplying the animals with 250mg/ kg body weight /day of Arab, UKMR-1 and UKMR-2 extract give results to insignificant different in the increasing of body weight compared to normal control group. This result parallel to the finding obtained by Vilasinee *et al* in 2005 where induced hypercholesterolemic rats treated with various doses of roselle extract give result to insignificant different on the body weight when compared to normal control.

5.4.2 Triglyceride Measurement

The average level of triglyceride concentration in serum for normal control, cholesterol control, Arab, UKMR-1 and UKMR-2 groups are shown in Table 4.12. The different in triglyceride level in all groups of animal before treatment were statistically insignificant. The triglyceride concentration in serum for normal control group was record a declining pattern during 60 days of study. The triglyceride level was decrease to nearly 15 % on day 30^{th} (day 0^{th} = 0.9 mmol/L, day 30^{th} = 0.87 mmol/L) and continues to markedly decrease to 32 % at the end of study (day 60^{th} = 0.62 mmol/L). The declining of the triglyceride concentration over the time was due to the growth of the animals. As the experiment progress, the animal also growth and increase in the body weight. After 60 days of study, the animals gained approximately 0.38 kg (16.7%) in body weight. As the animals' growth, the utilization of triglyceride in body metabolism will also increase. However, the diet was fixed to remain at 100g/day throughout the study. Thus, the declining in triglyceride concentration was expected. These reasons also explain the decreasing in concentration of total cholesterol in normal control group.

In contrast, animals fed with diet containing 1% (1 gram) of cholesterol daily in cholesterol control, Arab, UKMR-1 and UKMR-2 groups were recorded markedly increment in triglyceride concentration. The animals in cholesterol control, Arab, UKMR-1 and UKMR-2 groups were purposely fed with high cholesterol diet to induce hypercholesterolemia therefore atherogenesis or the development of atherosclerosis can occur. Animals in cholesterol control group exhibit the highest increment in triglyceride concentration. The triglyceride level increase from 0.91 mmol/L at the beginning of the experiment to 1.20 mmol/L (24%) on day 30^{th}. After 60 days animals were daily fed with 1% cholesterol diet, triglyceride concentration increase significantly to 1.52 mmol/L or

67% higher than concentration at first week. This tremendous increment make the different in triglyceride concentration in cholesterol control group statistically significant compared to normal group.

Triglyceride concentration in serum animals in Arab, UKMR-1 and UKMR-2 groups were also shows increment pattern. On day 30th of study, triglyceride level in Arab group increase 2.2% (0.95 mmol/L) from first week and continue to increase to 1.10 mmol/L or 18.3% on day 60th. While for UKMR-1 and UKMR-2 groups, triglyceride level were recorded reduction to 9.6% (0.85 mmol/L) and 5.6% (0.85 mmol/L) respectively on day 30th of treatment. But at the end of study, triglyceride level increase again nearly 6.4% and 5.6% in both group. At the end of the experiment, triglyceride concentration in Arab group shows highest increment (1.10 mmol/L) followed with UKMR-1 (1.00 mmol/L), and UKMR-2 (0.90 mmol/L) groups.

Tough that, the increasing of triglyceride level in Arab, UKMR-1 and UKMR-2 groups was not high as was recorded in cholesterol control group and not significantly different compared to normal group. These results prove that daily administration of 250mg/kg body weight of Arab, UKMR-1 and UKMR-2 extract help to lessen the triglyceride increment. The ability of *H. sabdariffa* extract in this research to reduce the elevation of triglyceride concentration in induced hypercholesterolemic animal is consistent with the previous study done by El-Saadany *et al.,* 1991, Hirunpanich *et al.,* 2006 and Fernández-Arroyo *et al.,* 2011. According to Fernández-Arroyo *et al.,* 2011, triglyceride concentration reduction could be because of reduction in VLDL concentrations, the major triglyceride-containing lipoprotein. This effect could be observed in other polyphenol-rich plant extracts (Beltrán-Debón *et al.,* 2010).

5.4.3 Total Cholesterol Measurement

Total cholesterol concentrations for normal control, cholesterol control, Arab, UKMR-1, and UKMR-2 groups at beginning of study, on day 30th and on day 60th were present in Table 4.13. The different in total cholesterol concentration in all groups of animal before treatment were statistically insignificant. Total cholesterol level in control group was exhibit reduction throughout the study period. Total cholesterol level was reduced nearly 11.0% to 1.05 mmol/L from 1.18 mmol/L on day 30^{th} and continue reduces to 0.93 mmol/L on day 60^{th}. Same as triglyceride, total cholesterol level experience a declining pattern because the usage of cholesterol in body metabolism increases as the animal growth.

After 30 days treated with 250 mg/kg of Arab extract, the induced hypercholesterolemic animals recorded elevation in total cholesterol concentration from 1.23 mmol/L to 16.53 mmol/L. At the end of study, the level of total cholesterol in this group was 21.23 mmol/L. Animals treated with UKMR-1 and UKMR-2 extracts also exhibit huge elevation in total cholesterol concentration in their blood serum. Total cholesterol level in UKMR-1 group was increased to 18.75 mmol/L on day 30^{th} of experiment from 1.25 mmol/L. On day 60^{th} of treatment, total cholesterol level in UKMR-1 group was 25.48 mmol/L. While for UKMR-2 group treated animals, total cholesterol level on day 30^{th} was 18.65 mmol/L and the level was continues to increase to 25.45 mmol/L.

As expected, feeding the animals with diet containing 1% cholesterol daily will induce hypercholesterolemia in cholesterol control, Arab, UKMR-1, and UKMR-2 groups. Total cholesterol level on day 30^{th} was highest in UKMR-1 group (18.75 mmol/L) followed with UKMR-2 group (18.65 mmol/L) and Arab group (16.53 mmol/L). Total cholesterol level continues to increase at the end of study with animals in UKMR-1 recorded the highest reading (25.48 mmol/L), while UKMR-2 and Arabs groups with 25.45 mmol/L and 21.23 mmol/L respectively. These tremendous increment makes the total cholesterol level in Arab, UKMR-1 and UKR-2 groups significantly different compared to normal control group.

Despite markedly increase in total cholesterol level, administration of 250 mg/kg body weight of Arab, UKMR-1 and UKMR-2 extract on induced hypercholesterolemic animals was observed to give hypocholesterolemic effect in total cholesterol level. Although the reduction was small and makes insignificant different compared to cholesterol control group, animal treated with *H. sabdariffa* extract exhibit increment lower than recorded by cholesterol control group. Furthermore, it is impossible to get hypercholesterolemic under normal condition. Thus, *H. sabdariffa* extract would be good supplement to reduce cholesterol in blood under normal condition.

The calyx of roselle has been known to contain many phytochemical compounds such as ß-sitosterol and pectin. Since phytochemical compounds such as ß-sitosterol and pectin have been reported to possess hypocholesterolemic effect in vivo, it may be speculated that the hypocholesterolemic effects of *H. sabdariffa* are attributed to these compounds (Hirunpanich *et al.,* 2006).

5.4.4 HDL and LDL Measurement

Even though HDL and LDL involve in cholesterol transportation, but both have opposite roles in cholesterol regulation. LDL transport cholesterol from liver to peripheral cells. It's always labeled as 'bad' lipoprotein because elevated levels of LDL are directly related to increased cardiovascular risk. This is because oxidation of LDL was proved to be an important step in the atherogenic process. Thus it's become primary target of lipid-lowering therapy.

As opposed to LDL, HDL involve in reverse transportation of cholesterol, the process whereby cholesterol is brought from peripheral cells to the liver for excretion in the bile. This primary role of HDL in cholesterol transportation contributes to the protective effect to coronary heart disease (CHD). Besides that, anti-inflammatory activity of HDL, which may be mediated in part by enzymes paraoxonase, can prevent the oxidative modification of LDL (Gabay & Kushner, 1999). The relationship between reduction of LDL level and protection against CHD risk is well established from clinical trials. The drugs that most effectively decrease levels of atherogenic LDL are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (Statin). Statins reduce LDL levels by blocking cholesterol biosynthesis (Antonio *et al.*, 2002).

HDL and LDL concentration in each groups of animal throughout this study period are present in Table 4.14 and 4.15. . The baseline level of HDL and LDL before the experiment was not significantly different within the treated groups. Without any treatment HDL and LDL concentration in Normal group remains at stable level with no significant on the different level of HDL and LDL at beginning of study compared to the level on day 30th and day 60th. HDL concentration records a marginal increase to 0.46 mmol/L at the end of study from 0.43 mmol/L. As for LDL, the concentration exhibit reduction from 0.25 mmol/ to 0.15 mmol/L.

On the other hand, induction of hypercholesterolemia on animals also promotes the increasing of HDL and LDL level in Cholesterol, Arab, UKMR-1 and UKMR-2 groups. HDL and LDL level in UKMR-1 treated group exhibit highest increment throughout the study period. Concentrations of HDL and LDL on day 30th in UKMR-1 group were 1.04 mmol/L and 17.63 mmol/L. At the end of study, the levels of HDL and LDL in this treated group were 9.15 mmol/L and 22.02 mmol/L respectively. Meanwhile, animals treated with Arab variety extract recorded lowest elevation in HDL and LDL concentration. After 60 days of treatment, HDL and LDL concentrations were increased to 7.12 mmol/L and 19.54 mmol/L respectively. Nevertheless, Statistical analysis shows the different concentrations of HDL and LDL level in Arab, UKMR-1 and UKMR-2 animals groups after day 30th and 60th of treatment are significant compared to normal control.

5.4.5 Atherosclerotic Plagues Analysis

The present study showed that 60 days induction of hypercholesterolemia in Cholesterol, Arab, UKMR-1 and UKMR-2 groups lead to development of atherosclerosis in the aorta between its origins into the iliac arteries. Further analysis discovered 80% of aorta surface region of Cholesterol group are covered with atheromatous plaque. The large formation of atheromatous plaque in Cholesterol group is not surprising as the increment of triglyceride and total cholesterol level throughout the study period are tremendously high.

The anticholesterol action of *H. sabdariffa* (0.5% or 1%) was confirmed in rabbits fed cholesterol for 10 weeks. This treatment was effective in reducing the serum concentrations of triglycerides, total cholesterol and low-density lipoprotein cholesterol, and in mitigating atherosclerosis in the aorta. Histopathologically, it was found that feeding *H. sabdariffa* had reduced foam cell formation and inhibited smooth muscle cell migration and calcification in the blood vessel of treated rabbits (Chen *et al*., 2003).

5.4 Phytochemical Analysis

5.4.1 Thin layer Chromatography

Chromatographic method has frequently been applied in the determination of chemical compounds. It is a popular method for solving some analytical problems encountered in the analysis of complex samples of natural origin. In this study, phytochemical compounds in *H. sabdariffa* extracts of Arab, UKMR-1 and UKMR-2 variety were prescreens using Thin Layer Chromatography (TLC) method before each extracts were further analyze using HPLC-MS that gives the possibility to confirm the structures of the separated compounds. TLC analysis for Arab, UKMR-1 and UKMR-2 Variety are presented in Table 4.17, 4.18 and 4.19 respectively. The phytochemical compounds in extracts samples of Arab and UKMR-1 variety were separate to produce seven spots on silica coated plate when acetone and methanol in ratio 3: 7 were used as mobile phase. Most of the spot gave positive result for phenolic compound when tested with vanillin. Meanwhile, the same mobile and stationary phase separates the phytochemical compounds in extract sample from UKMR-2 into 8 groups of bands. The spot also gave positive result for phenolic compound and flavonoids. These phenolic compounds most probably anthocyanins.

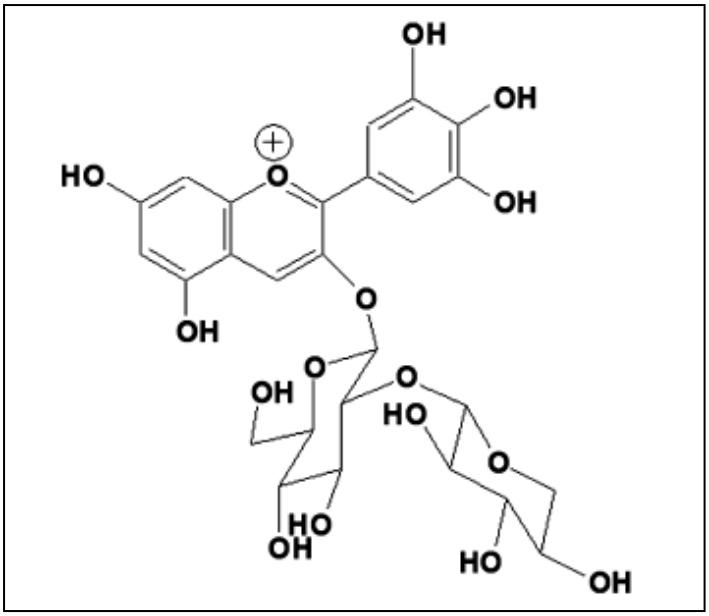
In this method, phytochemical compounds are separate base on their size or solubility to mobile phase. A spot or band produced may consist of several phytochemical compounds with nearly same size or solubility to mobile phase. Most of phytochemical compounds presences in Arab, UKMR-1 and UKMR-2 extracts are from phenol and flavonoids group. Three spots from TLC analysis of Arab extract were positive for phenol. Five spots from both UKMR-1 and UKMR-2 extracts were also contains phytochemical compounds from phenol group. Meanwhile, Flavonoids group presence in Arab, UKMR-1

and UKMR-2 extracts hypotheses to has smaller molecular size compared to phenol group due to larger R_f value they produces on TLC analysis.

5.4.2 LC-MS/MS Analysis

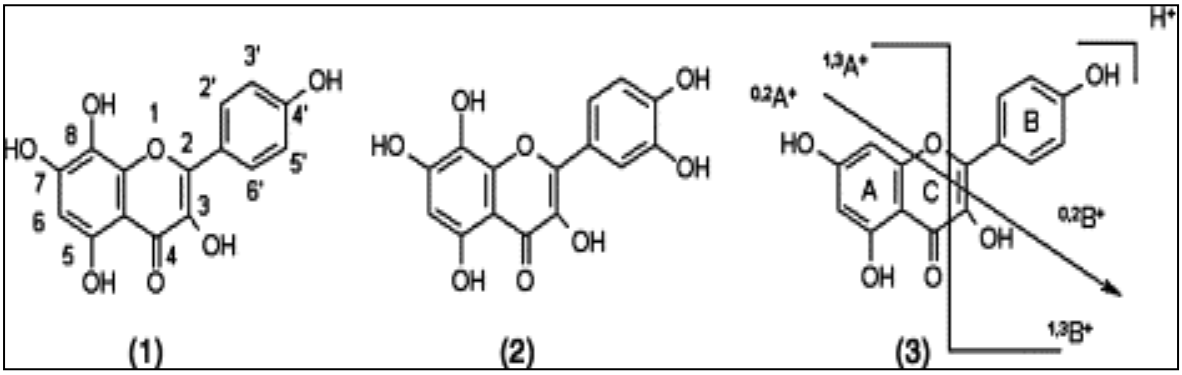
The phytochemical compounds in the three aqueous preparations of *H. sabdariffa* variety were further analyzed by UV and MS spectral analysis. As expected, flavonoids were the main constituents in calyx parts of *H. sabdariffa*. Gossypetin-3-O-glu-7-O-xylo, herbacetin-8-o-xylo-3-o-glu, delphinidin, delphinidin-3-sambubioside, cyanidin 3-sambubioside, and kaempferol-3-o-rutinoside were present in arab extracts. phytochemical analysis on UKMR-1 extract found the present of delphinidin-3-sambubioside, hibiscetine, delphinidin, gossypetin, herbacetin-8-o-xylo-3-o-glu, and quercetin rutinoside. While UKMR-2 sample extract contains hibiscetine, delphinidin, herbacetin-8-o-xylo-3-o-glu and quercetin rutinoside.

Figure 5.1: Chemical structure of delphinidin 3-sambubioside



These phytochemical compositions results are in parallel with the previous research finding. Most of the previous chemical constituent investigation towards characterization of *H. sabdariffa* calyx pigments reported the present of delphinidin and cyanidin. The first documented investigation on chemical constituent in *H. sabdariffa* was done by Yamamoto and Oshima (1932). They isolated an anthocyanin, to which they assigned the structure, cyanidin-3-glucoside. Seshadri and Thakur (1961) was isolated delphinidin-3-sambubioside (also known as hibiscin), the major anthocyanin in *H. sabdariffa* flowers. Shibata and Furukawa (1969) studied the pigments of Taiwanese roselle and also reported the presence of delphinidin-3-sambubioside, along with small amounts of delphinidin-3-monoglucoside, cyanidin-3-monoglucoside and delphinidin.

Figure 5.2: Structures of herbacetin (**1**), gossypetin (**2**) and kaempferol (**3**).



Research done by Gaet (1999), reported the isolation of cyanidin-3-rutinoside, delphinidin, delphinidin-3-monoglucoside, cyanidin-3-monoglucoside, cyanidin-3-sambubioside, cyanidin-3,5-diglucoside and delphinidin-3-sambubioside. Delphinidin, Delphinidin-3-sambubioside (hibiscin) and cyanidin were also found to be present in Arab, UKMR-1 and UKMR-2 extracts. Other chemical constituents that were reported in the previous research and also proved to be exist in extracts of Arab, UKMR-1 and UKMR-2 were gossypetin (Subramanian & Nair, 1972, Milletti *et al*., 1959 and Salah *et al*, .2002), chlorogenic acid (Salah *et al*, .2002), hibiscus acid (Khafaga & Koch, 1980), myricetin (Milletti et al., 1959) , kaempferol-3-O-rutinoside, herbacetin-3-O-glu-7-O-xyloara, quercetin (Milletti *et al.*, 1959, Takeda & Yasui, 1985 and Salah *et al*., 2002,). The isolation of hibiscetine from *H. sabdariffa* was also reported by Milletti *et al.* (1959) and Subramanian (1972).

The calyx and flowers of *H. sabdariffa* also have been known to contain many chemical constituents such as alkaloids, ascorbic acid, ß-carotene, anisaldehyde, arachidic acid, citric acid, malic acid, tartaric acid, glycinebetaine, trigonelline, gossypetin-3-glucoside, gossypetin-7-glucoside, gossypetin-8-glucoside and sabdaritrin, protocatechuic acid, pectin, polysaccharides, mucopolysaccharides, stearic acid and wax (Hirunpanich *et al*., 2005).