Potential of *Tinospora crispa* as a Hypocholesterolemic Agent in Rabbits

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ABSTRACT

**Introduction:** Hypercholesterolemia is the major cause of cardiovascular disturbances. The influence of *Tinospora crispa* on atherosclerotic plaque formation in rabbits fed with high cholesterol diet was investigated. **Methods:** Thirty male New Zealand White rabbits were divided into 6 groups. The negative control (NC) and positive control (PC) groups were used as a negative and positive (0.5% cholesterol) control. The simvastatin control (SC) group was given a high cholesterol diet (HCD) with 5mg/kg simvastatin. Treatment groups T150, T300 and T450, were given HCD with supplementation of 150, 300 and 450 mg/kg of *T.crispa* extract respectively for 10 weeks. Blood was collected from ear vein for plasma analysis while the aortas were excised and examined microscopically. **Results:** Comparison within groups showed that PC, T300 and T450 had a significant increase (p<0.05) in total cholesterol level throughout the study. The groups supplemented with *T.crispa* (T150, T300 and T450) were significantly higher (p<0.05) in high density lipoprotein (HDL) level by 10.7-fold, significantly higher (p<0.05) in total antioxidant activity and had a significantly lower (p<0.05) LDL level compared to PC at week 10. At week 10, T450 had significantly highest (p<0.05) glutathione peroxidase and superoxide dismutase levels compared to PC. No foam cell formation was visible in the aorta of rabbits in groups NC, SC and T450. However, there was visible foam cell formation in the aorta of groups PC, T150 and T300. **Conclusion:** This study suggests that supplementation of 450mg/kg of *T.crispa* extract would be able to reduce or retard the progression of atherosclerotic plaque development induced by dietary cholesterol.

**Keywords:** Antioxidant, atherosclerosis, hypercholesterolemia, *Tinospora crispa*

INTRODUCTION

Hypercholesterolemia, resulting from lipid metabolic changes, is the major cause of cardiovascular disturbances \(^1\) such as atherosclerosis. Studies in hypercholesterolemic animal models indicate that oxidation of low density lipoprotein (LDL) is likely to play an important role in atherogenesis.\(^2\) Macrophages, in an environment of oxidised LDL, will
avidly remove LDL from the interstitium and generate macrophage foam cells, a major cell type present within the fatty streaks and fibrous plaque.\textsuperscript{[3]}

\textit{Tinospora crispa} contains a wide variety of compounds with antioxidant activity, such as flavonoids, lignans, alkaloids and terpenes.\textsuperscript{[4]} In Thai traditional medicine, \textit{T. crispa} is one of the ingredients used for maintaining good health.\textsuperscript{[5]} It has been reported that the \textit{T. crispa} extract, used as an antipyretic\textsuperscript{[6]} agent, is able to cause a reduction in blood glucose level due to its insulinotropic activity.\textsuperscript{[7]}

Its mechanism of action in the biological system is not well understood as scientific reports on this plant are scant; however bitter tonics like \textit{T. crispa} are postulated to have antioxidative effects because of their rich polyphenolic compounds.\textsuperscript{[7]} Thus, in this study, we investigated the effect of \textit{T. crispa} extract on the development of experimental atherosclerosis.

\section*{METHODS}

\textbf{Preparation of Extract}

Fresh stem of \textit{T. crispa} were collected from Universiti Putra Malaysia (UPM) after being identified and confirmed by a plant taxonomist. A voucher specimen was deposited in the Institute of Bioscience, UPM (SK1550/07). The stems were cut into small pieces, dried and pulverised. Ten percent of \textit{T. crispa} aqueous crude extract of the stem was prepared by soaking 100g of the powdered stem in 900ml distilled water and incubated in shaking water bath at 60\textdegree C for 6 hours. On completion of filtration, the filtrates were freeze dried and kept at -20\textdegree C until used.

\textbf{Animals and Experimental Design}

Thirty male New Zealand white rabbits were individually housed in metabolic cages. They were acclimatised under control condition of humidity with regular light and dark cycles and free access to food and water for a week. Following acclimatisation, the rabbits were randomly divided into 6 groups. Group NC and PC were used as the negative and positive (0.5\% cholesterol) control. Cholesterol was purchased from Sigma (St. Louis, USA). Group SC was given high cholesterol diet (HCD) with 5mg/kg simvastatin, an anti-hypercholesterolemic agent. Groups T150, T300 and T450 served as treatment groups and were given HCD with supplementation of 150, 300 and 450mg/kg of \textit{T. crispa} extract respectively. Blood was taken from ear vein at weeks 0 and 10. At the end of week 10, the rabbits were euthanised. A midline thoracotomy was performed and the aorta was excised for histomorphometric analysis. All procedures for laboratory animal handling were reviewed and approved by the Faculty’s Animal Care and Use Committee (ACUC No: 2004/BM/1).

\textbf{Lipid Profile Measurement}

Blood samples were collected at weeks 0 and 10. Analysis of lipid profile included measuring serum total cholesterol (TC), triglyceride (TG), high density lipoprotein (HDL) and low density lipoprotein (LDL) levels using a Roche kit (Penzberg, Germany). All parameters were measured spectrophotometrically with Hitachi Chemistry Analyser. The tests utilised the principle of enzymatic colorimetric assay to read the sample.
Antioxidant Activity
Total antioxidant activity (TAA), glutathione peroxidase (GSH-Px) and superoxide dismutase level were determined to assess the antioxidant activity. Total antioxidant status was measured by monitoring radical cation formation from 2,2-azino-di-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) incubated with a peroxidase (metmyoglobin) and $\text{H}_2\text{O}_2$ to produce a radical cation with a stable blue colour, which was measured at 600nm. The colorimetric method was programmed into a Cobas Mira autoanalyser, using a Randox kit (County Antrim, UK). Glutathione peroxidase (GSH-Px) is an important antioxidant enzyme involved in the detoxification of peroxides and the protection of cells from lipid peroxidation. The GSH-Px catalyses the reduction of $\text{H}_2\text{O}_2$ to water. The role of SOD is to accelerate the dismutation of the toxic superoxide radical ($\text{O}_2^-$), produced during oxidative energy processes, to hydrogen peroxide and molecular oxygen. This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2(4-iodophenyl)-3-(4-nitropheno1)-5-phenyltetrazolium chloride (I.N.T) to form a red formazan dye. The superoxide dismutase activity is then measured by the degree of inhibition of this reaction. GSH-Px and SOD were measured with a Cobas Mira autoanalyser using a Roche kit (Penzberg, Germany).

Evaluation of Atherosclerosis Lesions
For histological analysis, paraffin-embedded tissue sections of aortic arch were stained with hematoxylin and eosin (H & E) stain. The thickness of foam cells and fatty streak were measured with a light microscope equipped with an image analyser system (Olympus, Germany).

Statistical Analysis
Analysis of variance (ANOVA) and Tukey HSD were performed to compare the mean between groups. Significance was accepted at $p<0.05$.

RESULTS
Lipid Profiles
The concentrations of total cholesterol, HDL and LDL at weeks 0 and 10 are shown in Table 1. The administration of a high cholesterol diet to rabbits in PC group produced a significant increase ($p<0.05$) in total cholesterol as compared to normal control. The level of total cholesterol in group T150 and T300 was found to be significantly lower than in PC by end of week 10.

HDL level was significantly higher ($p<0.05$) in groups T300 and T450, which were supplemented with 300 and 450mg/kg of T. crispa respectively, compared to other groups at week 10. Supplementation of T. crispa to rabbits fed with high cholesterol diet also reduced LDL level. At week 10, all treatment groups showed significantly lower ($p<0.05$) LDL level at 76%, 59% and 50% respectively compared to PC. Table 1 shows the LDL to HDL ratio at the beginning of the study and after 10 weeks for all groups. Atherosclerotic index (AI), which is the ratio of LDL to HDL level, significantly decreased in T300 and T450.
Table 1. Lipid profile and total antioxidant activity of the rabbits after a 10-week experimental period

<table>
<thead>
<tr>
<th>Groups</th>
<th>TC (mmol/l)</th>
<th>HDL (mmol/l)</th>
<th>LDL (mmol/l)</th>
<th>TAA (mmol/l)</th>
<th>LDL:HD</th>
<th>GSH-Px (U/l)</th>
<th>SOD (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>1.25</td>
<td>0.78</td>
<td>0.35</td>
<td>0.95</td>
<td>0.46</td>
<td>495.677</td>
<td>336.11</td>
</tr>
<tr>
<td></td>
<td>±0.18*</td>
<td>±0.14*</td>
<td>±0.06*</td>
<td>±0.04</td>
<td>±0.09*</td>
<td>±76.58*</td>
<td>±12.65</td>
</tr>
<tr>
<td>PC</td>
<td>20.59</td>
<td>0.86</td>
<td>16.46</td>
<td>1.11</td>
<td>20.00</td>
<td>220.407</td>
<td>415</td>
</tr>
<tr>
<td></td>
<td>±1.85</td>
<td>±0.11</td>
<td>±1.60</td>
<td>±0.05</td>
<td>±4.36</td>
<td>±19.69</td>
<td>±44.23</td>
</tr>
<tr>
<td>SC</td>
<td>0.47</td>
<td>0.15</td>
<td>0.35</td>
<td>1.09</td>
<td>2.44</td>
<td>272.507</td>
<td>451</td>
</tr>
<tr>
<td></td>
<td>±0.09*</td>
<td>±0.03*</td>
<td>±0.03*</td>
<td>±0.08</td>
<td>±0.66*</td>
<td>±28.58</td>
<td>±81.19</td>
</tr>
<tr>
<td>T150</td>
<td>3.19</td>
<td>2.78</td>
<td>4.21</td>
<td>1.03</td>
<td>1.52</td>
<td>342.893</td>
<td>203</td>
</tr>
<tr>
<td></td>
<td>±0.46*</td>
<td>±0.62</td>
<td>±0.68</td>
<td>±0.03</td>
<td>±0.10*</td>
<td>±29.56</td>
<td>±42.57*</td>
</tr>
<tr>
<td>T300</td>
<td>14.84</td>
<td>8.25</td>
<td>6.81</td>
<td>0.99</td>
<td>0.84</td>
<td>376.847</td>
<td>299</td>
</tr>
<tr>
<td></td>
<td>±2.10*</td>
<td>±0.98*</td>
<td>±1.54*</td>
<td>±0.02</td>
<td>±0.25*</td>
<td>±59.90</td>
<td>±20.42</td>
</tr>
<tr>
<td>T450</td>
<td>29.60</td>
<td>9.23</td>
<td>8.38</td>
<td>1.14</td>
<td>0.92</td>
<td>494.323</td>
<td>567</td>
</tr>
<tr>
<td></td>
<td>±6.04</td>
<td>±1.36*</td>
<td>±0.99*</td>
<td>±0.04</td>
<td>±0.25*</td>
<td>±96.72*</td>
<td>±33.41*</td>
</tr>
</tbody>
</table>

Values are expressed as mean ±SD (n=5). * P<0.05 in comparison to positive control group. NC = negative control, PC = positive control, SC = 0.5% cholesterol + simvastatin, T150 = 0.5% cholesterol + 150mg/kg of T.crispa, T300 = 0.5% cholesterol + 300mg/kg of T.crispa, T450 = 0.5% cholesterol + 450mg/kg of T.crispa.

Antioxidant Activity
The total antioxidant activity (TAA) was slightly higher in the group fed with high cholesterol diet as compared to the group fed with a basal diet after treatment period, as shown in Table 1. The results showed that the GSH-Px activity significantly decreased (p<0.05) by 56% in rabbits fed with high cholesterol diet compared to NC group. Meanwhile, at week 10, supplementation of 450 mg/kg of T.crispa caused a significantly higher (p<0.05) GSH-Px activity compared to PC group (Table 1). The supplementation of 150 and 300mg/kg/day of T. crispa showed a significantly lower (p<0.05) SOD activity compared to PC group. The SOD activity was significantly higher (p<0.05) in PC group compared to NC. The supplementation of 450 mg/kg of T.crispa resulted in significantly higher (p<0.05) SOD activity compared to PC group, as shown in Table 1.

Thickness of Foam Cells
The extent of atherosclerosis in the aorta was evaluated as the area of fatty region using the detection of formation of foam cells in the atherosclerotic lesions. The thickness of intima in the positive control and group receiving high cholesterol diet supplemented with 150 and 300mg/kg of T. crispa aqueous extract on day 70 were 172.34 ± 24.24μm, 106.44 ± 12.98μm and 72.48 ± 6.12μm respectively with significant difference at p<0.05 (Figure 1). The number of foam cells in atherosclerotic lesions in the thoracic aorta decreased in T150 and T300 groups (Figure 2). There was no foam cell layer observed in group T450 (Figure 2). The lesions in most rabbits were relatively uniform in appearance and consisted of initial
Figure 1. Foam cells thickness (μm) after a 10-week experimental period. Each value represents the mean ± SD (n=5). Values are significantly different (p<0.05) between groups. NC = negative control, PC = positive control, SC = 0.5% cholesterol + simvastatin, T150 = 0.5% cholesterol + 150mg/kg of T.crispa, T300 = 0.5% cholesterol + 300mg/kg of T.crispa, T450 = 0.5% cholesterol + 450 mg/kg of T.crispa

Figure 2. Photomicrographs of aorta stained with H&E at LM x40. I=intima, M=media, A=Adventitia, L=Lumen. Group; (A): NC, (B):PC, (C): SC, (D):T150, (E): T300, (F):T450
foam cell, smooth muscle cells and calcification. Smooth muscle cell migration was observed in the atherosclerotic lesions in T150 group, but very few smooth muscle cell migrations in T300 group (Figure 2).

DISCUSSION

Lowering of serum lipids levels through dietary or drug therapy seems to be associated with a decrease in the risk of vascular disease. The supplementation of 450mg/kg of T. crispa showed a significantly 30% higher (p<0.05) TC level against PC at week 10. A similar result was observed in a study by Chawanya et al. They found that patients with type 2 diabetes supplemented with 3g/day of T. crispa had significant cholesterol elevation. Another study has also found that TC level was higher in rabbits fed with a high cholesterol diet and flax seed than in those fed with high cholesterol diet alone. Flax seed alone produced a slight rise in serum cholesterol. These findings show that consuming a high cholesterol diet with treatment at a certain level might produce higher total cholesterol level compared to consuming a high cholesterol diet alone. Several epidemiologic studies have demonstrated that HDL is a strong, independent, inverse predictor of CHD risk.

The plasma HDL level in the group supplemented with 300 and 450mg/kg of T. crispa was significantly higher (p<0.05) than in other groups at week 10. From this study, it was also found that supplementation of T. crispa to rabbits fed with a high cholesterol diet caused a reduction in plasma LDL but increased HDL. A high HDL level may compete with LDL receptor sites on arterial smooth muscle cells and thus inhibit the uptake of LDL. Weggemans and Trautwein reported that flavonoid intake increases HDL, which may hasten the removal of cholesterol from peripheral tissue to liver for catabolism and excretion.

Atherosclerotic index (AI), defined as the ratio of LDL and HDL, is believed to be an important risk factor of atherosclerosis. Since LDL was significantly suppressed and administering aqueous extract of T. crispa resulted in increased HDL, the value of AI was significantly decreased. The decrease in AI can be said to be another positive change after T. crispa treatment. Wan et al. reported that flavonoid may decrease the risk of cardiovascular disease by lowering LDL: HDL ratio.

Triglyceride (TG) level is closely related to obesity problems. It is transported by very low density lipoprotein and chylomicron. Supplementation of 150 and 450mg/kg of T. crispa had significantly decreased (p<0.05) TG level against PC. Patients with low HDL level will have high TG level. Since HDL level in the group supplemented with 150 and 450 mg/kg of T. crispa was high, the TG level was low.

Although a close relationship exists between high blood cholesterol levels and atherosclerosis, it has been suggested that this relationship might be dependent on enhanced oxidative stress. The increase in the antioxidant activity observed in this study could be due to increased activity of antioxidant enzymes in response to oxidative stress.

To counteract the oxidants, an important endogenous antioxidant system exists in vivo, which includes antioxidant compounds such as vitamin E and antioxidant enzymes such as SOD, CAT, GSH-Px, glutathione reductase, glutathione transferase and glucose-6-phosphate dehydrogenase.
Glutathione peroxidase (GSH-Px) is an important antioxidant index, which is active in removing hydrogen peroxide, thereby preventing the generation of free hydroxyl radicals.\textsuperscript{[21]} The activities of antioxidant enzymes like GSH-Px form the first line of defense against ROS and the decrease in their activity contributes to oxidative stress.\textsuperscript{[22]} In this study, in rabbits fed with high cholesterol diet and orally administrated \textit{T. crispa} extract (group T450), it was found that the addition of \textit{T. crispa} extract increased the antioxidant potential and antioxidant enzyme activities (SOD and GSH-Px) suggesting a protective mechanism role against oxidative stress.\textsuperscript{[23]} Oral administration of \textit{T. crispa} reversed the changes induced by feeding a high cholesterol diet, supporting the hypothesis that plant products are effective chemopreventive agents.\textsuperscript{[23]} Oral administration of \textit{T. cordifolia} root extract to alloxan diabetic rats for 6 weeks in an earlier study also experienced an increase in GSH-Px in blood plasma.\textsuperscript{[24]}

Superoxide is considered an important free radical contributing to oxidative stress. It is dismutated to hydrogen peroxide, a much less harmful product by the family of SOD enzymes.\textsuperscript{[25]} In this study, the SOD activity was significantly higher (\textit{p}<0.05) in rabbits fed with high cholesterol diet. SOD activity has been reported to increase in hypercholesterolemic rabbits.\textsuperscript{[12]} The SOD catalyses the removal of superoxide radicals, thus reducing the degree of oxidation.\textsuperscript{[26,27]} Therefore, the increased activity of this enzyme suggests a greater level of endogenous antioxidant defense associated with the supplemental intake of \textit{T. crispa}.\textsuperscript{[26]} A increase in SOD activity would protect GSH-Px and CAT against inactivation by superoxide anion.\textsuperscript{[29]}

Hypercholesterolemia and high cholesterol diet are associated with the development of atherosclerosis. The results showed that the thickness of foam cells in the group fed with a high cholesterol diet was significantly higher compared to any other group. These findings are consistent with other reports.\textsuperscript{[30,31]} Atherosclerosis caused by hypercholesterolemia is associated with an increase in serum and aortic MDA, which suggests an increase in oxygen radicals. Increased levels of oxygen radicals are known to produce endothelial cell injury\textsuperscript{[32]} which represents a critical initiating event in the development of atherosclerosis.\textsuperscript{[33]} Therefore, inhibiting oxidative modification of LDL is considered an important therapeutic approach.

The physiological effect of flavonoids includes possible antioxidant activity, therefore suggesting a role in the prevention of coronary heart disease\textsuperscript{[34]} including atherosclerosis. In this study, it was observed that the thickness of foam cells in the group receiving a high cholesterol diet supplemented with 150 and 300mg/kg of \textit{T. crispa} extract was significantly lower than in the group without supplementation. Interestingly, there was no presence of foam cells in the aorta of rabbits supplemented with 450mg/kg of \textit{T. crispa}. The reduction in the extent of atherosclerosis could be due to the lipid lowering activity.\textsuperscript{[35,36]}

\textbf{CONCLUSION}

\textit{Tinospora crispa} extract is able to reduce or retard the progression of atherosclerotic plaque development induced by dietary cholesterol. The enhanced serum HDL cholesterol level and the increased circulating antioxidant status may be the possible underlying mechanisms of antiatherogenic effect of \textit{Tinospora crispa in vivo}. Future research should be carried out in order to strengthen the properties of this herb by screening the bioactive compounds in this herb that may contribute to the hypocholesterolemic effect.
ACKNOWLEDGEMENT
The author would like to thank all who have contributed to this project. The research was supported and funded by Ministry of Science, Technology and Innovation, Malaysia (Project number: 06-02-04-0880-EA 001).

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