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Hypocholesterolemic effect of red pitaya *(Hylocereus* sp.*)* on hypercholesterolemia induced rats

[1,2*]Mohd Adzim Khalili, R., [1,3]Norhayati, A.H, [4]Rokiah, M.Y., [4]Asmah, R., [5] Siti Muskinah, M. and [2]Abdul Manaf, A.

[1] *Department of Food and Safety, Faculty of Health and Life Sciences, Management and Science University (MSU), 40100 Shah Alam, Selangor, Malaysia*
[2]*Faculty of Agriculture and Biotechnology, Universiti Darul Iman Malaysia, City Campus, 20040 Kuala Terengganu, Terengganu, Malaysia*
[3] *Faculty of Medicine and Health Sciences, Universiti Darul Iman Malaysia, City Campus, 20040 Kuala Terengganu, Terengganu, Malaysia*
[4]*Department of Nutrition and Dietetic, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[5]*Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

Abstract: This study was carried out to evaluate the total phenolic content and anti-oxidant activity of methanolic extract of red pitaya, and hypocholesterolemic effect of red pitaya (*Hylocereus sp.*) on lipid profiles status on hypercholesterolemia induced rats. From the analysis, total phenolic content in red pitaya is 46.06 ± 1.77 mg GAE/100 g fresh weight and antioxidant activity is 76.10% using FTC method. TBA analysis also showed red pitaya extract had high antioxidant effect (72.90%). An in-vivo study also showed red pitaya has hypocholesterolemic effect on induced hypercholesterolemia rats. After 11 weeks of study, total blood cholesterol significant decrease ($p<0.05$) in the groups supplement with red pitaya. The total cholesterol (TC) level for group PF1 were reduced from 3.356 mmol/L to 1.707 mmol/L (49.14%), group PF2 reduced from 3.435 mmol/L to 1.487 mmol/L (56.72%) and group PF3 reduced from 3.448 mmol/L to 1.412 mmol/L (59.06%) as compare to baseline respectively. The mean total cholesterol level in both negative (N – 5.12%) and positive controls (group HC – 13.79%) were not significantly different ($p<0.05$). The mean triglycerides (TG) level for all groups had shown a reduction ($p<0.05$) with value of 23.87% (group N), 22.674% (group HC), 42.81% (group PF1), 52.82% (group PF2) and 59.52% (group PF3) as compare to baseline levels. The mean HDL level increased by about 2.12% (group N), 19.31% (group PF1), 21.93% (group PF2) and much higher increase in group PF3 (34.42%). The mean LDL decreased by about 39.06% (PF3), 15.10% (PF2), 1.50% (PF1) and 4.33% (group N). The positive control has showed significantly increase with the mean value for 25.68%. In conclusion, all groups that received red pitaya supplementation has high antioxidant properties and showed a good results in managing of lipid profile. It was suggested that the consumption of red pitaya demonstrated the potential to reduce dyslipidemia and play a role in the prevention of cardiovascular disease.

Keywords: Hypocholestrelomic effect, red pitaya fruit and hypercholesterolemia induced rats

*Corresponding author.
Email: ps0049@putra.upm.edu.my
Tel.: +609-6275548 ; Fax: +609-6666943

Introduction

Red pitaya fruit is believed to be native to Southern Mexico, the pacific side of Guatemala, Costa Rica and El-Salvador. Currently, this fruit is being cultivated on a large scale in Malaysia. It is organically grown without the use of any pesticide and chemical fertilizers. Red pulp of pitaya fruit has generated a lot of interest as a source of natural red color for the food coloring, cosmetic industry and health potential for improving eyesight and preventing hypertension and combat anemia (Raveh *et al.,* 1998; Lee, 2002; Stintzing *et al.,* 2002). The medicinal qualities of red pitaya range from alleviating common stomach ailment and have been recommend for hypercholesterolemia, diabetic and anemic persons (Raveh *et al.,* 1998; Lee, 2002). Consumption of fruits and vegetables is associated with lowered risk of cancer and cardiovascular diseases (Raveh *et al.,* 1998; Lee, 2002; Stintzing *et al.,* 2002). Epidemiological studies also suggest that a high intake of fruits and vegetables (5-7 serving/day) reduces the risk of coronary heart disease (Wybraniec *et al.,* 2001). The importance of diets rich in vegetables and fruits for prevention of atherosclerosis has been already reported, considering that fruits and vegetables are rich sources of a variety of nutrients including vitamins, trace minerals, dietary fiber and many other classes of biologically active compounds. One such approach has been to use naturally occurring plant sterols (phytosterols) as cholesterol-lowering adjunct in foods (Block *et al.,* 1992). Consuming diet rich in plant foods will provide a milieu of phytochemical, nonnutritive substances in plants that possess health-protective benefits. Based on the belief that it could reduce cardiovascular disease risk through the beneficial combination of micronutrients, antioxidants, phytochemical and fiber content in this food the American Heart Association has recommended a diet with 5-9 servings of fruits and vegetables daily (Block *et al.,* 1992).

Previous studies on the effect of cholesterol-rich diet on the development of arthrosclerosis in rabbits have shown that atherosclerosis can be easily induced by feeding with 1-2% cholesterol. The most convincing evidence is the fact that, in animal species, atherosclerosis may be experimentally induced by the use of hypercholesterolemic diets (Wybraniec *et al.,* 2001). In rats and other species administration of dietary cholesterol increases low density lipoprotein cholesterol (LDL-C) and decreases high density lipoprotein cholesterol (HDL-C). It is now well established that elevated plasma cholesterol, especially LDL-C, is a predisposing factor for atherosclerosis and cardiovascular disease, frequently occurring in both industrialized and developing countries. In contrast to this, HDL-C may exert a protective effect. It has been reported that hypercholesterolemic atherosclerosis is associated with an increase in tissue concentration of lipid peroxidation products, malondialdehyde (MDA) and conjugated dienes (Lorgeril *et al.,* 1994). Since, there are no reported on the aspect of health potential of this fruit, despite the many claims that have been made. Therefore, this study objectives aim to investigate total phenolic content, anti-oxidant activity and hypocholesterolemic effect of red pitaya (*Hylocereus sp.*) on lipid profiles status on hypercholesterolemia induced rats.

Materials and Methods

Chemicals

Ammonium thiocyanate and ferrous chloride were purchased from Merck (Merck

KGaA, Germany). Ferric chloride, linoleic acid (99.5%), potassium ferricyanide butylated hydroxytolune (BHT), butylated hydroxyanisole (BHA), thiobarbituric acid (TBA) and trichloroacetic acid (TCA) were obtained from Sigma Chemical Co. (St. Louis, USA), folin ciocalteu reagent (APS Chemical Limited, Can, Australia) and Sodium Bicarbonate (BDH Chemicals, Poole, England).

Antioxidant activity and phenolic content
Extraction method

Red pitaya fruit were obtained from red pitaya plantation in Lembah Bidong Setiu, Terengganu, Malaysia. The fruits were carefully washed under running tap water, dried with a soft cloth and the skin peeled; the fresh flesh was then cut into small pieces (1.5 cm x 1.5 cm x 1.5 cm) and macerated in methanol for 5 days. Sample was extracted twice. The methanolic extract was then concentrated under reduced pressure at 40^0C and store at -20^0C until further analysis.

Total phenolic content

Total phenolic content was determined using Folin-ciocalteu reagent following the method of Velioglu *et al.* (1998) with slightly modification using garlic acid as a standard. A total of 100 µL of extract solution (0.2 mg/ml) was added in test tube than 0.75 m of Folin-ciocalteu reagent and left the mixture under room temperature for 5 min. After 5 min 0.75 ml natrium carbonate (60 g/l) was added and the mixture was allowed to stand at room temperature for 90 min. The absorbance was measured at 725 nm using a UV-Vis spectrophotometer (Secoman, France). Total phenolic content was expressed as mg of garlic acid equivalent (GAE) using an equation obtained from the standard garlic acid calibration graph.

Ferric thiocyanate (FTC) method

The standard method with slightly modification as described by Kikuzaki and Nakatani (1993) was used. A mixture of 4.0 mg of red pitaya extract in 4.0 ml of absolute ethanol, 4.1 ml of 2.52% linolenic acid in absolute ethanol, 8.0 ml of 0.05 M phosphate buffer (pH 7.0), and 3.9 ml of distilled water was placed in test tube with a screw cap and then placed in dark oven at 40^0C. To 0.1ml of this solution were added 9.7 mL of 75% ethanol and 0.1 ml of 30% ammonium thiocyanate. Precisely 3 min after the addition of 0.1 ml of 0.02 M ferrous chloride in 3.5% HCl to the reaction mixture, the absorbance of red color was measured at 532 nm every 24 hours until one day after the absorbance of the control reached its maximum. BHT and α-tocopherol were used as positive controls and mixture without red pitaya extract was used as the negative control.

Thiobarbituric acid (TBA) method

The combination method of Ottolenghi (1959) and Aqil *et al.* (2006) with slightly modification was followed. Approximately 1 ml of sample solution from FTC method was added with 2.0 ml of 20% trichloroacetic acid (TCA) and 2.0 ml of 0.67% thiobarbituric acid (TBA) in the test tube. The mixture was placed in water bath (95^0C) for 10 min. After cooling the mixture was centrifuged at 3000 rpm for 20 min. Absorbance of the supernatant was measured at 532 nm. Antioxidant activity was based on the absorbance on the final day of the FTC method.

In-vivo study
Rats

Thirty *Sprague Dawley* rats (200-250 g) were divided randomly into five groups which is normal group (N) and hypercholesterolemic control (HC) and other three groups were given red pitaya diet

supplements (0.5 %, 0.83% and 1.17% / Daily diet). The normal group (N) was fed with the basal diet, while four other groups (HC, PF1, PF2, and (PF3) were given basal diet added with 1% cholesterol (Sigma) and 0.1% cholic acid (Sigma), 30 g daily. The hypercholesterolemic diet was given for 4 weeks as hypercholesterolemic induced weeks. After 4 weeks of inducing hypercholesterolemia the total cholesterol plasma level was confirmed using kit determination by Chemistry Auto-analyzer machine (Roche Diagnostic, USA). If the total plasma cholesterol level in rats measured above 2.3 mmol/l rats are assumed hypercholesterolemia. The hypercholesterolemic diet was continued plus treatment with the red pitaya according to the treatment required. All animals were kept in individual stainless steel metabolic cages at temperatures $27^0C \pm 1^0C$ with a 12 hour light and dark cycle. The intake of the diets was monitored daily, water, feed were provided ad libitium, and the diets were given once daily at 10.30 AM. The study period was 11 weeks. About 10 ml blood was taken from the aorta three times during the study which was during baseline, first and final week of treatment. The blood sample was placed in K_3 + Na EDTA tubes and was centrifuged at 3000 rpm for 10 min to obtain blood plasma. The blood plasma was decanted into eppendorf tubes and immediately frozen at -20^0C stored until plasma was ready for further biochemistry analysis. Protocol of the experiment was approved by the Animal Care and Use Committee at Universiti Putra Malaysia, Malaysia.

Preparation of red Pitaya supplement

The edible portion of red pitaya were blended and homogenized before being frozen at -80^0C for two days, after the fruits which well freeze-dried using freeze dryer machine (BEWHAY/SB4, United Kingdom) at Food Engineering Processing Laboratory, Faculty of Engineering, Universiti Putra Malaysia, Malaysia for 3 days, the fruits were freeze-dry in non light exposure to avoid nutrient losses such as vitamins and phenolics compound. The supplementation were used in this study are 0.5% (PF1), 0.83% (PF2) and 1.17% (PF3) red pitaya per daily diet (30 g) per day. The correct amount supplement of freeze dried red pitaya was diluted accordingly with 2 ml of distilled water and given by force-feeding according to the treatment required. The supplement duration was carried out for 5 weeks.

Preparation of diet

The control group (N) was fed with the basal diet, while four other groups (HC, PF1, PF2, and PF3) were given basal diet added with 1% cholesterol and 0.1% cholic acid. The hypercholesterolemia diet was prepared using the following procedure; normal rat chow was grounded using an automatic grinder to pass through a sieve of 18 cm radius to produced rat chow powder. After that rat chow powder will be added with 1% cholesterol and 0.1% cholic acid. Distilled water was added to the mixture and then the mixture was shaped into pellets using biscuit shaper. After shaping, pellets were dried at 75^0C in an oven for 48 hours. The nutritional composition of the basic diet and hypercholesterolemic diet is presented in the Table 1.

Biochemistry tests

Concentrations of total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-C) and low density lipoprotein (LDL-C) in plasma were analyzed colorimetrically using kit determination (Roche, USA) by chemistry auto-analyzer machine (Roche Diagnostic, USA).

Table 1. The composition (%) of diet used to feed rats according to their treatment group of study.

	Treatment Groups				
Ingredient	**1(N) g**	**2(HC) g**	**3(PF1) g**	**4(PF2) g**	**5(PF3) g**
Casein	20	20	20	20	20
Wheat starch	60	58.5	58.5	58.5	58.5
Potato starch	5	5	5	5	5
Sunflower oil	10	10	10	10	10
Mineral mix*	3.5	3.5	3.5	3.5	3.5
Vitamin mix**	1.3	1.3	1.3	1.3	1.3
Choline	0.2	0.2	0.2	0.2	0.2
Cholesterol	0	1	1	1	1
Cholic acid	0	0.5	0.5	0.5	0.5

* Mineral mix composition (g kg^{-1}): $CaHPO_4*2H_2O$, 27.89; K_2PO_4, 2.43; K_2SO_4, 2.04; NaCl, 0.92; $NaHPO_4*12H_2O$, 1.61; MgO, 0.75; $C_3H_4(OH)(COO)3Fe*3H_2O$, 37.85; $Zn(CH_3COOH)2*2H_2O$, 7.88; $MnCO_3$, 23.41; $Cu(CH_3COO)2*H_20$, 4.95; KJ, 0.04; $C_3H_4(OH)(COOH)_3$, 25.87.

** Vitamin mix composition (units or g kg-1): vit.A, 2000 000 U;D3, 2000 000U; E, 10 000 U; para amino benzoic acid, 10 g; inositol, 10 g; vit. PP, 4 g; pantothenic acid, 4 g; vit.B2, 0.8 g; vit.B1,0.5 g; vit.B6, 0.5 g; vit.B12, 0.003g; folic acid, 0.2 g; biotin, 0.04 g; wheat starch Q.S.P. 1000g.

Group N: normal group receiving basal diet, Group HC: Control group receiving hypercholesterolemic diet, Group PF1: Pitaya supplementations group receiving 0.5% red pitaya supplement/body weight, Group PF2: Pitaya supplementations group receiving 0.83% red pitaya supplement/body weight and Group PF3: Pitaya supplementations group receiving 1.17% red pitaya supplement/body weight.

Statistical analysis

Data were reported as means ± SD. One-way analysis ANOVA was applied to find the difference between the groups. Duncan's multiple range tests was used to find the significant difference among means. Results are considered significantly different at the level of $p<0.05$.

Results and Discussion

Antioxidant activity and phenolics content

The FTC method measures the amount of peroxide value in the beginning of the lipid peroxidation, where ferric ion was formed upon reaction of peroxide with ferrous chloride. The ferric ion will then unite with ammonium thiocyanate producing ferric thiocyanate (a red color substance). The darker the color the higher will be the absorbance (Huda-Faujan *et al.,* 2009). From the FTC analysis it was found that the antioxidant activity of red pitaya and α-tocopherol in methanolic extract are 76.10% and 73.56% (*Figure 1*). Its shows that sample extract and α-tocopherol had been oxidized when stored for seven days at 40-45^{0}C. Previous study done by Maznah *et al.* (2000) also reported that the organic extract sample had the highest antioxidant activity compared with α-tocopherol, the same finding also come from Huda-Faujan *et al.* (2009) and Aqil *et al.* (2006). The TBA test is used to measure the secondary product of oxidation such as aldehyde and ketone (Farag *et al.,* 1989). The result of TBA analysis of the methanolic red pitaya extract and α-tocopherol at sevent day storage, the percentage of antioxidative activity for red pitaya and α-tocopherol are 72.90% and 67.28% (*Figure 2*). This result was in line with other previous study (Maznah *et al.,*

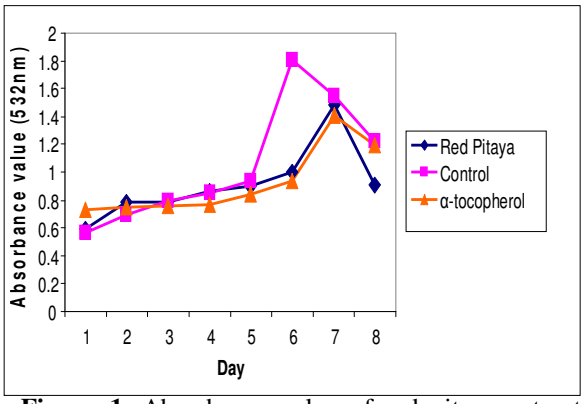

Figure 1. Absorbance value of red pitaya extract (4.0 mg/ml concentration) using FTC method. The values are compared with the absorbance values of the control and α-tocopherol at 532 nm. Each value represents the mean of three replications and betaxanthins (Wybraniec *et al.,* 2001). Anthocyanin is the antioxidant component which involved giving the red, blue and indigo colour pigment to the fruits (Wang *et al.,* 1997). Highest content of anthocyanin also reflect the highest present of phenolics content in the fruits and vegetables, which anthocyanin is the phenolics component which existing in human diet (Javanmardi *et al.,* 2003).

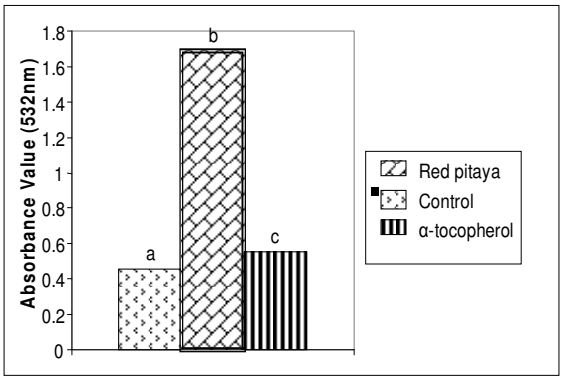

Figure 2. Total antioxidant activity of methanolic extract of red pitaya, α-tocopherol and control using TBA method. Value represents the mean of three replications

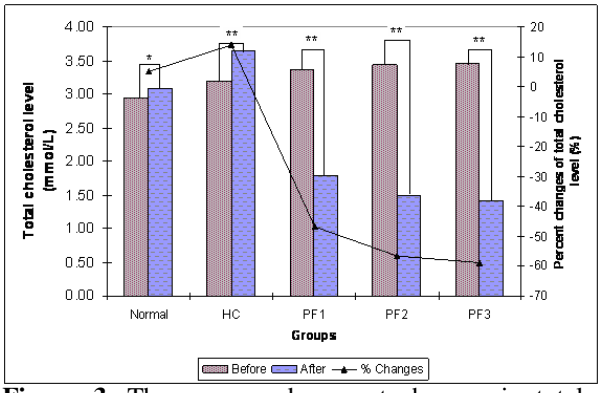

Figure 3. The mean and percent changes in total cholesterol level for the normal, hypercholesterolemic, hypercholesterolemic + red pitaya supplementation (PF1, PF2 and PF3) before and after 5 weeks of treatment

*Means are not significantly different between in weeks ($p<0.05$).

** Means are significantly different between in weeks ($p<0.05$).

Group N: normal group receiving basal diet, Group HC: Control group receiving hypercholesterolemic diet, Group PF1: Pitaya supplementations group receiving 0.5% red pitaya supplement / daily diet (30 g), Group PF2: Pitaya supplementations group receiving 0.83% red pitaya supplement / daily diet (30 g) and Group PF3: Pitaya supplementations group receiving 1.17% red pitaya supplement / daily diet (30 g).

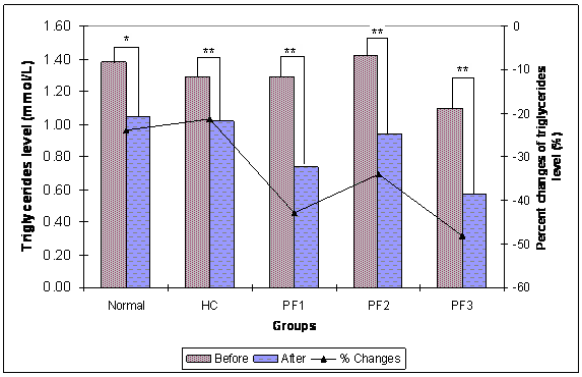

Figure 4. The mean and percent changes in triglycerides level for the normal, hypercholesterolemic, hypercholesterolemic + red pitaya supplementation (PF1, PF2 and PF3) before and after 5 weeks of treatment

*Means are not significantly different between in weeks ($p<0.05$).

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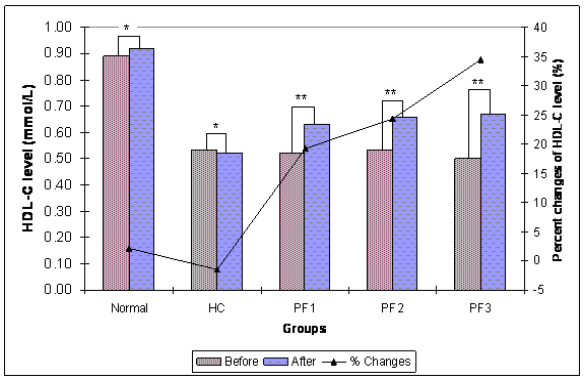

Figure 5. The mean and percent changes in HDL-C level for the normal, hypercholesterolemic, hypercholesterolemic + red pitaya supplementation (PF1, PF2 and PF3) before and after 5 weeks of treatment

*Means are not significantly different between in weeks ($p<0.05$).

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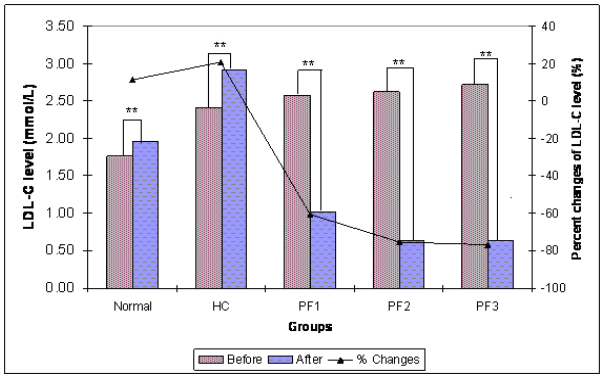

Figure 6. The mean and percent changes in LDL-C level for the normal, hypercholesterolemic, hypercholesterolemic + red pitaya supplementation (PF1, PF2 and PF3) before and after 5 weeks of treatment

*Means are not significantly different between in weeks ($p<0.05$).

** Means are significantly different between in weeks ($p<0.05$).

Group N: normal group receiving basal diet, Group HC: Control group receiving hypercholesterolemic diet, Group PF1: Pitaya supplementations group receiving 0.5% red pitaya supplement / daily diet (30 g), Group PF2: Pitaya supplementations group receiving 0.83% red pitaya supplement / daily diet (30 g) and Group PF3: Pitaya supplementations group receiving 1.17% red pitaya supplement / daily diet (30 g).

2000; Huda-Faujan *et al.,* 2009). From this study also we have found that the total phenolic content in red pitaya methanolic extract is 46.06 ± 1.77 mg GAE/100 g fresh weights. Red pitaya are rich in anthocyanin especially betacyanins and betaxanthins (Wybraniec *et al.,* 2001). Anthocyanin is the antioxidant component which involved giving the red, blue and indigo colour pigment to the fruits (Wang *et al.,* 1997). Highest content of anthocyanin also reflect the highest present of phenolics content in the fruits and vegetables, which anthocyanin is the phenolics component which existing in human diet (Javanmardi *et al.,* 2003).

In-vivo study

Some authors have claimed that a diet rich in vegetables and fruits can prevent atherosclerosis (Lorgeril, *et al.,* 1994; Zulet *et al.,* 1999). According to Koshy *et al.* (2001) the action of HMG CoA reductase is inhibitory in normolipidemic rats after receiving fruits supplementation, thus eliciting highly favorable hypocholesterolemic action. However, in rats fed with a high cholesterol diet and HMG CoA reductase showed no any inhibitory effect probably due to the high concentration of cholesterol in liver and plasma because of cholesterol feeding (Lorgeril, *et al.,* 1994; Zulet *et al.,* 1999). Hyperlipidemia, including hypercholesterolemia and hypertriglyceridemia, is a major risk factor for the development of cardiovascular disease. Oxidatively damage LDLs are taken up by macrophages, which accumulate in the endothelial wall as lipid-laden foam cell in initial phases of atherosclerotic fatty streak

lesions. Therefore, reduction of circulating TGs and total cholesterol and LDLs is primary in prevention of vascular disease. Also prevention of LDL oxidation by dietary antioxidants could delay the development of atherosclerosis (Koshy *et al.,* 2001).

In the present study, feeding rats with diets rich in cholesterol resulted in increased TC, TG and LDL cholesterol levels. This model was used to study the potential of hypolipidemic effect of different supplementations of red pitaya that contained significant amounts of antioxidants properties and useful minerals (Mohd Adzim Khalili *et al.,* 2006). From this study, we found that daily oral administration red pitaya supplements shows a positive result on significantly reduced total cholesterol levels (Figure 3) in plasma after 5 weeks of supplementation. Group that received 1.17% red pitaya diet showed a higher reduction (59.06%) in total cholesterol levels followed by the groups that received 0.87% (56.72%) and 0.5% (49.14%) red pitaya diets respectively. The triglycerides levels in plasma for all groups also showed decreased in value after 5 weeks of treatment (Figure 4*)*. The highest reduction in triglycerides levels was in the group supplemented with 1.17% red pitaya diet. The HDL-C levels in plasma increased (Figure 5) in all groups except for the hypercholesterolemic groups which showed a decrease (-3.61%). The highest increment HDL-C value is in group supplement with 1.17% of red pitaya diet. The LDL-C levels in plasma for all groups' showed a reduction in value except for the hypercholesterolemic group (Figure 6*)*, which showed an increase in value (25.68%) after 5 weeks of treatment. The higher reduction of total cholesterol level in groups with supplementation of red pitaya diets may be due to the increased excretion of bile acid. Red pitaya was reported to have high crude fibre and mineral content, especially potassium, sodium, magnesium, phosphorus, zinc and iron (Mohd Adzim Khalili *et al.,* 2006). The dietary components in red pitaya which have been shown to have a hypocholesterolemic effect through increased excretion of bile acids are soluble fiber, unsaturated fatty acids and minerals especially potassium, sodium, magnesium, phosphorus and zinc (Partiff *et al.,* 1994; Mohd Adzim Khalili *et al.,* 2006).

In conclusion, this study demonstrates that red pitaya fruit rich in phenolics content and antioxidant properties that has a significant influence on altering rat's lipid metabolism. The red pitaya supplement diet has potential in reducing TC, TG and LDL-C and Increasing HDL-C levels. Red pitaya is suggested to have certain antioxidant effect based on high antioxidant activity and phenolic content, therefore the supplementation of red pitaya in diet may be beneficial in the prevention of dyslipedemia; however, further more study is needed to confirm on the findings.

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