An aqueous extract of *Citrus mitis* possesses antioxidative properties and improves plasma lipid profiles in rat induced with high cholesterol diet

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Accepted 19 November, 2009

The *in vitro* antioxidant activity of *Citrus mitis* aqueous extract (CME) and its effects on antioxidative status and lipid profiles of rat fed with high cholesterol diet were examined. The *in vitro* antioxidant activity was assessed by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ferric-reducing antioxidant power (FRAP), while the total phenolic content was measured as gallic acid equivalent. The antioxidative status in the plasma was further assessed by thiobarbituric acid reactive substances (TBARS) assay whereas plasma lipid profile was analysed spectrophotometrically. The result showed that both 5 and 10% extracts possessed antioxidant activities in concentration dependent manner in all tested methods which positively correlated with high phenolic content. The supplementation of 5 mg/kg of both 5 and 10% CME respectively reduced plasma total cholesterol (TCHOL), low-density lipoprotein (LDL) and triglycerides (TG) levels concomitantly with an increased level of high-density lipoprotein (HDL) in rat induced hypercholesterolemia (p < 0.05). The lipid parameters were comparable with statin. The atherogenic index (AI) and sdLDL values were found to be lower in CME-treated groups compared to the control (p < 0.05). Microsomal lipid peroxidation indicated with TBARS estimation was found to be lower in both CME-treated groups. The results obtained suggest that *C. mitis* aqueous extract possesses lipid lowering and antioxidative effect in hypercholesterolemic-induced model and could potentially be used as therapeutic regimen in managing hypercholesterolemia.

Key words: *Citrus mitis*, lipid profile, lipid peroxidation, malondialdehyde, antioxidant.

INTRODUCTION

The use of plants as medicaments either in the form of traditional preparations or as derivatives from pure active principles is widely practiced in ethnomedicine. In every region, based on the climate and geographic conditions, special medicinal plants grow and many of them have unique medicinal properties in the treatment of diseases. Musk lime (*Citrus mitis* Blanco) is used in different con-
and antiphlogistic. When combined with pepper, the infusion is prescribed by the old folk as a laxative and also used to expel phlegm. The peel was also used as a mosquito repellent whereas the juice concentrate is topically applied as a medication of insect bites and prevention of irritation. According to Fisher and Phillips (2008), citrus oils not only lend themselves to use in food but also are generally recognized as safe and have been found to be inhibitory, both in direct oil and vapor form, against a range of both Gram-positive and Gram-negative bacteria. Peels of some fruits including that of Citrus species are also known to have antioxidative, anti-inflammatory, anticancer, and antibacterial activities (Anagnostopoulou et al., 2005; Higashi-Okai et al., 2002; Hakim et al., 2000; Parish et al., 2003; Murakami et al., 2000). In addition, the potential use of citrus flavonoids in cancer treatment has been suggested by some investigators (Siok-Lam and Lee-Yong, 2006; Gharagozloo et al., 2002). The fruits contain several classes of phytochemicals and flavonoids, and act as antioxidants which have been reported to possess multi-beneficial effects in disease prevention.

Increased oxidative stress, resulting from increased reactive oxygen species (ROS) production appears to play an important role in the chronic inflammatory responses to hypercholesterolemia and atherosclerosis (Ross, 1993; Saratho et al., 1996). Although precise mechanism of atherogenesis still need further investigation, oxidative modification of low-density lipoprotein (LDL) is considered to be an essential process in the activation of the inflammatory pathway leading to the formation of atheromatous plaque in the intimal layer of the artery. In contrast to the adverse effects of LDL elevation, the concentration of high-density lipoprotein (HDL) correlates inversely with atherosclerosis development. Apart from hypocholesterolemic drug intervention in reducing blood cholesterol level, the prophylaxis of atherosclerosis using antioxidant therapy and herbal sources has been extensively evaluated in animal experiments (Pratico, 2001; Witztum and Steinberg, 1991). The beneficial effect of plant antioxidants on atherosclerosis is most often attributed to their protective mechanism on LDL oxidation (Bjerkhem et al., 1991). It is therefore of interest to examine whether C. mitis influences lipid parameters.

In the present study, a more detailed examination about the beneficial effects of C. mitis freeze-dried powder on some lipid parameters in the plasma of rat fed with diets supplemented with and without cholesterol is examined, and it is demonstrated that this dietary supplement possesses antioxidative properties both in vivo and in vitro and demonstrated a favourable effect on the concentration of plasma lipids in rat induced hypercholesterolemia. The scientific elucidation of the health-promoting effect of C. mitis extract will provide important information that can contribute to the effective management of atherosclerosis and hypercholesterolemia in humans.

MATERIALS AND METHODS

Raw materials

C. mitis fruits were procured from Universiti Putra Malaysia (UPM) botanical garden and authenticated by plant botanist. The voucher specimen was kept in the university’s Herbarium Unit (SK35421). The fruits were washed thoroughly using a flow tap water, cut into pieces and allowed to dry in oven at 60°C for seven days. The dried fruits were pulverised into powder and kept in airtight container for a maximum of seven days before further use.

Preparation of extracts

C. mitis extracts (5 or 10%) were prepared by soaking 5 and 10 g of dried sample respectively, in 100 ml of distilled water. The solution was kept in a shaking water bath at 60°C for 48 h. The extract was filtered using a Whatman filter paper No.1 to remove debris and insoluble products followed by freeze drying. The crude extracts were reconstituted with distilled water into the desired concentrations for animal supplementation.

Assessment of antioxidant activity

The determination of free radical scavenging activity of the extract was assessed using 1,1-diphenyl-2-picrylhydrazyl (DPPH) method as previously described with minor modification (Masuda et al., 1999). Fifty microlitres of DPPH solution in methanol (5 mM) were mixed with 2.45 ml solution of plant extract and allowed to stand in room temperature for 30 min. The change in colour from normal purple to light yellow was measured at 517 nm wavelength. The decrease in absorbance was converted to percentage antioxidant activity and evaluated as: percent scavenging = [A0 - (A1 - A2)]/A0 x 100, where A0 is the absorbance of DPPH alone, A1 is the absorbance of DPPH + extract and A2 is the absorbance of the extract only. Antioxidant activity of the sample was compared with standard butylated hydroxytoluene (BHT) and vitamin C (both from Sigma Chemicals, USA).

Reducing power

The reducing power of the extract was determined spectrophotometrically according to the protocol of Benzie and Strain (1996). Test solution was freshly prepared by mixing together 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) and 20 mM ferric chloride in 0.25 M acetate buffer, pH 3.6. One hundred micro litre of the extract was added to 300 µl of distilled water followed by 3 ml of FRAP reagent (25 ml acetate buffer, 300 mM/L, pH 3.6 + 2.5 ml 10 mM/L TPTZ in 40 mM/L HCl + 2.5 ml 20 mM/L FeCl36H2O). The absorbance was read at 593 nm after 4 min incubation at room temperature against a blank. The standard curve was constructed using ferrous sulfate (0.2-1.5 mM/L). Antioxidant activity could be determined from the standard curve using its measured absorbance. The results were expressed in millimole per litre. Antioxidant activity of the sample was compared with standard BHT and vitamin C.

Total phenolic content

Total phenolic content was estimated colorimetrically using the Folin-Ciocalteu method (Singleton et al., 1999) with minor modification. A 1.0 ml of appropriately diluted (using 80% ethanol)
Experimental animals

Thirty adult male Sprague Dawley rats (gross body weight 150-250 g) were procured from the University of Putra Malaysia Animal House and were acclimatized under controlled conditions of humidity with regular light/dark cycle and had free access to food and water for one week before use. Following acclimatization, the animals were segregated into 5 groups (n=6) and labelled as A, B, C, D and E. Group A was given 20 g/head/day of normal rat chow, groups B – E were given normal rat chow encoated with 1.5% cholesterol to induce hypercholesterolemia. Groups C and D were supplemented with 5 mg/kg of 5 and 10% C. mitis extract respectively via oral gavage daily whereas group E was supplemented with 20 mg/kg of simvastatin (Sigma Chemicals, USA). Drinking water was given ad libitum. The experimental period was designed for ten weeks. Body weights were monitored at the beginning of the experiment and at week ten (w10). Approximately, 5 ml of blood was withdrawn into an ethylene-diethyl-tetraacetic acid (EDTA) tubes at week 0 and 8 through cardiac puncture procedure after an overnight fast, and the plasma obtained was kept in -70°C for a maximum of 7 days before analysis. All animal handling is in accordance to the institutional Animal Care Committee.

Plasma lipid profile estimation

Full plasma lipid concentrations were estimated spectrophotometrically using COBAS Mira (Roche, Switzerland) chemistry analyser and commercially available kit (Roche, Switzerland). Plasma total cholesterol (TCHOL) and triacylglycerol (TG) concentrations were estimated by enzymatic colorimetric test whereas the high-density lipoprotein (HDL) and low-density lipoprotein (LDL) levels were estimated by precipitation technique as instructed by the manufacturer protocol provided. The atherogenic index (AI) was calculated by dividing the concentration of LDL to the HDL whereas the small dense LDL particle size (sdLDL) was calculated by dividing the concentration of TG to the HDL (Haglund et al., 1992).

Microsomal lipid peroxidation

Plasma lipid peroxidation indicated by thiobarbituric acid reactive substances (TBARS) concentration was estimated spectrophotometrically at 532 nm as malondialdehyde (MDA) equivalents (Ledwozyw et al., 1986) whereas the plasma protein was assessed by Biuret method (Kingsley, 1942).

Statistical analysis

All data were expressed as mean ± standard deviation. Statistical analysis was carried out by one-way ANOVA using the SIGMAStat version 2.01 computer software. Tukey post-tests were performed for multiple group comparison. In all cases, statistical significance was set at p<0.05.

RESULTS

Effect of C. mitis extract on body weight

No significant difference in body weight was observed in all groups at week zero (w0). Supplementation with C. mitis extract (CME) at both 5 and 10% concentration had no significant effect on reducing the body weight of the animals throughout the experimental period. No significant difference was observed in all groups at week ten (Figure 1).

Free radical scavenging, reducing power and total phenolic content

The free radical scavenging activity assay was selected to be used as the first step in this investigation, because intrinsic antioxidant potential of an extract could be measured, taking antioxidant constituents as free radical scavenger in a redox-linked colorimetric reaction. In this study, CME at 5% concentrations showed a weak scavenging power relative to both BHT and vitamin C (p>0.05) but at higher concentrations (10%), CME demonstrated a strong free radical scavenging ability at 93 % and the value was found to be comparable with BHT and vitamin C (Figure 2). Meanwhile, for the FRAP assay, reducing power of CME at concentration of 10% was 1.03 mmol/L and was significantly higher compared with CME at 5% (p < 0.05). There was no significant difference between 10% CME with BHT and vitamin C. The small difference in the FRAP value of CME with vitamin C and BHT, which was 0.32 ± 0.06 mmol/L and 0.18 ± 0.05 mmol/L, respectively, proves that the extract possesses high level of antioxidant properties (Figure 2). The total phenolic content of CME at both 5 and 10% concentration was 32.23 ± 0.84 and 57.45 ± 0.74 mg of GAE/g, respectively. The differences in total phenolic content between concentrations were significantly different (p < 0.05). The level of total phenolic in 10% CME was almost double-fold compared to 5% CME.

Plasma TBARS

The concentration of TBARS as microsomal lipid peroxidation marker is shown in Figure 3. No significant difference was observed in the level of TBARS in all groups at w0. However, TBARS level increased in all groups after the experiment (p < 0.05). The increased TBARS level was highest in group B, compared with other groups (p < 0.05) with almost 4-fold increment than its level at w0 and 1.6 fold than that in group A at w10. Interestingly, groups treated with C. mitis extract demonstrated a statistical significant low level of TBARS when compared with high cholesterol fed group without C. mitis treatment (p < 0.05). This reduction of TBARS le-
Figure 1. Effects of 5 and 10% C. mitis extract supplementation for 10 weeks period on body weight in animals fed with high cholesterol diet. Bar represent mean ± SD. *p < 0.05 compared to its control at week zero, n = 6. A = normal control; B = 1.5 % of high cholesterol diet (HCD); C = HCD + 5 % C. mitis extract; D = HCD+10 % C. mitis extract; E = HCD+20 mg/kg simvastatin.

vel in C. mitis treated groups after 10 weeks treatment was found to be dose-dependent; there was a lower TBARS level in 5% CME which markedly attenuated in the group treated with 10 % CME. The reduced TBARS level in group receiving 10% CME was also comparable with the simvastatin group. The level of TBARS in high cholesterol fed group was highest throughout the experimental period, indicating that 1.5% cholesterol load to rats for 10 weeks was able to cause marked hypercholesterolemia. This made microsomal lipid peroxidation process to be overwhelming in vivo, as suggested by increased TBARS plasma level while supplementation of C. mitis extract provides a defensive mechanism against free radical lipid peroxidation indicated with reduced TBARS concentration in the plasma.

**Effect of C. mitis extract on plasma lipid levels**

The results of the plasma lipid profiles and AI are shown in Table 1. All lipid fractions at w0 demonstrated a low baseline level in all groups. When fed with 1.5% high cholesterol diet for 10 weeks, hypercholesterolemia occurred in the experimental animals as indicated by marked increase of TCHOL, LDL and TG levels in group B rats (p<0.05) in comparison with the levels at w0. The increase of TCHOL, LDL and TG levels at w10 was found to be about 38, 25 and 48% higher than that at w0, respectively. Feeding with high cholesterol diet also increased AI value significantly at w10 when compared to the value before the experiment (Table 1).

Conversely, supplementation of C. mitis extract at both 5 and 10% concentrations showed a favourable effect in improving plasma lipid parameters as shown by a significant decrease in the levels of TCHOL and LDL (p < 0.05) or a significant increase in the level of HDL (p < 0.05) after 10 weeks of treatment when compared with group B (Table 1). The levels of TCHOL and LDL in C. mitis treated groups were also comparable with that of the statin treated group. In this experiment, C. mitis treated groups exhibited a significantly lower AI value at w10 (p < 0.05) when compared to the group B. No statistically significant difference of AI value was obtained in all groups at w0. 1% cholesterol load to the experimental animals was found to be able to statistical significantly increase TCHOL, TG and LDL levels in cholesterol and C. mitis treated groups. The observation that animals exhibited low AI value and high HDL concentration after C. mitis treatment suggests that the supplement may have possibly facilitated the hepatic HDL biosynthesis *in vivo*, rather than the overall total cholesterol concentration.

**DISCUSSION AND CONCLUSION**

Reactive oxygen species (ROS) generated *in vivo* is responsible for the oxidative damage of lipids, proteins, DNA and small molecules. Recent evidence also indicates that oxidative stress is the main mechanism responsible for cardiovascular diseases while hypercholesterolemia under oxidative stress is the predisposing factor for the progression of atherosclerosis and abnor-
Figure 2. Antioxidative capacity of *C. mitis* extract at 5 and 10% respectively indicated with scavenging activity of DPPH radicals (A) and reducing power (B) *in vitro*. Data are mean value ± SD. *p<0.05 vs vitamin C, *p<0.05 vs. BHT, **p<0.05 vs CME 5%.

Mal lipid metabolism. On the other hand, antioxidants might be able to prevent the impairment originated by excessive oxidative stress. Since endogenous antioxidants may not be sufficient to prevent damage, diet-derived antioxidants could be important for maintaining health and preventing free radical-mediated diseases. Their beneficial health effects have been attributed, in part, to the presence of antioxidant polyphenols and flavonoids (Tavarini et al., 2008; Robak and Gryglewski, 1996). These observations led to the hypothesis that dietary antioxidants might reduce the risk of degenerative diseases by reduction of oxidative stress and inhibition of macromolecule oxidation (Collins et al., 2003; Rush et al., 2002).

In this experiment, 1.5% cholesterol load to the animals could generate hypercholesterolemia. Studies in both animals and humans have demonstrated that prolonged high cholesterol concentration in the circulating blood positively correlates with developing atherosclerosis (Tohru et al., 2005; Pratico, 2001). Result from
Figure 3. The thiobarbituric acid reactive substances (TBARS) concentration as malondialdehyde equivalents in animal treated with 5 and 10% of \( C. \) mitis extract. Bar represent mean \( \pm \) SD. *p<0.05 compared to its control at week zero; different superscript letters are significantly different (p<0.05) at week 10. A= normal control; B= 1.5% of high cholesterol diet (HCD); C= HCD + 5% \( C. \) mitis extract; D= HCD+10% \( C. \) mitis extract; E=HCD+20 mg/kg simvastatin.

Table 1. Blood lipids (mmol/l) and the atherogenic index, AI of the animals prior to the experiments and at week 10 (n=6; mean \( \pm \) SD; p<0.05; CME = \( C. \) mitis extract).

<table>
<thead>
<tr>
<th>Group/ Lipid profiles</th>
<th>TCHOL ( w0 )</th>
<th>TCHOL ( w10 )</th>
<th>HDL ( w0 )</th>
<th>HDL ( w10 )</th>
<th>TG ( w0 )</th>
<th>TG ( w10 )</th>
<th>LDL ( w0 )</th>
<th>LDL ( w10 )</th>
<th>AI ( w0 )</th>
<th>AI ( w10 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (normal chow)</td>
<td>1.71( \pm )0.16</td>
<td>1.75( \pm )0.20</td>
<td>1.12( \pm )0.20</td>
<td>1.15( \pm )0.11</td>
<td>0.65( \pm )0.09</td>
<td>0.68( \pm )0.15</td>
<td>0.47( \pm )0.09</td>
<td>0.58( \pm )0.04</td>
<td>0.42( \pm )0.05</td>
<td>0.50( \pm )0.11</td>
</tr>
<tr>
<td>B (normal chow + 1.5% cholesterol, HCD)</td>
<td>1.75( \pm )0.15</td>
<td>2.13a( \pm )0.18</td>
<td>0.85( \pm )0.17</td>
<td>0.89( \pm )0.22</td>
<td>0.54( \pm )0.17</td>
<td>0.79a( \pm )0.14</td>
<td>0.45( \pm )0.05</td>
<td>0.93( \pm )0.10</td>
<td>0.53( \pm )0.07</td>
<td>1.04( \pm )0.09</td>
</tr>
<tr>
<td>C (HCD + 5 mg/kg 5% CME)</td>
<td>1.73( \pm )0.21</td>
<td>1.28c( \pm )0.18</td>
<td>1.34( \pm )0.25</td>
<td>1.39( \pm )0.24</td>
<td>0.76( \pm )0.09</td>
<td>0.63( \pm )0.06</td>
<td>0.45( \pm )0.08</td>
<td>0.27a( \pm )0.07</td>
<td>0.34( \pm )0.05</td>
<td>0.17a( \pm )0.04</td>
</tr>
<tr>
<td>D (HCD + 5 mg/kg 10% CME)</td>
<td>1.68( \pm )0.18</td>
<td>1.06b( \pm )0.19</td>
<td>1.27( \pm )0.20</td>
<td>1.54b( \pm )0.20</td>
<td>0.74( \pm )0.07</td>
<td>0.53b( \pm )0.11</td>
<td>0.49( \pm )0.07</td>
<td>0.23b( \pm )0.08</td>
<td>0.39( \pm )0.08</td>
<td>0.18b( \pm )0.07</td>
</tr>
<tr>
<td>E (HCD + 20 mg/kg simvastatin)</td>
<td>1.59( \pm )0.23</td>
<td>1.42b( \pm )0.19</td>
<td>1.44( \pm )0.17</td>
<td>1.51b( \pm )0.18</td>
<td>0.67( \pm )0.07</td>
<td>0.83( \pm )0.10</td>
<td>0.45( \pm )0.09</td>
<td>0.34b( \pm )0.04</td>
<td>0.31( \pm )0.07</td>
<td>0.23b( \pm )0.04</td>
</tr>
</tbody>
</table>

*p<0.05 within the same group at w0.*p<0.05 compared with the control at w10.
the plasma lipid profiles of 1.5% high cholesterol fed animals for 10 weeks showed concentrations of plasma TCHOL and LDL, to have increased in w10 compared to their baseline levels, whereas HDL concentration was not changed. The results of the present study are consistent with that from previous studies (Maria et al., 2008; Cotelle et al., 1996). These changes are associated with the phenomenon that excessive load of cholesterol to the liver, above the acceptable level of its normal physiological limit, causes the liver to be unable in metabolizing the lipids, thus resulting in high cholesterol return in the circulating blood (Kushi et al., 1996). In this study, however, the treatment of high cholesterol fed animals concomitantly with CME showed not only a protective effect against free radical attack as indicated by a reduced plasma lipid peroxidation biomarker, but also a remarkable reduction of lipid profiles towards the level of the control group. The lipid lowering effect of citrus extract was also found to be comparable with that of statin. The low level of plasma lipids in CME treated groups indicates that besides its antioxidative effect, C. mitis may also possess lipid lowering properties. The mechanism on how exactly citrus extract could lower blood cholesterol requires further investigation but it is postulated that high polyphenolic and flavonoid compounds concentrated in the citrus herbal preparation could partly explain the underlying mechanism of its lipid lowering properties. Citrus plants and fruits are rich in naturally-occurring flavanoids which have been demonstrated to possess a wide spectrum of biological activities such as antibacterial, antioxidant and antiproliferative effects in cancerous cell lines (Siok-Lam and Lee-Yong, 2006; Gharagozloo et al., 2002).

Another important application of plasma lipid markers that is relevant to CVD risk is the association between the LDL to HDL ratio known as atherogenic index (AI). Our results showed that CME had a strong hypocholesterolemic effects in plasma of rats with a reduction of plasma TCHOL levels and an increase of HDL levels (Table 1). Furthermore, the atherogenic index markedly decreased due to a significant decrease of LDL levels in CME treated groups (Figure 4). The observed increase in HDL is one of the most important criteria of an anti-hypercholesterolemic agent. Moreover, numerous studies have demonstrated that high levels of HDL are associated with a lower incidence of cardiovascular diseases (CVD) (Catherine et al., 2004; Yusuf et al., 2004). The increase in HDL levels observed in our study might be due to the stimulation of pre-β HDL and reverse cholesterol transport as shown by previous findings (Gupta et al., 1993). In this respect, it is suggested that the lipid lowering effect of citrus extract in this study can be attributed mainly to a combination of the enriched phenolic contents in the extract and enhanced biosynthesis of hepatic-HDL resulting in increased plasma HDL concentration.

Another aspect that can be used in assessing the CVD risk is the estimation of the small dense LDL (sdLDL). Despite having direct method in estimating the LDL subfraction, the value from TG to HDL ratio also can be
used as an indirect measurement of sdLDL particle size in the circulating blood (Ayyobi et al., 2003; Demacker, 2000). sdLDL particles are thought to be more susceptible to oxidative modification and subsequent uptake by scavenger receptors on activated macrophages leading to the development of lipid-laden foam cells. Our study revealed an increased TG to HDL ratio in high cholesterol diet group after 10 week treatment (p < 0.05) which was reduced upon supplementation with CME (Figure 5). The observed increased LDL concentration is mainly due to high TG to HDL ratio. The intrinsic properties of sdLDL particles have been suggested to be biologically responsible for increasing the risk of developing CVD (Gentile et al., 2008; Stampfer et al., 1996) with numerous studies reporting that the presence of sdLDL particles is associated with a more than three-fold increase in the risk of coronary artery disease (Krauss and Dreon, 1995). sdLDL also penetrates the arterial wall more easily and have a higher capacity to bind to intimal proteoglycans, all properties that are associated with greater atherogenicity (Anber et al., 1997).

Although the present data do not allow the conclusion that C. mitis extract supplementation could prevent atherosclerosis-free radical activity despite its lipid lowering ability, the data are in agreement with a model in which antioxidant supplementation may contribute to a reduction of bad cholesterol in the circulatory system. Because of the mixture of bioactive components present in C. mitis extract, it is possible that more than one mechanism underlying this reduction in lipids is involved.

ACKNOWLEDGEMENT

Author wishes to extend sincere gratitude to the Government of Malaysia and University Putra Malaysia for granting the research works and Mrs Siti Muskinah Mansor for her technical assistance.

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