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Anti-atherogenic effects of morelloflavone from *Garcinia dulcis* leaves in cholesterol fed rabbits

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Abstract

A biflavonoid, morelloflavone isolated from leaves of *Garcinia dulcis* Kurz was investigated for its ability to reduce plasma lipids and prevent progression of atherosclerotic lesions in hypercholesterolemic rabbits. Male rabbits were divided into 6 groups. Group 1 received a regular diet, Group 2 received a regular diet with 0.02% (w/w) of *G. dulcis* derived morelloflavone, Group 3 was fed a diet containing 1% (w/w) cholesterol to induce hypercholesterolemia, and Group 4 to Group 6 had their 1% cholesterol diets supplemented with morelloflavone at concentrations of 0.005%, 0.01% and 0.02%, respectively. During a 4 month-experimental period, rabbits were monitored for their body weights and plasma cholesterol, triglycerides and thiobarbituric acid-reactive substances (TBARS) levels monthly and the extent of aortic atherosclerotic lesions were analysed at the end of the period. The levels of plasma cholesterol, triglycerides and TBARS as well as intimal thickening within aortas were increased in all animals fed with cholesterol diets. Such increases, however, were reduced in the presence of morelloflavone. The concentrations of morelloflavone that showed inhibitory effects in all cases were 0.005% and 0.01%, whereas the 0.02% dosage was ineffective.

Keywords: atherosclerosis, atherosclerotic lesion, *Garcinia dulcis*, hypercholesterolemic rabbit, hypolipidemic effect, morelloflavone.

1. Introduction

Hypercholesterolemia is a major risk factor for atherosclerosis and related occlusive vascular disease [1]. Several lines of evidence have shown that hypercholesterolemia may increase production of oxygen free radicals (OFRs) leading to oxidation of low-density lipoprotein (LDL), which sets the stage for development of atherosclerosis [2]. Thus, apart from avoiding

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high-cholesterol intake, protecting LDL against oxidative modification by antioxidant supplementation should be effective in inhibiting or at least suppressing atherosclerosis. A number of lipophilic antioxidant drugs such as probucol [3], statins [4], lacidipine [5] etc., have been previously shown to inhibit atherogenesis by limiting the oxidative modification of LDL and/or exerting hypolipidemic effects in experimental models. Despite their beneficial effects, there is some concern of the side effects of these synthetic antioxidants. Therefore, finding drugs of natural origin with no side-effect or with low toxicity is of high interest. *Garcinia dulcis* (Roxb.) Kurz (Guttiferae) is a sub-woody tropical tree, grown mostly in Southeast Asia. Different parts of this plant have been used traditionally to cure several ailments. Its bark is used as an antiseptic, the fruit juice as an expectorant and antitussive agent, leaf and seed extracts are used for mumps, lymphatitis and struma remedies [6]. Phytochemical studies have identified abundant flavonoids and xanthones in various parts of this plant [7-10]. Morelloflavone (Figure 1), the biflavonoid with a potent anti-inflammatory activity [11], is present in the leaf [12], fruit [9], flower [10] and seed (W. Mahabusarakam, personal communication) of *G. dulcis*. We also found that this compound effectively scavenged free radicals [9] and increased the resistance of human LDL to oxidation *in vitro* [13]. In this study, an ethyl acetate fraction containing morelloflavone as the major constituent, was prepared from *G. dulcis* leaves. Its ability to reduce plasma cholesterol as well as to prevent progression of atherosclerotic lesions was then investigated in rabbits fed a high-cholesterol diet, a test-animal for atherosclerosis.

2. Materials and Methods

2.1 Plant material

Garcinia dulcis leaves were collected in the Tar-Kham region, Hat Yai. After verification of identity, they were air-dried and then used to prepare morelloflavone. A voucher specimen (Collection No. 02, Herbarium No. 0012652) was also deposited at the Herbarium of Faculty of Science, Prince of Songkla University.

2.2 Preparation of morelloflavone

A total of 10 kg of dried *G. dulcis* leaves was finely powdered and extracted with acetone. After removal of the insoluble matter by filtration, the filtrate was concentrated *in vacuo*. A second extraction was achieved with hexane and the hexane-insoluble fraction was subsequently extracted with ethyl acetate. The ethyl acetate extract was then subjected to solvent partitioning with distilled water. Finally, the ethyl acetate fraction was concentrated *in vacuo*, dried and ground. A total of 500 g of the sample was obtained. Fractionation of the sample was achieved with a high-performance liquid chromatography (HPLC) system (Agilent 1100 Series, Germany), equipped with a solvent delivery pump (BinPump G1312A), an autosampler (ALS G1313A), photodiode-array detector (DAD G1315B) and data output (LC Chemstation, Rev. A.10.02). An ODS-2 column (5 mm particle size, 4.6 x2 50 mm i.d.; Inertsil™, Shimadzu, Japan) was used. The mobile phase consisting of 45% (v/v) acetonitrile and 55% (v/v) 1% acetic acid was pumped at a flow rate of 1 mL/min. The effluent was monitored at 289 nm. Morelloflavone in the sample was identified by comparing its spectral data with that of a standard, previously purified from *G.dulcis* fruits [9]. The peak analysis also revealed that the sample contained mostly morelloflavone (82.3%). The same preparation was then used throughout this study.

2.3 Animals and treatments

Male New Zealand albino rabbits weighing 2.0-2.5 kg were purchased from the National Laboratory Animal Center (Nakhon Prathom,

Thailand). They were housed individually in the Southern Animal Laboratory Facility Building at Prince of Songkla University under the standard environmental conditions (temperature 25 ± 1°C, relative humidity 55 ± 5% and a 12 h light and dark-cycle) and used in the experiment after a 7-day quarantine period. Rabbits were randomly divided into six groups of four each. The control group received 100 g of normal rabbit chow (Lee Feed Mill, Bangkok, Thailand) daily. The high-cholesterol diet group was maintained on the same commercial diet, supplemented with 1% (w/w) cholesterol (Fluka, Switzerland). Three treatment groups received the same cholesterol-containing diet with an additional supplement of 0.005%, 0.01%, and 0.02% (w/w) *G. dulcis* morelloflavone. The last group received only the commercial diet supplemented with 0.02% (w/w) of *G. dulcis* morelloflavone. Each diet and drinking water was provided *ad libitum*. The food consumption and body weight were measured daily and monthly, respectively. After a 4-month (16 weeks) period of diet administration, the overnight fasted animals were injected with pentobarbitone sodium solution (60 mg/mL) into the marginal ear vein. After collecting blood, aortas were excised and kept in 10% (v/v) formalin for histological examination. All procedures were conducted in accordance with international guidelines for animal research. This study was approved by the University's Animal Ethics Committee.

2.4 Analysis of plasma lipids

Fasting blood samples were collected in EDTA tubes from the marginal ear vein of each rabbit, just before the onset of the diet administration period and then monthly until termination. The plasma was obtained by centrifugation at 3000 x g for 15 min at 4°C. Fresh plasma samples were stored at -20°C and used for plasma lipid determination. The plasma cholesterol and triglycerides concentrations were measured enzymatically using a commercial kit (CPT Diagnostics, Spain) based on a modification of the cholesterol oxidase method [14] and lipase-glycerol phosphate oxidase method [15], respectively.

2.5 Determination of TBARS levels

Plasma lipoprotein oxidation markers, malondialdehyde (MDA) and MDA-like substances, were determined on the basis of a spectrophotometric absorbance measurement of the pink colored product of the thiobarbituric acid-reactive substances (TBARS) complex as described previously [16] with a slight modification. Briefly, 0.5 mL of plasma was mixed with 2.5 mL of 20% (w/v) trichloroacetic acid. After standing for 10 min, the mixture was centrifuged at 12000 x g for 10 min. The pellet was mixed with 2.5 mL of 50 mM H_2SO_4 and 3.0 ml of 0.2% thiobarbituric acid (TBA) in 2 M Na_2SO_4, and the mixture was boiled for 30 min. After cooling, the mixture was extracted into 4.0 ml of *n*-butanol and then centrifuged at 12000 x g for 10 min. The butanol layer was read at 530 nm and the TBA-MDA adduct was calculated from the calibration curve using standard MDA.

2.6 Analysis of the extent of atherosclerosis

Immediately after each rabbit was sacrificed, the portion of the aorta from the aortic valves to the renal arteries was removed and rinsed in 0.15 M NaCl. The washed vessel was then cut longitudinally. The areas covered with plaques on the luminal surface of the proximal aorta, where atherosclerotic lesions have been found mostly in cholesterol-fed rabbits [17], was then examined. For histological examination, sections of the aortic arch were, fixed in 10% (v/v) formalin overnight, embedded in paraffin and serially cut into 10-μm-thick slices. Each tissue slide was stained with Oil Red O and Harris's

haematoxylin and observed with a microscope at a magnification of 100. An image of each slice was captured and used for the calculation of the percent intimal thickening by comparing the ratio between the intima and the intima plus media areas as described previously [18]. The data presented for each animal is the mean value for five segments.

2.7 Statistical Analysis

All data was expressed as the mean ± S.D. Significant differences among the groups were determined as one-way ANOVA using the SPSS 11.0 package program for Windows. Scheffe's test was performed if differences were identified between groups. Values of $p < 0.001$, $p < 0.01$, and $p < 0.05$ were considered to be statistically significant at the 99.9%, 99%, and 95% confidence levels, respectively.

3. Results and discussion

3.1 Effects on food intake and body weight gain

During the 4-month period, no abnormal appearance was observed in any rabbits. In addition, the average daily water and food intake did not differ significantly among the groups (data not shown). Prolonged consumption of *G. dulcis* derived-morelloflavone seems to have no effect on the growth of rabbits since those fed with a normal diet plus 0.02% morelloflavone gained body weight at the same rate as the control animals (Figure 2). For the rabbits on a high cholesterol diet, however, their body weights were lower than those of the normal controls ($p < 0.001$) after three months of experiment (Figure 2). At the termination, they were significantly less than at the start ($p < 0.05$). Such result might be due to a suppressive effect of accumulated choleston-3-one and its 3-oxo derivatives, produced by bacteria in the gut from unabsorbed cholesterol [19]. Rabbits on a cholesterol diet with 0.01% and with 0.02% morelloflavone, however, maintained their weight throughout the course of the experiment. At the 4th month, they were significantly heavier than those in non-supplemented cholesterol diet group ($p < 0.05$) (Figure 2). Rabbits fed a cholesterol diet with or without 0.005% morelloflavone showed no significant differences in weight over four months of experiment.

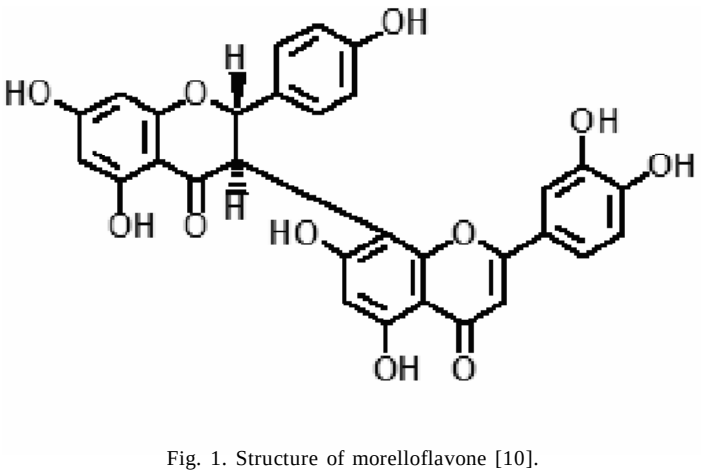

Fig. 1. Structure of morelloflavone [10].

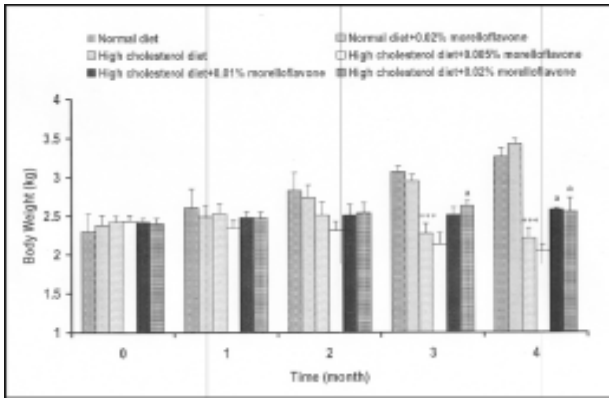

Fig. 2. Body weights of rabbits in the different experimental groups during the 4-months of study. Values are Means ± S.D. (n = 4). At the same time point, *** = $p < 0.001$ significantly different from the control (normal diet) group, whereas a = $p < 0.05$ significantly different from the hypercholesterolemic diet (untreated) group.

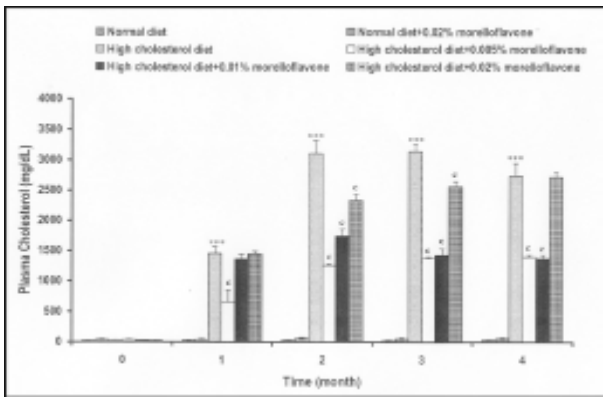

Fig. 3. Plasma total cholesterol levels of rabbits in the different experimental groups during the 4-months of study. The bars represent Means ± S.D. (n = 4). At the same time point, *** = $p < 0.001$ significantly different from the control (normal diet) group, whereas c = $p < 0.001$ significantly different from the hypercholesterolemic diet (untreated) group.

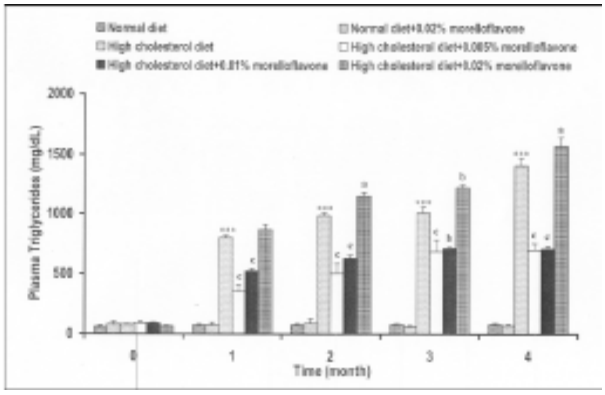

Fig. 4. Plasma triglycerides levels of rabbits in the different experimental groups during the 4-months of study. The bars represent Means ± S.D. (n = 4). At the same time point, *** = $p < 0.001$ significantly different from the control (normal diet) group, whereas a = $p < 0.05$, b = $p < 0.01$, c = $p < 0.001$ significantly different from the hypercholesterolemic diet (untreated) group.

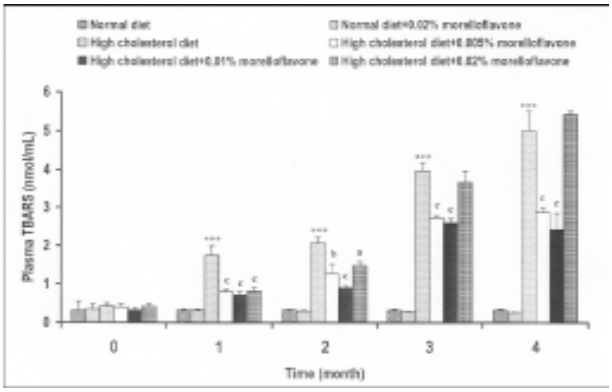

Fig. 5. Plasma TBARS levels of rabbits in the different experimental groups during the 4-months of study. The bars represent Means ± S.D. (n = 4). At the same time point, *** = $p < 0.001$ significantly different from the control (normal diet) group, whereas a = $p < 0.05$, b = $p < 0.01$, c = $p < 0.001$ significantly different from the hypercholesterolemic diet (untreated) group.

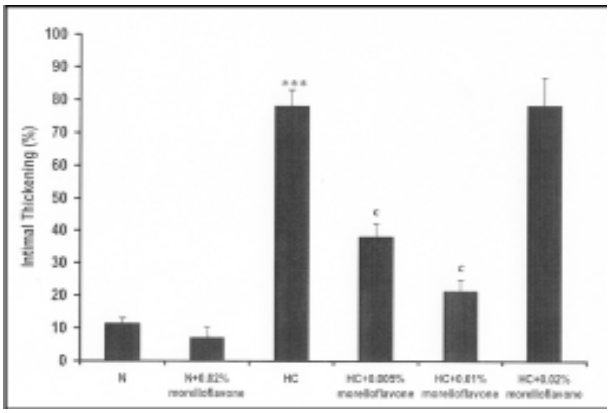

Fig. 6. Percent intimal thickening in the aortic arch from the rabbits in the different experimental groups after the 4-month feeding period. The bars represent Means ± S.D. (n = 4). N = normal diet, HC = high cholesterol diet. [*** = $p < 0.001$ significantly different from the control group, c = $p < 0.001$ significantly different from the hypercholesterolemic diet (untreated) group].

3.2 Plasma lipid levels

Within one month of the experiment, all rabbits in the four cholesterol diet groups developed hypercholesterolemia (Figure 3). Plasma cholesterol levels in the high cholesterol groups with or without morelloflavone at 0.01% and at 0.02% increased by a similar amount, whereas the increase in the group dosed with 0.005% morelloflavone was significantly less ($p < 0.001$) (Figure 3). Since there was very little difference in the uptake of food by the different groups, such variation may have been due to a decreased dietary cholesterol absorption or a higher excretion of cholesterol from their gut in the form of fecal sterols, in the presence of 0.005% morelloflavone. At the 2nd month, the plasma cholesterol levels in all cholesterol diet groups had continued to increase but at a slower rate in

the presence of morelloflavone. One month later (month 3), the group supplemented with 0.01% morelloflavone showed a significant reduction in the plasma cholesterol level when compared to its value at the 2nd month ($p < 0.05$), while there was almost no change in the others. At the 4th month, a slight reduction in the plasma cholesterol was observed in the cholesterol group without morelloflavone but its level was not significantly different ($p > 0.05$) from those in the previous periods (month 2 and month 3). Based on these results, morelloflavone can exert an anti-hypercholesterolemic activity in cholesterol-fed rabbits but surprisingly it was effective at 0.005% and 0.01% only. The actual mechanism of action of morelloflavone is still unknown. Owing to their stereoconfiguration, most flavonoids are highly lipophilic and thus have fast intestinal absorption. Previous studies have found that the reduction of plasma cholesterol in hypercholesterolemic animals by flavonoids from plant sources seems to be based on their ability to increase cholesterol degradation and excretion activities [20-21] and this might be the same case for morelloflavone. However, the ability of this compound to inhibit endogenous cholesterol biosynthesis as well as to block intestinal absorption of dietary cholesterol as exhibited by the citrus bioflavonoids in rats [22] might also be involved.

In this study, the consumption of cholesterol also induced an elevated level of plasma triglycerides in our experimental rabbits (Figure 4) but the increase was less than for cholesterol (Figure 3), as found by others [23-24]. At present, we still have no good explanation for such an increase of circulating triglycerides in the cholesterol-treated rabbits. The mechanism by which it elicits this effect may be complex, but seemingly connected to a homeostatic response following the increased plasma total cholesterol level in these animals. Considering that blood cholesterol in the cholesterol-fed rabbits is transported mainly in β-VLDL and LDL [25] that also contain triglycerides but in lower quantities, a large increase of cholesterol in plasma may unavoidably enhance the production of both the lipoprotein fractions, resulting in an increased level, in the circulation, of their components including triglycerides. In a similar way to its ability to lower plasma cholesterol levels (Figure 3), morelloflavone can reduce the rise of plasma triglyceride levels, when given along with the cholesterol-rich diet (Figure 4) and its effective concentrations over the 4 month-period were also 0.005% and 0.01%. The plasma triglyceride reducing activity of morelloflavone might be related to the decreased absorption as well as increased excretion of this lipid via feces, as it has been postulated by another group [26] or perhaps to different factors, including reduced VLDL secretion by the liver, repressed hepatic lipogenesis or accelerated clearance by peripheral tissues [27].

3.3 TBARS levels

Elevation of cholesterol, especially LDL-cholesterol, in plasma, is known to provoke free radical production leading to accelerated lipid peroxidation which is an essential step in atherogenesis [28]. In agreement with those reports by others [17, 23-24, 29], we also observed a large increase in plasma MDA content ($p < 0.001$), determined as the TBARS level, that indicates an enhanced lipid peroxidation process in our rabbits made hypercholesterolemic by feeding a high-cholesterol diet (Figure 5). Administration of *G. dulcis* morellolavone, on the other hand, showed a suppressive effect in a biphasic manner. Concomitantly with those of cholesterol (Figure 3), both the low dose (0.005%) and middle dose (0.01%) of morelloflavone significantly decreased the concentrations of plasma TBARS ($p < 0.001$), whereas the high dosage (0.02%) was least

effective. The ability of morelloflavone to prevent the lipid peroxidation and thus result in a decreased content of MDA in plasma, may be mainly attributed to its antioxidant properties [9, 13]. In addition, its hypolipidemic and hypocholesterolemic effects could also have led to decreased lipid peroxidation, by decreasing the availability of lipid substrates. A recent report on the ability of a flavonoid-rich extract of *Hypericum perforatum* to enhance the antioxidant enzyme activity in rats fed a cholesterol-rich diet [26], also indicates a possibility that morelloflavone would also act on the antioxidant defense mechanisms, which in turn slowed down the lipid peroxidation process in those hypercholesterolemic rabbits.

3.4 Aortic Atherosclerosis

Cellular events related to the progression of atherosclerosis that occur frequently in hypercholesterolemic animals, include the endothelial foam cell formation, the development of fatty streak and eventually the appearance of complicated atherosclerotic lesions [30]. In the present study, the continuous consumption of dietary cholesterol resulted in severe athrosclerosis development in most rabbits of the cholesterol-rich diet group. Macroscopic examination of their proximal aortas at the time of sacrifice found atheromatous plaques covering almost the entire intimal surface area between the aortic arch and thoracic aorta. These lesions were however diminished with the morelloflavone supplementation (data not shown). The findings from both intimal thickening analysis (Figure 6) and histological examination of the aortic arch sections (Figure 7) also confirm such anti-artherosclerotic effects of morelloflavone. Compared with the normal rabbits, aortic intima of the hypercholesterolemic rabbits were significantly thicker (Figure 6). Due to a large accumulation of the lipids, observed as orange materials from Oil Red O staining which localised mainly in the areas subjacent to the endothelium (Figure 7F), the percent intimal thickening in the aortic arch of those animals fed with high cholesterol diet only (78.2 ± 9.1%), was about seven fold greater than that of the control (Figure 6). However, in the morelloflavone treated groups, they became less enlarged. At doses of 0.005% and 0.01%, this compound caused a significant reduction of intimal thickening (38.1 ± 3.7% and 21.3 ± 2.8%, respectively) ($p < 0.001$), whereas at a dose of 0.02%, the value was not different from that of the untreated group (Figure 6). The important role of the inhibtion of LDL oxidation in reducing the atherosclerosis process has been widely accepted. Obviously, the anti-oxidation properties of morelloflavone on this lipoprotein have important implications for its inhibition on the progression of atherosclerotic lesions in those hypercholesterolemic rabbits. Moreover, both hypolipidemic activity (Figure 3 and Figure 4) and the known anti-inflammatory mechanisms of this compound [11] would have some protective effects in connection with development of atherosclerosis in the same rabbits.

In conclusion, the present study has demonstrated an anti-atherogenic effect of morelloflavone isolated from *G. dulcis* leaf in the cholesterol-induced hypercholesterolemic rabbits. This effect of morelloflavone may be due to its ability to reduce plasma cholesterol level as well as the susceptibility of LDL to oxidation which are known risk markers for atherosclerosis.

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