Antioxidant Activity of *Rhodomyrtus tomentosa* (Kemunting) Fruits and Its Effect on Lipid Profile in Induced-cholesterol New Zealand White Rabbits

(Aktiviti Antioksida Buah *Rhodomyrtus tomentosa* (Kemunting) dan Kesannya terhadap Profil Lemak di dalam Kolesterol-Induksi Arnab Putih New Zealand)

Muhamad Fahrin Maskam, Jamaludin Mohamad*, Mahmood Ameen Abdulla, Adlin Afzan & Isa Wasiman

ABSTRACT

The objective of this study was to determine the antioxidant activity of Rhodomyrtus tomentosa *fruit extract and its effect on triacylglycerides (TG), total cholesterol (TC), low density lipoprotein (LDL), high density lipoprotein (HDL) and lipid peroxidation in induced-cholesterol New Zealand White Rabbits. In DPPH assay, at concentration of 200 ug/mL methanol extract give 62.13% inhibition of DPPH free radicals with IC_{50} of 107 µg/mL. Similarly, in FRAP assay the methanol extract at concentration of 500 ug/mL showed the highest absorbance (0.16) for antioxidant activity. Whereas, in metal chelating assay the methanol extract at concentration of 100 mg/mL exhibited 36% inhibition of metal chelating ions. The antioxidant activities were due to the presence of phenolics compounds of quinic acid, gallic acid and caffeic acid, which were identified with Q-TQF MS. Total phenolic and total flavonoid content was the highest in water extract at 66.515 mg of GAE/g and 1.828 mg of QE/g, respectively. The water extract of* R. tomentosa *was non-toxic at $LC_{50} = 616.083$ µg/ mL. The white New Zealand rabbits group A was fed by oral gavages with normal diet; Group B cholesterol 1% diet; group C cholesterol 1% diet with fruit extract 50 mg/kg and Group D cholesterol 1% diet with simvastatin standard drug 5 mg/kg. The rabbits in group C has a significantly reduced (p<0.05) total cholesterol (TC), low density lipoprotein (LDL) and significantly increased (p<0.05) high density lipoprotein (HDL) and triacylglycerides (TG) as compared with group B. The lipid peroxidation was reduced significantly (p<0.05) as indicated by the low TBARs-MDA level in group C. Thus, these results showed that the* R. tomentosa *fruit extracts was able to reduce cholesterol level and increased HDL level which can prevent the formation of atherosclerosis in New Zealand white rabbits.*

Keywords: Antioxidant; atherosclerosis; high density lipid; low density lipid; Rhodomyrtus tomentosa

ABSTRAK

Objektif kajian adalah untuk menentukan aktiviti antioksidan ekstrak buah Rhodomyrtus tomentosa *dan kesannya terhadap triasilgliserida (TG), jumlah kolesterol (TC), lipoprotein ketumpatan rendah (LDL), lipoprotein ketumpatan tinggi (HDL) dan peroksidaan lipid. Di dalam asai DPPH, pada kepekatan 200 ug/mL ekstrak metanol memberikan 62.13% perencatan radikal bebas dengan LC_{50} 107 µg/mL. Begitu juga asai FRAP, ekstrak metanol pada kepekatan 500 ug/ mL menunjukkan serapan tertinggi (0.16) untuk aktiviti antioksidan. Manakala di dalam asai pengkelat logam, ekstrak metanol pada kepekatan 100 mg/mL menunjukkan 36% perencatan ion logam pengkelat. Aktiviti antioksidan adalah disebabkan kehadiran sebatian fenolik asid quinik, asid galik dan asid kafeik yang telah dikenal pasti dengan Q-TQF MS. Jumlah kandungan fenolik dan kandungan flavonoid adalah tertinggi di dalam ekstrak air masing-masing pada 66.515 mgGAE/g dan 1.828 mgQE/g. Ekstrak air* R. tomentosa *adalah tidak toksik pada $LC_{50} = 616.083$ µg/mL. Arnab putih New Zealand kumpulan A telah diberi secara gavaj oral diet normal; kumpulan B diet 1% kolesterol; kumpulan C diet 1% kolesterol dengan ekstrak buah 50 mg/kg dan kumpulan D diet 1% kolesterol dengan dadah simvastatin piawai 5 mg/ kg. Jumlah kolesterol (TC) arnab dalam kumpulan C berkurangan dengan ketara (p<0.05), lipoprotein ketumpatan rendah (LDL) dan peningkatan yang ketara (p<0.05) lipoprotein ketumpatan tinggi (HDL) dan triasilgliserida (TG) jika dibandingkan dengan kumpulan B. Peroksidaan lipid menurun dengan ketara (p<0.05) seperti yang ditunjukkan oleh paras TBARs-MDA yang rendah dalam kumpulan C. Oleh itu, semua keputusan ini menunjukkan ekstrak buah* R. tomentosa *mampu merendahkan paras kolesterol dan meningkatkan paras HDL yang boleh mencegah pembentukan arteroklorosis di dalam arnab putih New Zealand.*

Kata kunci: Antioksida; arteroklorosis; lipid ketumpatan rendah; lipid ketumpatan tinggi; Rhodomyrtus tomentosa

INTRODUCTION

Medicinal plants have become the most potential sources of natural antioxidant (Keli Chen et al. 2005). It contained phytochemical compounds such as flavonoid, phenolic acids, tannins, terpenoids and alkaloids with significant antioxidant activity and good for maintaining health (Exarchou et al. 2002). Researchers have showed that a phytochemicals compound from medicinal plants

possesses antioxidant activity for the treatment of various diseases induced by free radicals (Hou et al. 2003). Reactive oxygen species (ROS) initiated various health disorders such as atherosclerosis, inflammatory injury, cancer and cardiovascular disease (Halliwell 1977). The presence of antioxidant can help in scavenging free radicals from ROS and prevent oxidative damage by interrupting the free radical chain reaction of lipid peroxidation (Halliwell & Guttridge 1999). Thus, it can become an effective precautionary defence strategy against various human diseases. Natural antioxidant extracted from medicinal plants is low-cost, safe and has no toxic effect compared with synthetic antioxidant BHA that is known to have toxic and carcinogenic effects on human health (Barlow 1990; Chan 1987; Imadia et al. 1983). Nutritional antioxidants can prevent the formation of atherosclerosis by reducing the susceptibility of LDL from oxidation by free radicals (Harris 1992).

Many studies have showed that the risk of cardio vascular disease and atherosclerosis is closely related to high level of cholesterol in the blood which may caused increased in low-density lipoprotein (LDL) in circulating blood and get deposited in the vascular system to form atherosclerosis (Diaz et al. 1997)**.** The elevation of LDL is easily attacked by natural occurring free radical in body into oxidized form of LDL (De Groot & Noll 1987). Gutteridge (1989) showed that free radicals play an important role in the development of tissue damage and pathological events. The conformational changes of lipoprotein of oxidized LDL will lead to deposition in blood vessel of aorta. Thus, antioxidant plays a significant role by delaying or reducing the oxidation of LDL and be able to prevent the formation of atherosclerosis.

Rhodomyrtus tomentosa or commonly known as Kemunting by the Malays in Malaysia is a large evergreen shrub native originated from South East Asia (Verheij & Coronel 1992). It belongs to the family of Myrtaceae and is an evergreen shrub native to South East Asia (Latiff 1992). It has been used as one of the components in traditional medicines to treat urinary tract infections (Wei 2006). The blueberry-like fruit are edible and contains sugars, vitamins and minerals. The roots and leaves were used to treat diarrhoea, wounds, stomach aches and as a tonic after childbirth. The fruits have the effect on reducing cholesterol level and it has antioxidative effect due to rich in chemical compounds (Asadhawut & Wilawan 2008; Crow et al. 1971; Dachriyanus et al. 2004). There are many cholesterol lowering effect of dietary plants which has been studied from plants (Hamendra & Anand 2007; Shela et al. 2003). However, there are no studies on the antioxidant activities have been reported in *Rhodomyrtus tomentosa* fruits. In this study, antioxidant activities of *Rhodomyrtus tomentosa* and its effect is investigated on the lipid profile in induced-cholesterol New Zealand White rabbits.

MATERIALS AND METHODS

PREPARATION OF FRUIT EXTRACTION

The fruits of *Rhodomyrtus tomentosa* were collected from Kuala Rompin, Pahang. It was cleaned and dried at room temperature for two days. The dried fruits were grinded to powder form and extracted with 300 mL of petroleum ether, chloroform, methanol and water consecutively. The extraction mixtures were incubated in water bath at 40°C for 2 h. The mixture was filtered and the filtrate evaporated to dryness using vacuum rotary evaporator at 40°C. The dried crude extracts were kept in air tight sample bottles until further used.

SEPARATION AND IDENTIFICATION OF PLANT CHEMICAL COMPOUNDS

The chemical compounds presence in the *Rhodomyrtus tomentosa* extract was separated using thin layer chromatography. The presence of phenols, terpenoids and alkaloids were detected by using folin-calticeau, vannilin-H_2SO_4 and Dragendorf reagent, respectively. The identification chemical compounds were identified using high pressure liquid chromatography (HPLC) and gas chromatography mass spectroscopy (GCMS) with referenced to known standard compounds.

DETERMINATION OF ANTIOXIDANT ACTIVITY 1,1-DIPHENYL-2-PICRYLHYDRAZYL (DPPH) RADICAL SCAVENGING ASSAY

The DPPH test was adopted from Yen and Hsieh (1998). DPPH of 8 mg/mL was prepared by adding 0.04 g of DPPH in 5 mL of methanol. A stock solution of ascorbic acid in methanol was prepared at the concentration of 400 μg/mL and kept in flask wrapped in aluminium foil. The reaction mixtures consist of ascorbic acid, DPPH and fruit extracts were incubated at room temperature for 30 min. The DPPH radical was used without ascorbic acid as control. The quenching of free radicals by ascorbic acid is measured spectrophotometrically at 517 nm. The degree of discoloration indicates the free radical scavenging efficiency of ascorbic acid. The percentage of inhibition of DPPH was determined using the formula:

$$\% \text{ of DPPH Inhibition} = [(OD_{control} - OD_{sample}) / OD_{control}] \times 100,$$

where $OD_{control}$ is the absorbance value of control and OD_{sample} is the absorbance value of sample or crude extract.

A graph of percentage of DPPH inhibitions against concentration was plotted to determine the LC_{50} value which was defined as the concentration at which 50% of DPPH radicals is inhibited.

REDUCING POWER ASSAY

The ferric reducing power assay was evaluated following the method as described by Benzie and Strain (1996). Different concentration of crude extracts i.e. 5, 10, 15, and 20 mg were dissolved in 1 mL methanol and stirred until it was completely mixed. Then 1 mL of crude extracts was added with 2.5 mL of 0.2 M phosphate buffer (pH6.6) and later with 2.5 mL of 1% (w/v) potassium ferricyanide. The mixtures were incubated in water bath at 50ºC for 20 min. Following incubation, 2.5 mL of 10% trichloroacetic acid (TCA) solution was added to each mixture and then centrifuged at 1000 rpm for 10 min. 2.5 mL of aliquot of the upper layer was transferred into test tubes and added with 2.5 mL of distilled water and 0.5 mL of 0.1% (w/v) ferric chloride solution. The mixtures are then transferred into cuvetts and the absorbance was taken using spectrophotometer at 700 nm. Increased absorbance of the reaction mixtures indicates greater reducing power. Butylated hydroxyanisole (BHA) was used as standard reference. All tests were carried out in triplicates. Mean values for three independent samples were calculated for each extract.

METAL CHELATING ASSAY

Ethylenediaminetetraacetic acid (EDTA) was used as a standard reference. EDTA stock solution of 0.1 g/mL was prepared by dissolving 1 g of EDTA in 4 mL deionized water. The pH was adjusted with NaOH solution. The standard and crude extract samples were added with ferrous ($FeCl_2$) and ferrozine in centrifuge tubes.

The reaction mixture was shaken vigorously and left incubated in the room temperature for 10 min. Then, 1 mL of the mixture was transferred into cuvette. The absorbance reading is measured at 562 nm. Deionized water was used as blank. All of the samples were tested in triplicate. The percentage inhibition of ferrozine Fe^{2+} complex was determined using the formula:

$$\% \text{ Inhibition} = [\, (\text{Abs control} - \text{Abs sample}) \, / \, \text{Abs control} \,] \times 100$$

where Abs control is the absorbance reading of control and Abs sample is the absorbance reading of sample.

The crude extracts were assayed at concentration of 1, 2, 3, 4 and 5 mg/mL. They were prepared by dissolving 20 mg/mL of crude extract in 1 mL methanol.

EXPERIMENTAL DETAILS

A total of 24 New Zealand White male rabbit with body weight of 2 kg were used as the experimental model and were divided into 4 groups (*n*=6). Group A was fed by oral gavages with normal diet; Group B with cholesterol 1% diet; group C with cholesterol 1% diet with fruit extract 50 mg/kg and Group D cholesterol 1% diet with simvastatin (standard drug) 5 mg/kg for 10 weeks. The animals were acclimatized under control condition of humidity 70–80% with 12 h light/dark cycle and free access to food and water under room temperature in individual cages for one week before use. The animals used were in concordance with procedure accepted by Animal Care and Use Committee, Faculty of Medicine, University of Malaya (Animal Ethic No: ISB/11/03/2009/MFM(R)).

PREPARATION OF SIMVASTATIN DRUG AND PLANTS EXTRACT FOR ANIMAL TREATMENTS

Commercial Simvastatin drug was purchased from Pharmaniaga. The dosage given to the treatment of rabbit in group D and water extract of *R. tomentosa* was 5 and 50 mg/kg, respectively. Treatment was given to the subject orally through force-feeding needle.

PREPARATION OF ANIMALS BLOOD SAMPLE

Blood were taken from ear vein of non-anaesthetized rabbit at week 0, week 5 and week 10 of the experimental period. Rabbits were kept under fasting condition at least 12 h before blood sampling to allow the relevant estimation of lipid profile levels. Blood volume was collected minimally at 7 mL using 21 G syringe. The animals were kept in rabbit restrainers and the ears were disinfected by wiping the central vein area thoroughly with 10% alcohol swab. Then, the needle was inserted at ¼ the length of the needle distally into the central vein, with the tips of the needle pointing toward the base of the ear. When needle is in place, blood was collected into open plain tube, as it should begin to flow immediately through the needle. EDTA tube for serum collection and Gel Tube for plasma collection was used. In order to obtain the serum, blood in the tube were leave clot for 30 min. Then, the tubes were centrifuged at 3000 rpm for 10 min. Serum and plasma obtained from the centrifugation was separated into 3 appendorf tubes for duplicate and kept under -80ºC before further analysis.

ANALYSIS OF LIPID PEROXIDATION WITH TBARS-MELONDIALDEHYDE (MDA)

The standard MDA was prepared by adding together 0.01 mL 1,1,3,3-tetraetoxipropane (malondialdehyde tetraetil asetal) 4.05 M or MDA reagent into 1 L distilled water to form 0.04 mM MDA. Five different MDA standard concentrations (0.25, 0.5, 1.0, 2.0 and 4.0 nmol/mL) were prepared.

0.5 mL of standard MDA was pippetted out into 5 test tubes. Then, 2.5 mL trichloroasetic acid 1.22 M (TCA 1.22 M in HCl 0.5 M) and 1.5 mL tiobarbituric acid (0.67% TBA in 0.05 M NaOH) was added into each test tubes. Test tubes were tightly closed and heated in water bath at 100ºC for 30 min to allow the formation of MDA-TBA complex. Then the test tubes were taken out and allowed cooling at room temperature.

The MDA-TBA complex was extracted with 4 mL n-butanol and vortexes vigorously for 3 min. The mixture was centrifuged for 10 min at 3000 rpm (to separate

between n-butanol and aqueous layers). The supernatant layer was removed and the absorbance was read at 532 nm using spectrophotometer. The MDA concentration in serum sample was determined by adding 0.1 mL diluted serum to 2.5 mL TCA and 1.5 mL TBA at room temperature for 15 min. The MDA concentration in the sample was determined using the MDA standard curve formula:

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

where Vn is the final volume and V_O is the early volume. MDA are then divided by protein (mg/mL) to find the MDA in nmol/mg protein.

LIPID PROFILE ANALYSIS

The lipid profiles observed were the total cholesterol, triglycerides, high-density lipoprotein (HDL) and low-density lipoprotein (LDL). The Commercial kit (SIGMA) was used to run the analysis using Hitachi Chemistry Analyzer at clinical diagnostic laboratory, University Hospital of University Malaya, Kuala Lumpur.

STATISTICAL ANALYSIS

The data collected were in triplicate and presented as average ± standard deviation (SD). Data were statistically evaluated using one-way ANOVA, followed by Dunnett test using STAT software. The values were considered significant when $p<0.05$.

RESULTS AND DISCUSSION

IDENTIFICATION OF PLANT CHEMICAL COMPOUNDS

The present of chemical compounds were separated using Thin Layer Chromatography (TLC). The phenolic compounds were detected with folin reagent gives purple colours. The compositions of the phenolic compounds were identified with Q-TQF MS. Table 1 shows that it contained phenolic compounds of quinic acid, gallic acid and caffeic acid. The content of malic acid in *R. tomentosa* was elucidated with mass charge of 133.0139. Gallic acids are

plant polyphenols that widely distributed in plant kingdom and its presence was detected in *R. tomentosa* fruit with mass charge of 169.0142. Gallic acid (Rice-Evans et al. 1996), quinic acid and caffeic acid (Santos et al. 2010) have been shown to possess antioxidant properties.

DPPH ANTIOXIDANT ASSAY

The DPPH assay is commonly used to screen antioxidant activity of plant crude extract and be able to be detected at low concentration (Sanchez-Moreno 2002). The DPPH radical scavenging activity of the fruit crude extracts of *R. tomentosa* were investigated. As shown in Figure 1, the highest activity was observed in the methanol followed by water, chloroform and petroleum ether extracts. At the concentration of 200 ug/mL, the DPPH radical inhibition of fruit crude extract of *R. tomentosa* decreased in the following order: methanol (62.13%) > water (59.17%) > chloroform (34.19%) and petroleum-ether (20.29%). Ascobic acid (Vitamin C), a well-known antioxidant which is used as appositive controls shows 95% inhibition on DPPH radical at a concentration of 200 ug/mL. As shown in Table 2, the IC_{50} values of ascorbic acid, water, methanol, chloroform and petroleum-ether extracts were 0.51, 154, 107, 230 and 250 ug/mL, respectively. The methanol fruit extract showed the highest antioxidant activity in the DPPH assay. This results indicated that *R. tomentosa* have better performance against DPPH radicals. The high antioxidant activity of the methanol fruit extract was due to the presence of gallic acid, quinic acid and caffeic acid (Table 1) as these compounds have been known for its high antioxidant properties (Catherine et al. 1996; Sonia et al. 2010).

FERRIC REDUCING POWER ASSAY

The reducing power activity of a compound could be used as a useful indicator for antioxidant (Meir et al. 1995). In this assay, the yellow colour of the reaction solution changes to green depending on the reducing power of the test sample. The presence of antioxidant in the solution causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, the formation of Fe^{3+} can be determined by measuring its absorbance at 700 nm (Zhou

TABLE 1. Identification of chemical compounds in crude extracts from *Rhodomyrtus tomentosa* by direct infusion Q-TOF MS

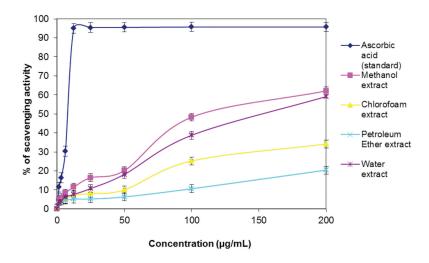| No	Mass/Charge (Da)	MS2	Compounds detected	Class of compound
1.	191.0565	171, 137, 127, 109, 93, 87, 85, 81, 67, 59	Quinic acid	Carboxylic acid
2.	169.0142	151, 125, 124, 97, 79, 69	Gallic acid	Hydrobenzoic acid
3.	331.0663	313, 295, 271, 239, 211, 169,168, 125, 124, 107, 89, 71, 59	Galloyl glucose	Hydrolysable tannin
4.	291.0140	247, 219, 203, 191, 175, 171, 125, 80	Brevifolin carboxylic acid	Hydrolysable tannin
5.	133.0139	115, 89, 73, 71, 59	Malic acid	Organic acid
6.	179.0558	161, 135, 134, 99, 87, 75, 71, 59	Caffeic acid	Hydrocinnamic acid

FIGURE 1. Free-radical scavenging activity of crude extract from fruits of *Rhodomyrtus tomentosa* measured by DPPH assay. Results were mean ± SD (*n*=3)

TABLE 2. IC$_{50}$ values (DPPH free radical scavenging activity assay), total phenolic content, total flavonoid content and LC$_{50}$ of BSLA from crude fruit extracts of *Rhodomyrtus tomentosa*

Extract Sample	IC$_{50}$ (ug/mL)	Total Phenols (ug of GAE/g)	Total Flavonoid (ug of QE/g)	LC$_{50}$ BSLA (ug/mL)
Water	154	66.52 ± 0.01	66.52 ± 0.01	616.083
Methanol	107	40.00 ± 0.01	1.60 ± 0.01	316.228
Chloroform	230	13.99 ± 0.01	1.50 ± 0.01	100.012
Petroleum ether	250	12.99 ± 0.01	0.40 ± 0.01	31.623

Results were mean ± SD (*n*=3)

et al. 2004). As shown in Figure 2, the reducing power of the fruit crude extracts of *R. tomentosa* at 500 ug/mL were as follow: methanol (O.D value=0.16)>chloroform (O.D value= 0.12)>water (O.D value=0.11)>petroleum-ether (O.D value=0.08). The reducing power of methanol crude extract was the highest of all the crude extracts and it increased linearly with increasing concentration. The high absorbance of methanol fruit crude extract supports the evidence that gallic acid and caffeic acid (Table 1) have high reducing power activity that able to donate electron and can react with free radicals to convert them to more stable products and terminate radical chain reaction as described by Gow-Chin and Hui Yin (1995) and Hideo et al. (1990), respectively.

METAL CHELATING ASSAY

EDTA was used as a standard due to its strong metal chelator. The results in Figure 3 shows that the methanol crude extract give a better inhibiting effect with 36% of inhibition at a concentration of 100 mg/mL, followed by water extract (17.6%), chloroform extract (14%) and petroleum-ether (12%), respectively. These results indicated that the fruit crude extract of *R. tomentosa* showed poor metal chelating activity. The presence of gallic acids (Table 1) in the methanol crude extract could be the possible explanation

for its low antioxidant activity towards ferrous ions due to its inability to chelate ferrous ion and similar observation has been reported by Duh et al. (2001). Many authors have reported that metal chelating potency play a minor role in the overall antioxidant activities of some polyphenols (Rice-Evans et al. 1996). However, the ability of the phenolic compounds to chelate iron was far lower than that of EDTA (Andjelkovic et al. 2006).

TOTAL PHENOLS AND FLAVONOIDS CONTENT OF *RHODOMYRTUS TOMENTOSA*

The total phenols in the fruit crude extract of *R. tomentosa* were determined according to the Folin-Ciocalteu method as described by Spanos et al. (1990). As shown in Table 2, the total Phenolic content in water extract give the highest amount (66.52 mg of GAE/g), followed by methanol extract (40 mg of GAE/g), chloroform extract (13.99 mg of GAE/g) and petroleum-ether extract (12.99 mg of GAE/g). The Phenolics contents in plants have been shown related with their antioxidant activity due to their redox properties to act as reducing agent, hydrogen donor and singlet oxygen quenchers (Chang et al. 2001). The results from Table 2, showed that the total flavonoids content was highest in the water extract (1.83 mg/mL) followed by methanol extract (1.60 mg/mL), chloroform
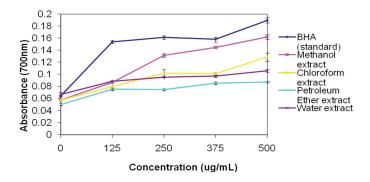
FIGURE 2. Ferric reducing power of crude extract from fruit of *Rhodomyrtus tomentosa*. Results were mean ± SD (*n*=3)
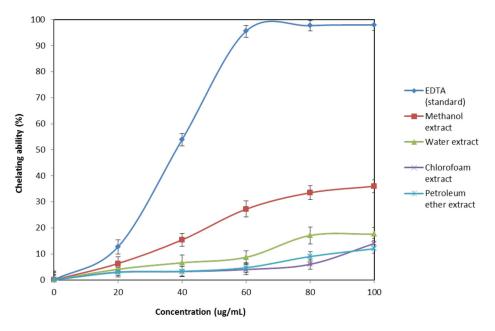

FIGURE 3. Metal chelating ability of the crude extract from the fruit of *Rhodomyrtus tomentosa*. Results were mean ± SD (*n*=3)

extract (1.50 mg/mL) and petroleum-ether extract (0.14 mg/mL). The results obtained showed that the antioxidant activity give a good correlation with the total phenolis and flavonoids content. Yun Shen et al. (2009) have shown that phenolic and flavonoids content correlated with antioxidant activity.

BRINE SHRIMP LETHALITY ASSAY OF *RHODOMYRTUS TOMENTOSA*

Brine Shrimp Lethality Assay (BSLA) is a general bioassay which was used to evaluate the toxicity of plants extract (Meyer et al. 1982). A study by Hlywka et al. (1997) has shown that there was a correlation between the number of dead shrimps and concentration of the plant extract. In this study, the results showed that the water extract of *R. tomentosa* at LC_{50} of 616.08 µg/mL was nontoxic and no mortality of brine shrimp was observed. The most toxic extract was petroleum ether with LC_{50} at 31.62 µg/mL. Since the water extract is non-toxic it was used to evaluate

its activity on the lipid profile in induced-atherosclerosis in New Zealand white rabbits.

TREATMENT OF WATER EXTRACT ON ANIMALS

In this study water extract of *R. tomentosa* together with 1% cholesterol diet was administered via oral gavage into New Zealand white rabbits following the procedure as described by Tijburg et al. (1997). Water extract was used to evaluate the effect on the cholesterol level and the lipid profile in rabbits due to its non-toxic effect on Brine Shrimp Lethality Assay (BSLA). The results of antioxidant from Figures 1, 2 and 3 also show that methanol extract of *R. tomentosa* possessed a good antioxidant activity. This will give a good indication for its effect on the lipid profile in rabbits. Beob-Jin et al. (2003) have showed that there were correlation between antioxidant activity with the cholesterol level and the development of atherosclerosis in rabbits. The ability of plant extracts to reduce cholesterol and prevent atherosclerosis could be due to the presence of flavonoids

rich nutrient (Michael Aviram 2004). The maximum tolerated dose (MTD) of water extract of *R. tomentosa* given to the rabbits was 50 mg/kg/day. There was no sign of toxicity and no mortality was observed during the test period.

EFFECT OF *RHODOMYRTUS TOMENTOSA* WATER EXTRACT ON BODY WEIGHT

Figure 4 shows that at week 5, there was significant increased ($p<0.05$) on the body weight in cholesterol, tomentosa group and simvastatin group compared to the normal group. However, at week 10, the entire group showed significant increased in body weight ($p<0.05$) with the cholesterol group has the highest body weight at 2.873±0.50 kg. The significant in body weight ($p<0.05$) within group at week 10 was observed in normal and simvastatin group. This showed that *Rhodomyrtus tomentosa* water extract, cholesterol diet and simvastatin have effect on the rabbit body weight.

EFFECT OF *RHODOMYRTUS TOMENTOSA* WATER EXTRACT ON TRIACYLGLICERIDES

Figure 5 shows that there was a significant increase ($p<0.05$) of TG level in cholesterol diet groups at week 5. The increase of TG with supplementation of cholesterol diet was reported by Mohamedain and Fred (2000). At week 5, triacylglicerides in cholesterol diet group increased up to 225% and 13% increase in *R. tomentosa* group compared with the normal group rabbit. In simvastatin group triacylglicerides reduced significantly ($p<0.05$) by 22% compared with cholesterol group at week 10. The reduction of triacylglicerides in hypercholesterolemic induced rabbit treated with simvastatin has been reported by Hernandez-Presa et al. (2003). This showed that *R. tomentosa* has the potency to reduce triacylglicerides level significantly in rabbits.
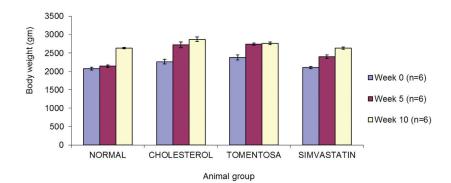

FIGURE 4. The effect of water extract of *Rhodomyrtus tomentosa* on rabbit body weight. Data are expressed as mean ± S.E.M. (*n*=6)
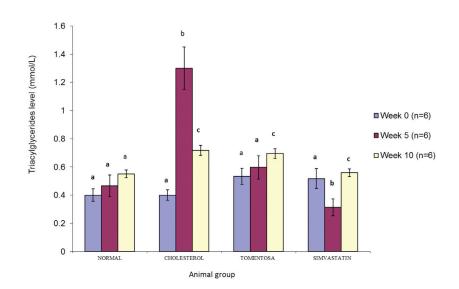

FIGURE 5. The effect of water extract of *Rhodomyrtus tomentosa* on Triglycerides (TG). Data are expressed as mean ± S.E.M. (*n*=6). Treatment groups that do not share the same letter were significantly different ($p<0.05$)

EFFECT OF *RHODOMYRTUS TOMENTOSA* WATER EXTRACT ON TOTAL CHOLESTEROL (TC)

The average of serum total cholesterol level (TC) for all groups was taken at week zero (w 0) as baseline. The average level at week 0 was found to be 1.204±0.362 mmol/L with range between 0.950 and 1.383 mmol/L. As shown in Figure 6, the TC level at week 0 showed no significant difference in normal, cholesterol, *tomentosa* and simvastatin group. At week 5, cholesterol group showed the highest level of TC (24.833±0.337 mmol/L), followed by simvastatin group (15.967±8.333 mmol/L) and *tomentosa* group (12.633±4.121 mmol/L). At week 10, *tomentosa* group (17.05±1.025 mmol/L) and simvastatin group (16.067±1.594 mmol/L) showed significant decreased of TC level compared to cholesterol group (25.983±1.409 mmol/L). This showed that the supplementation of high cholesterol diet has increased the total cholesterol (TC) level significantly in the cholesterol group. This support the previous study that high intake of cholesterol in diet can induce high level of serum cholesterol in rabbit as it absorbs cholesterol efficiently (Chin et al. 1990).

In the *tomentosa* extract group, there was a significant decreased ($p<0.05$) in TC level to 32% when compared with the cholesterol group. While the simvastatin group reduced significantly to 36% of TC level. This showed that *tomentosa* extract has good effect in lowering the TC level in hypercholesterolemic rabbits. Gould et al. (1998) has shown that 10% reduction of total serum cholesterol can reduced the mortality rate of CVD to 15%. This indicated that tomentosa extract has the potential to prevent cardiovascular diseases.

EFFECT OF *RHODOMYRTUS TOMENTOSA* WATER EXTRACT ON LOW DENSITY LIPOPROTEIN (LDL)

As shown in Figure 7, the estimation of LDL level at week 0 can be used as baseline value as all level in each group showed no significant difference. The average level of LDL is 0.36±0.241 mmol/L with the range of 0.232 to 0.448 mmol/L. At week 5, all groups except normal group showed increased level of LDL. Cholesterol group have the highest level of LDL (23.295±0.334 mmol/L), followed by *tomentosa* group (11.647±4.244 mmol/L) and simvastatin group (15.305±7.740 mmol/L). The LDL level at week 10 showed significant decreased in *tomentosa* group (17.572±0.036 mmol/L) and simvastatin group (15.293±1.654 mmol/L) compared with cholesterol group.

The cholesterol group showed a significantly higher LDL level as compared with normal group, *tomentosa* group and simvastatin group. The high LDL level can be attributed to the down regulation of LDL receptor by cholesterol in the liver as reported by Mustad et al. (1997). Thus it can be postulated that high cholesterol diet intake can induce elevation level of TC, LDL and decreased the HDL level.

The LDL level of both *tomentosa* and simvastatin group were reduced to 29 and 38%, respectively, compared with cholesterol group at week 10. The reduction of the LDL could be due to the presence of antioxidant compounds in tomentosa extract that attacked free radical species and prevented it from interacted with LDL. Studies by Weggemens and Trautwein (2003) have shown that flavonoids intake decreased LDL level by removing cholesterol from peripheral liver tissue for catabolism. The possible explanation of the reduction of the LDL level in the simvastatin group could be due to its antioxidant action (Aviram et al. 1998; Chaudiere & Ferrari-lliou 1999) and inhibition of hepatic reductase (Nobuhiro et al. 2006; Roberto et al. 2005).

EFFECT OF *RHODOMYRTUS TOMENTOSA* WATER EXTRACT ON HIGH DENSITY LIPOPROTEIN (HDL)

As shown in Figure 8, the estimation of HDL level at week 0 is used as baseline as each group showed no significant
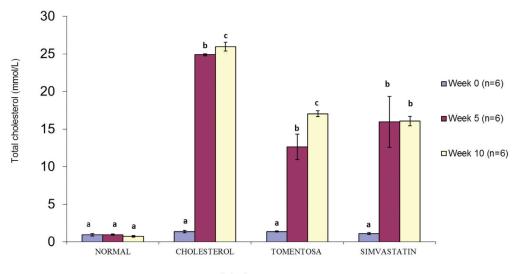

FIGURE 6. The effect of water extract of *Rhodomyrtus tomentosa* on total cholesterol (TC). Data are expressed as mean ± S.E.M. (*n*=6). Treatment groups that do not share the same letter were significantly different ($p<0.05$)
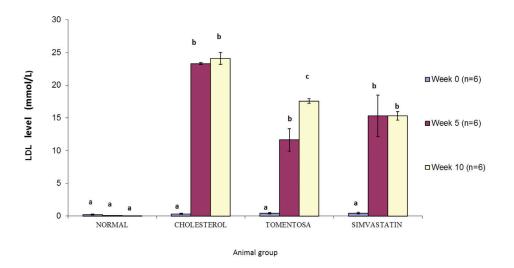
FIGURE 7. The effect of water extract of *R. tomentosa* on low density lipoprotein (LDL). Data are expressed as mean ± S.E.M. (*n*=6). Treatment groups that do not share the same letter were significantly different (*p*<0.05)
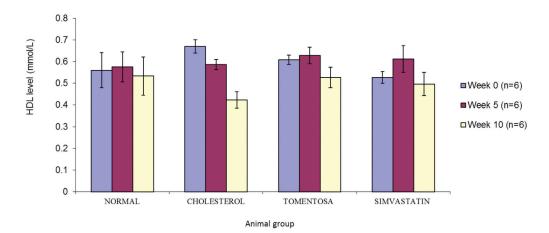

FIGURE 8. The effect of water extract of *Rhodomyrtus tomentosa* on high density lipoprotein (HDL). Data are expressed as mean ± S.E.M. (*n*=6)

different. The average of HDL level is 0.591±0.119 mmol/L in range of 0.527 to 0.670 mmol/L. At week 5, there is no significant difference in HDL level in all groups although the *tomentosa* group and simvastatin group showed slightly increased HDL level with 0.628±0.094 mmol/L and 0.612±0.151 mmol/L, respectively. After week 10, all groups showed no significant difference in HDL level. The cholesterol and *tomentosa* group showed the lowest level of HDL compared to the other group.
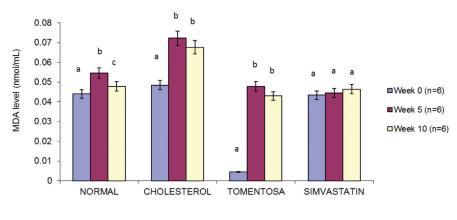
The level of HDL in the body indicates the risk factor for the developing of atherosclerosis. The lower the HDL level, the higher the risk of having atherosclerosis while increase in HDL may slow down the risk of atherosclerosis.

In this study, treatment of *tomentosa* extract with cholesterol diet 1% significantly (*p*<0.05) increase the HDL level. At week 10 both *tomentosa* group and cholesterol group have had almost similar HDL level of 0.420 and 0.423 mmol/L, respectively. The increased of HDL level

in *tomentosa* group at week 10 was 30% significantly (*p*<0.05) from the original baseline level at week 0. In simvastatin group, the level of HDL is increased by 30% compared with the cholesterol groups. This is in agreement from previous clinical studies on simvastatin that are able to moderately increased HDL level (Nobuhiro et al. 2006).

THE EFFECT OF WATER EXTRACT OF *RHODOMYRTUS TOMENTOSA* ON SERUM LIPID PEROXIDATION DETERMINED BY THE FORMATION OF TBARS-MALONDIALDEHYDE (MDA)

Malondialdehyde (MDA) is widely used to determine lipid peroxidation. MDA formed an adduct with thiobarbituric acid to produced TBA-MDA complex and determined spectrophotometrically. Lipid peroxidation occurred when cells were exposed to reactive oxygen species causing cell walls to rupture and membrane lipids to degrade to form the end-product that is MDA. As shown in Figure 9, the average level of MDA at week 0 is 0.452 ± 0.006 nmol. In week 5,

FIGURE 9. The effect of water extract of *R.tomentosa* on lipid peroxidation index, indicated by malondialdehyde (MDA). Data are expressed as mean ± S.E.M. (*n*=6). Treatment groups that do not share the same letter were significantly different (*p*<0.05)

cholesterol group showed significant increased (*p*<0.05) in MDA level compared with other groups. In week 10, the cholesterol group showed significantly higher (*p*<0.05) in MDA level compared with other groups. The *tomentosa* group showed the lowest level of MDA (0.043±0.002 mmol) followed by simvastatin group (0.046±0.003 mmol), normal group (0.048±0.006 nmol) and cholesterol group (0.068±0.003 nmol).

According to Selvan and Anuradha (1990), the increased level of TBARS-MDA level in cholesterol group was mainly due to free expulsion into the circulation of tissue lipid peroxides caused by pathological changes. Whereas in *tomentosa* and simvastatin groups might have protective effect against lipid peroxidation as the level of TBAR-MDA was decreased in hypercholesterolemic rabbits. The supplement of 50 mg/kg/day of *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 with cholesterol group at week 10. These suggested that the *tomentosa* extract has the potential to reduce oxidative stress and prevent lipid peroxidation. The result obtained from TBARS-MDA was correlated with the decreased of total cholesterol and LDL level in *tomentosa* extract to prevent the formation of atherosclerosis.

CONCLUSION

The phytochemical separation and isolation of the fruits *Rhodomyrtus tomentosa* extracts with thin layer chromatography showed the presence of phenolic compounds. The fruit extract has been shown to contain quinic acid, gallic acid, malic acid and caffeic acid with Q-TQF MS. The *Rhomyrtus tomentosa* extract showed antioxidant activity with DPPH, reducing power and metal chelating assay. The water extract of *Rhodomyrtus tomentosa* fruits has reduced significantly

the triacylgliserides (TG), total cholesterol (TC), low density lipoprotein (LDL) and increased significantly the high density lipoprotein (HDL) level in induced-cholesterol New Zealand White Rabbits. It also decreases the lipid peroxidation as it decreased the formation of the MDA-complex. This showed that the *Rhodomyrtus tomentosa* extract possessed antioxidant activity as well as be able to increase the HDL level to prevent the development of atherosclerosis in animals.

REFERENCES

Andjelkovic, M., Camp, J.V., Meulenaer, B.D., Depaemelaere, G., Socaciu, C. & Verloo, M. 2006. Iron-chelation properties of phenolic acids bearing catechol and galloyl groups. *Food Chemistry* 98: 23-31.

Asadhawut, H. & Wilawan, M. 2008. New acylphloroglucinols from the leaves of *Rhodomyrtus tomentosa*. *Tetrahedron* 64: 11193-11197.

Aviram, M., Rosenblat, M., Bisgaier, C.L. & Newton, R.S. 1998. Atorvastatin and emfibrozil metabolites, but not the parent drugs, are potent antioxidants against lipoprotein oxidation. *Atherosclerosis* 138: 271-280.

Barlow, S.M. 1990. Toxicology aspects of antioxidants used as food additives. In *Food Antioxidants,* edited by Hudson, B.J.F. New York: Elsevier. p. 253.

Benzie, I.F.F. & Strain, J.J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "Antioxidant Power": The FRAP Assay. *Analytical Biochemistry* 239(1): 70-76.

Beob-Jin Kim, Yeon-Kye Kim, Won-Hwan Park, Jeong-Heon Ko, Young-Choon Lee & Cheorl-Ho Kim. 2003. A water-extract of the Korean traditional formulation Geiji–Bokryung–Hwan reduces atherosclerosis and hypercholesteremia in cholesterol-fed rabbits. *International Immunopharmacology* 3(5): 723-734.

Chan, H.W.S. 1987. *Autoxidation of Unsaturated Lipids*. New York: Academic Press. pp. 1-16.

Chang, S-T., Wu, J-H., Wang, S-Y., Kang, P-L., Yang, N-S. & Shyur, L-F. 2001. Antioxidant activity of extracts from *Acacia confusa* bark and heartwood. *Journal of Agriculture and Food Chemistry* 49: 3420-3424.

Chaudiere, J. & Ferrari-Iliou, R. 1999. Intracellular antioxidants: From chemical to biochemical mechanisms. *Food Chemical Toxicology* 37(9-10): 949-962.

Chin, H.P., Liu, C.R. & Blankerhon, D.H. 1990. Very early aortic responses during arterosclerosis induction in rabbits: Measurement by duplex ultrasound: I. Non-invasive study of aortic hyperresponsiveness to serotonin. *Journal of Artherosclerosis* 83(1): 1-8.

Crow, W.D., Nicholls, W. & Sterns, M. 1971. Root inhibitors in eucalyptus grandis: Naturally occurring derivatives of the 2,3-dioxabicyclo[4.4.0]decane system. *Tetrahedron Letter* 12(18): 1353-1356.

Dachriyanus, Fahmi., R., Sargent, M.V., Skelton, B.W. & White, A.H. 2004. 5-Hydroxy-3,3',4',5',7-pentamethoxyflavone (combretol). *Acta Crystallography* E60: o86-o88.

De Groot, H. & Noll, T. 1987. The role of physiological oxygen partial pressures in lipid peroxidation. *Chemistry and Physic of Lipids* 44: 209-226.

Diaz, M.N., Frei, B., Vita, J.A. & Keaney, J.F. 1997. Antioxidants and atherosclerotic heart disease. *Journal of Medicine* 337: 408-416.

Duh, P.D., Yen, G.C., Yen, W.Y. & Chang, L.W. 2001. Antioxidant effects of water extracts from barley (*Hordeum vulgare* L.,) prepared under different roasting temperatures. *Journal of Agricultural and Food Chemistry* 49: 1455-1463.

Exarchou, V., Nenadis, N., Tsimidou, M., Gerothanassis, I.P., Troganis, A. & Boskou, D. 2002. Antioxidant activities and phenolic composition of extracts from Greek oregano, Greek sage, and summer savory. *Journal of Agriculture Food Chemistry* 50: 5294-5299.

Gould, A.L., Rossouw, J.E. & Santanello, N.C. 1998. Cholesterol reduction yields clinical benefit: Impact of statin trials. *Circulation* 97: 946-952.

Gow-Chin Yen & Hui-Yin Chen. 1995. Antioxidant activity of various tea extracts in relation to their antimut agenicity. *Journal of Agriculture Food Chemistry* 43: 27-32.

Gutteridge, J.M. 1989. Lipid peroxidation and antioxidants as biomarkers of tissue damage. *Clinical Chemistry* 41: 1819-1828.

Halliwell, B. 1997. Antioxidants and human diseases: A general introduction. *Nutrition Review* 55: S44-S52.

Halliwell, B. & Gutteridge, J.M.C. 1999. *Free Radicals in Biology and Medicine*. 3rd ed. Oxford, UK: Oxford University Press.

Hamendra, S.P. & Anand, K. 2007. Protective role of *Citrus sinensis*, *Musa paradisiacal*, and *Punica grantum* peels against diet-induced atherosclerosis and thyroid dysfunctions in rats. *Nutrition Research* 27(11): 710-718.

Harris, WS. 1992. The prevention of atherosclerosis with antioxidant. *Clinical Cardiology* 15(9): 636-640.

Hernández-Presa, M.A., Ortego, M., Tuˇnón, J., Martín-Ventura, J.L., Mas, S., Blanco-Colio, L.M., Aparicio, C., Ortega, L., Gómez-Gerique, J., Vivanco, F. & Egido, J. 2003. Simvastatin reduces NF-kappaB activity in peripheral mononuclear and in plaque cells of rabbit atheroma more markedly than lipid lowering diet. *Cardiovascular Research* 57: 168-177.

Hideo Iwahashi, Toshihiro Ishii, Ryojin Sugata & Ryo Kido. 1990. The effects of caffeic acid and its related catechols on hydroxyl radical formation by 3-hydroxyanthranilic acid, ferric chloride, and hydrogen peroxide. *Archives of Biochemistry and Biophysics* 276(1): 242-247.

Hlywka, J.J., Beck, M.M. & Bullman, L.B. 1997. The use of chicken embryo screening test and brine shrimp (Artemia Salina): Bioassay test to assess the toxicity of Fumasin $_{B1}$ mycotoxin. *Food and Chemical Toxicology* 35: 991-999.

Hou, W.C., Lin, R.D., Cheng, K.T., Hung, Y.T., Cho, C.H., Chen, C.H., Hwang, S.Y. & Lee, M.H. 2003. Free radical-scavenging activity of Taiwanese native plants. *Phytomedicine* 10: 170-175.

Imadia, K., Fukushima, S., Shirai, T., Ohtani, M., Nakanish, K. & Ito, N. 1983. Promoting activities of butylated hydroxytoluene on 2-stage urinary bladder carcinogenesis and inhibition of α-glutamyl transpeptidase-positive foci development in the liver of rats. *Carcinogenesis* 4: 895-899.

Keli Chen, Geoff W. Plumba., Richard N. Bennett & Yongping Bao. 2005. Antioxidant activities of extracts from five anti-viral medicinal plants. *Journal of Ethnopharmacology* 96: 201-205.

Latiff, A.M. 1992. *Rhodomyrtus tomentosa* (Aiton) Hassk. In *Plant Resources of South-East Asia: Edible Fruits and Nuts*, edited by Coronel, R.E. & Verheij, E.W.M. Indonesia: Prosea Foundation. pp. 276-277.

Meir, S., Kanner, J., Akiri, B. & Hadas, S.P. 1995. Determination and involvement of aqueous reducing compounds in oxidative defense systems of various senescing leaves. *Journal of Agriculture and Food Chemistry* 43: 1813-1815.

Meyer, B.M., Ferrigni, N.R., Putnam, J.E., Jacobsen, L.B., Nichols, D.E. & McLaughlin, J.L. 1982. Brine Shrimp: A convenient general bioassay for active plant constituents. *Journal of Medicinal Plant Research* 45: 31-34.

Michael Aviram. 2004. Flavonoids-rich nutrients with potent antioxidant activity prevent atherosclorosis development: The licorice example. *International Congress Series* 1262: 320-327.

Mohamedain, M.M. & Fred, A.K. 2000. Cholesterol-rich diets have different effects on lipid peroxidation, cholesterol oxides and antioxidant enzymes in rats and rabbits. *Journal of Nutritional Biochemistry* 5: 293-302.

Mustad, V.A., Etherton, T.D., Cooper, A.D., Maestro, A.M., Pearson, T.A., Jonnalagadda, S.S. & Kris-Etherton, P.M. 1997. Reducing saturated fat intake is associated with increased levels of LDL-receptors on mononuclear cells in healthy men and women. *Journal of Lipid Research* 38: 459-468.

Nobuhiro, T., Hisashi, K., Masatoshi, I., Hiroyuki, H., Kenkichi, B., Naofumi, H. & Tsutomu, I. 2006. Simvastatin attenuates plaque inflammation: Evaluation by fluorodeoxyglucose positron emission tomography. *Journal of the American College of Cardiology* 48(9): 1825-1831.

Rice-Evans, C.A., Miller, N.J. & Paganga, G. 1996. Structure–antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology & Medicine* 20: 933-956.

Roberto, C., Valentin, F., Zahi, A.F., Stephen, G.W., Gerard, H., William, F.C., Jörg, M., Jesse, W., Donald, A.S., Gabor, M. & Juan, J.B. 2005. Effects of aggressive versus conventional lipid-lowering therapy by simvastatin on human atherosclerotic lesions a prospective, randomized, double-blind trial with high-resolution magnetic resonance imaging. *Journal of the American College of Cardiology* 46(1): 106-112.

Sanchez-Moreno, C. 2002. Methods used to evaluate free radicals scavenging activity in foods and biological system. *Food Science and Technology International* 8: 121-137.

Selvam Ramasamy & Anuradha, C.V. 1990. Effect of oral methionine on blood lipid peroxidation and antioxidants in alloxaninduced diabetic rats. *The Journal of Nutritional Biochemistry* 1(12): 653-658.

Shela, G., Hanna, L., Maria, L., Antonin, L., Ryszard, K., Zofia, Z., Zenon, J. & Simon, T. 2003. Seed oils improve lipid metabolism and increase antioxidant potential in rats fed diets containing cholesterol. *Nutrition Research* 23(3): 317-330.

Santos, S.A.O., Pinto, P.C.R.O., Silvestre, A.J.D. & Neto, C.P. 2010. Chemical composition and antioxidant activity of phenolic extracts of cork from *Quercus suber* L. *Industrial Crops and Products* 31(3): 521-526.

Spanos, G.A., Wrolstad, R.E. & Heartherbell, D.A. 1990. Influence of processing and storage on the phenolic composition of apple juice. *Journal of Agriculture and Food Chemistry* 30: 1572-1579.

Tijburg, L.B., Wiseman, S.A., Meijer, G.W. & Weststrate, J.A. 1997. Effects of green tea, black tea and dietary lipophilic antioxidants on LDL oxidizability and atherosclerosis in hypercholesterolaemic rabbits. *Atherosclerosis* 135: 37-47.

Verheij, E.W.M. & Coronel, R.E. 1992. *Plant Resources of South East Asia 2: Edible Fruits and Nuts*. Bogor, Indonesia: Prosea Foundation. p. 446.

Wei, F. 2006. Manufacture of traditional Chinese medicine composition for treating urinary tract infection. (Gungxi Huahong Pharmaceutical Co., Ltd., Peop. Rep. China; Shanghai Fosun Pharmaceutical (Group) Co., Ltd.), Faming Zhuanli Shenqing Gongkai Shuomingshu. China Patent No. CN1853687.

Weggemens, R.M. & Trautwein, E.A. 2003. Relation between soy-associated isoflavones and LDL and HDL cholesterol concentration in humans: A meta-analysis. *European Journal of Clinical Nutrition* 57: 940-946.

Yen, G.C. & Hsieh, C.L. 1998. Antioxidant activity of extracts from Du-zhong (*Eucoma ulmoides*) toward various lipid peroxidation models *in vitro*. *Journal of Agricultural and Food Chemistry* 46: 3952-3957.

Yun Shen, Liang Jin, Peng Xiao, Yan Lu & Jinsong Bao. 2009. Total phenolic, flavonoids, antioxidant capacity in rice grain and their relations to grain color, size and weight. *Journal of Cereal Science* 49(1): 106-111.

Zhou, K., Su, L. & Yu, L. 2004. Phytochemical and antioxidant properties in wheat bran. *Journal of Agriculture and Food Chemisrty* 52: 6108-6114.

Muhamad Fahrin Maskam & Jamaludin Mohamad*
Institute of Biological Sciences
Faculty of Science
University Malaya
50603 Kuala Lumpur
Malaysia

Mahmood Ameen Abdulla
Department of Molecular Medicine
Faculty of Medicine
University of Malaya
50603 Kuala Lumpur
Malaysia

Adlin Afzan & Isa Wasiman
Herbal Medicine Research Centre
Institute of Medical Research, Jalan Pahang
50588 Kuala Lumpur
Malaysia

*Corresponding author; email: jamal@um.edu.my