Hypolipidemic effect of a novel biflavonoid from shells of *Camellia oleifera* (Abel.)

Y Ye*, H T Xing & Y Guo
Pharmaceutical Engineering Department, School of Chemistry and Chemical Engineering, South China University of Technology, Guangzhou 510 640, PR China

Received 22 October 2012

*Camellia oleifera* Abel. [C. oleosa (Lour.) Rehd.], an evergreen plant, is used for healthful oil production, but the shells are always discarded and need to be utilized. The present study was undertaken to explore the effect of extracts from the shells of *C. oleifera* on adjusting cardiovascular system. A flavonoid was obtained by reflux extraction of the shells in 70% methanol, hydrolysis in 2 M hydrochloric acid, and crystallization in acetone. Its structure was identified as a novel biflavonoid. Mice model of hyperlipidemia was setup by high fat diet for 30 d to evaluate the hypolipidemic effect of the biflavonoid at dose of 50, 100 and 200 mg/kg/d (ig). Antioxidative activity was determined by levels of malondialdehyde (MDA), superoxidase dismutase (SOD) and glutathione peroxidase (GSH-Px) in mice serum. The biflavonoid significantly controlled mice weight and liver coefficient, decreased the content of total cholesterol and triglyceride, promoted the level of high density lipoprotein in a dose dependent manner. The significant decrease of MDA content and increase of SOD and GSH-Px activity indicated it enhanced antioxidative capacity *in vivo* and was ascribed to hypolipidemic effect. The biflavonoid is useful in the prevention of high fat diet induced hyperlipidemia.

**Keywords:** Antioxidative activity, Biflavonoid, *Camellia oleifera*, Extracts, Hypolipidemic effect

Hyperlipidemia has been documented as potent risk factor for coronary heart diseases with high morbidity and mortality. Less healthy living habits and dietary preferences play significant roles in the onset of hyperlipidemia; now the morbidity of cerebrovascular and angiocardioathy disease is up to 8% and its mortality is almost 50% in China, making hyperlipidemia a leading cause of death. Therefore, natural medicines for hyperlipidemia with little side effect become the focus in current researches.

Flavonoids are polyphenols widely distributed in plants. There are about 5000 kinds of flavonoids such as flavones, biflavonoids, flavanones, flavanones, isoflavonones, isoflavones, flavan-3-ols, chalcones, flavan-3, 4-diols, dihydrochalcones, anthocyanidins, xanthones, etc. They have the functions of protecting cardiovascular, antioxidant, anti-tumor, relieving cough and phlegm, anti-inflammation, anti-virus and adjusting immunity, etc. Flavonoids were also found in *Camellia oleifera* Abel. [C. oleosa (Lour.) Rehd.], but have not been manufactured; their pharmacological activities are less reported.

The shells of *C. oleifera* are by-products of oil production, which are always discarded or used as fertilizer. Various bioactive compounds such as saponins, flavonoid glycosides and polysaccharides are found in the seeds, but little is known about the shells. In the present study a simple method has been applied to isolate the flavonoid from the shells to high purity, a biflavonoid is found and its hypolipidemic and antioxidative activities are further analyzed.

**Materials and Methods**

*Plant material*—The shells of *C. oleifera* were collected from Guangdong Tea Oil Company in Meizhou of Guangdong province, China during November, 2011. The fresh seeds of the title plant were obtained from the factory and authentified by Dr. Yun Chen from South China University of Technology, China.

*Chemicals and reagents*—Total cholesterol (TC) kit, total triglyceride (TG) kit and high density lipoprotein (HDL) kit were purchased from Beijing Beihuakangtai Clinical Reagent Company (Beijing, China); Superoxidase dismutase (SOD) kit, malondialdehyde (MDA) kit, and glutathione peroxidase (GSH-Px) kit were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China); Rutin was bought from Shanghai Chemical Co. Ltd; Simvastatin was procured from Guangzhou P.D. Pharmaceutical Co. Ltd.
Animals—Male Kunming mice, 10 weeks of age weighing 20±2 g were at 25±2 °C, 50±10% RH with a 12 h light/dark cycle, and received human care throughout the experiments. All animals were treated according to the guideline of animal handling in South China University of Technology.

Extraction and isolation—The shells of Camellia oleifera (1 kg) were refluxed in 10 L of 70% methanol at 80 °C for 2 h. The filtrate was concentrated and dried at 60 °C, 150 g of solid extract was hydrolyzed in 2.4 L of HCl (2.0 M) under reflux at 80 °C for 5 h. The precipitation was centrifuged in 3000 rpm for 10 min, and washed with 500 mL of water for 3 times, then crystallized in 150 mL of aqueous acetone. The crystal was air-dried in hood at room temperature; 21 g of yellow powder was obtained.

Determination of total flavonoid content—Total flavonoid content was determined according to Zhishen et al. Dried shell powder (0.1 g) was extracted with 250 mL of 75% methanol under reflux for 1 h. Filtrate (10 mL) was mixed with 1 mL of 10% aluminum chloride and 5% sodium nitrite, and a pink-colored flavonoid-aluminum complex was formed after 10 mL of 1 M NaOH was added to the solution. The absorbance at 510 nm was determined 5 min after 10 mL of 1 M NaOH was added to the solution. A reagent blank containing aluminum chloride and 5% sodium nitrite, and a pink-colored flavonoid-aluminum complex was formed after 10 mL of 1 M NaOH was added to the solution. The absorbance at 510 nm was determined 5 min after the mixing of the solution. A reagent blank containing methanol instead of sample was used. The total flavonoid content was calculated using a standard curve of rutin.

Determination of purity by HPLC—The analysis was run on HP 1100 high performance liquid chromatography apparatus (HPLC, Agilent Company, USA). The operating conditions were as follows: column, Hypersil ODS (250×4.6 mm, 5 µm); flow phase, methanol vs water (60:40); injection volume, 20 µL; flow rate, 1mL/min; temperature, 30 °C; wavelength, 366 nm.

Structure identification—UV spectra analysis was carried out on UV-3010 Ultra violet spectrometer (Hitachi Company, Japan) scanning from 200-600 nm; IR spectra were measured on Nicolet 380 FT-IR spectrograph (Nicolet apparatus company, USA) with KBr tablets from 4000 to 400 cm⁻¹ with resolution 2 cm⁻¹; Mass spectra were recorded on Bruker Esquire Hct Plus Mass spectrometer with ESI (Bruker Company, Germany) in m/z of cation model scanning from 150 ~1200 for 60 min; NMR spectra were determined on 400 MHz AM NMR (Bruker Company, Switzerland) in DMSO-d₆ operating at 101 MHz for ¹³C NMR and 400 MHz for ¹H NMR.

Hypolipidemic and antioxidative test—Hyperlipidemia animal model was set up according to the Guo et al. Mice (60) were divided into following 6 groups of 10 mice each: normal saline (NS), Veh, Control (simvastatin, 4 mg/kg/d), three flavonoid groups respectively at high (200 mg/kg/d), middle (100 mg/kg/d) and low dose (50 mg/kg/d). Simvastatin and flavonoid extract were suspended in normal saline, and administered mice intragastrically (0.2 mL/10g) for 30 days. Normal saline group received low fat diet (LFD) containing 5% fat, 20% protein and 75% carbohydrate, other groups were fed with high fat diet (1% cholesterol, 10% yolk, 10% lard oil and 79% LFD). Food intake was 5 g/animal/day. Mice were weighed every six-day, and fasted for 12 h after last administration, blood was taken from canthus vein of mice, and serum was separated and conserved at -80°C for subsequent analysis. Mice were sacrificed by decapitation, livers were weighed. Liver coefficient was calculated on the ratio of liver weight to body weight. TC, TG, HDL, MDA, SOD and GSH-Px were determined according to the kit description.

Statistical analysis—Data were presented as mean±SD. SPSS 11.0 software (SPSS Inc., USA) was used to analyze the data of animal tests in groups by one-way ANOVA and Dunnett’s t-test.

Results

Separation and identification of flavonoid—The total flavonoid content was about 2.3% on basis of standard curve of rutin. The linear regression equation was y (rutin, mg/mL) = 0.015A (absorbance) +0.0013 (r=0.9996). The flavonoid of C. oleifera was extracted by methanol, hydrolyzed and precipitated by hydrochloric acid. High purity of flavonoid could be obtained when the deposit was crystallized by aqueous acetone. Purity of the flavonoid was up to 93.8% which was calculated on peak area by HPLC, and yield was 2.1%. It meant that the extraction ration of flavonoid was 85.6%. General precipitation and extraction can purify the flavonoid from shells of C. oleifera without column chromatography, which is proper for industrial production.

The IR spectra showed the characteristic absorption band of hydroxyl (3423 cm⁻¹), aromatic ring (1514, 1459 cm⁻¹) and conjugated carbonyl (1648 cm⁻¹). There were 3 absorption peaks of UV (207, 265, 366 nm) due to chromophore of flavonoid. m/z [M]+ : 570.9, molecular formula could be deduced to...
In $^1$H NMR spectra, δ 12.48 (s, br, 2H), 10.78 (s, br, 2H), 10.11 (s, br, 2H), 9.37 (s, br, 2H) were 4 signals of hydroxyl protons on aromatic rings of kaempferol, δ 8.04 (d, J=8.8 Hz, 4H) and 6.93 (d, J=8.8 Hz, 4H) formed two AA'BB' systems, δ 6.44 (s, 2H) was 2 protons on benzene rings of kaempferol. $^{13}$C NMR spectra showed 2 carbonyl carbons, 14 double bonds with 28 carbons. The shift of C-8(8") with δ 4.2 compared to kaempferol suggested direct C-C bond of C-8 and C-8". The correlation of C and H was further confirmed by HMBC spectra. The compound could be deduced to bimolecular kaempferol structural biflavonoid (Fig. 1).

Comparison of mice body weight among groups—No obvious difference was found in body weight of mice among groups before high fat diet was fed, but body weight increased significantly in Veh group since the day 18th ($P<0.01$). All flavonoid groups gained less weight than Veh group especially from the day 18th on (Table 1). It proved that the biflavonoid had function of inhibiting the increment of body weight. Simvastatin also had the function, but reached the significant level ($P<0.01$) till the day 30th.

Variation of liver coefficient among groups—Liver coefficient always serves as the index of non-alcoholic fatty liver. The biflavonoid from C. oleifera has the effect of ameliorating liver weight at the same food intake as other groups. High fat diet caused significant increase of liver weight and liver coefficient ($P<0.01$), but they declined as the dose of biflavonoid increased. The correlation of liver coefficient between flavonoid groups and Veh group was significant after orally administration of biflavonoid at middle and high dose for 30 days. The results showed that the biflavonoid is beneficial to non-alcoholic fatty liver (Fig. 2).

Adjustment of blood lipid by the biflavonoid—TC, TG and HDL are important factors of evaluating the level of blood lipid. TC and TG in blood of mice were remarkably lifted, but HDL was suppressed after high fat diet for 30 days ($P<0.01$), it suggested that hyperlipidemia model was achieved. On the other hand, TC and TG in flavonoid groups obviously declined, and HDL increased, it is manifested that the biflavonoid from C. oleifera suppresses hyperlipidemia. Along with increment of dose, TC, TG and HDL changed a lot and reached the significant level ($P<0.01$) at high dose of biflavonoid. The hypolipidemic effect of biflavonoid is better than that of simvastatin (Fig. 3).

Effect of the biflavonoid on MDA content, SOD and GSH-Px activity—MDA, SOD and GSH-Px are always used to deduce the antioxidative capacity in vivo. MDA was higher, SOD and GSH-Px activity were lower in mice blood of Veh group than normal saline group, and these indicated that hyperlipidemia mice had a weaker antioxidative capacity. However, MDA was lower, SOD and GSH-

---

**Table 1—The change of body weight of mice during 30 days**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day 1</th>
<th>Day 6</th>
<th>Day 12</th>
<th>Day 18</th>
<th>Day 24</th>
<th>Day 30</th>
<th>Gain (%/30 d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS</td>
<td>22.18±1.11</td>
<td>24.86±1.98</td>
<td>28.58±2.12</td>
<td>29.72±2.18</td>
<td>30.83±2.24</td>
<td>30.24±2.26</td>
<td>36.34</td>
</tr>
<tr>
<td>Veh</td>
<td>21.67±1.19</td>
<td>27.16±3.35</td>
<td>31.16±3.61</td>
<td>35.18±3.43</td>
<td>37.32±3.48</td>
<td>38.60±3.34</td>
<td>78.13</td>
</tr>
<tr>
<td>Control</td>
<td>22.37±1.15</td>
<td>26.27±1.29</td>
<td>29.35±1.73</td>
<td>32.12±1.61</td>
<td>33.98±1.99</td>
<td>35.03±1.77</td>
<td>56.59</td>
</tr>
<tr>
<td>LD</td>
<td>21.86±1.06</td>
<td>26.44±1.92</td>
<td>29.10±2.47</td>
<td>31.42±2.43</td>
<td>33.03±2.76</td>
<td>34.11±2.69</td>
<td>56.04</td>
</tr>
<tr>
<td>MD</td>
<td>21.62±1.04</td>
<td>25.42±1.41</td>
<td>28.48±2.51</td>
<td>30.01±2.56</td>
<td>31.71±2.42</td>
<td>32.64±2.41</td>
<td>50.97</td>
</tr>
<tr>
<td>HD</td>
<td>22.10±1.05</td>
<td>25.22±1.52</td>
<td>27.70±2.33</td>
<td>29.20±2.58</td>
<td>30.73±2.38</td>
<td>31.91±2.19</td>
<td>44.39</td>
</tr>
</tbody>
</table>

NS=normal saline, Veh=hyperlipidemia, Control=Simvastatin, (4 mg/kg/d), LD=low dose (50 mg/kg/d), MD=middle dose (100 mg/kg/d), HD=high dose (200 mg/kg/d)

$P$ values: <0.01, compared with a NS; b Veh
Px activity was stimulated after oral administration of biflavonoid for 30 days (Table 2), and it revealed that the biflavonoid from *C. oleifera* could improve antioxidative capacity of mice especially at high dose. **Relationship between blood lipid and antioxidative capacity**—By ways of contrast of TC, TG and HDL content with MDA, SOD and GSH-Px levels, the data on both sides were related in certain degree (Table 3). The relative coefficients were not significant in groups of normal saline, simvastatin and the biflavonoid at low dose, but achieved a significant level (*P*<0.01) in groups of Veh, biflavonoid at middle and high dose. It suggests that the relationship between antioxidative ability in vivo and blood lipid is not sure at normal status, but is certain in the condition of hyperlipidemia. Simvastatin can reduce blood lipid through inhibiting cholesterol synthetase[^19^], but the correlation variation between biflavonoid and simvastatin implied their mechanisms were different.

**Discussion**

There are many flavonoid glycosides in the seeds of *C. oleifera*, whose basic part is kaempferol, but bimolecular kaempferol compound has not been found so far. Kaempferol glycosides can be obtained through separation[^20^], but pure kaempferol glycoside could not be easily gained unless column chromatography is applied. By ways of solvent extraction, hydrolysis, precipitation and crystallization, high purity of biflavonoid with bimolecular kaempferol structure was acquired. The method is suitable for isolation of biflavonoid from the shells of *C. oleifera* in industry.

The biflavonoid from *C. oleifera* shows good hypolipidemic effect. Hyperlipidemia is a disease of metabolic disorder of lipid, accompanying higher levels of TC, TG and LDL (low density lipoprotein), and lower level of HDL (high density lipoprotein) in blood. The abnormality causes thick blood and sediment of lipid and cholesterol on inner walls of blood vessel, leads to the hyperplasia of smooth muscle and atherosclerosis. Hyperlipidemia is an

### Table 2—MDA, SOD and GSH-Px activities in the groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose mg/kg/d</th>
<th>MDA nmol/mL</th>
<th>GSH-Px U/mL</th>
<th>SOD U/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS</td>
<td>3.76±0.78</td>
<td>132.38±25.82</td>
<td>98.92±9.99</td>
<td></td>
</tr>
<tr>
<td>Veh</td>
<td>5.38±0.81</td>
<td>90.78±12.14</td>
<td>82.80±7.06</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>4.89±0.37</td>
<td>101.24±11.29</td>
<td>82.23±3.48</td>
</tr>
<tr>
<td>LD</td>
<td>50</td>
<td>4.78±0.44</td>
<td>108.30±12.74</td>
<td>82.99±5.59</td>
</tr>
<tr>
<td>MD</td>
<td>100</td>
<td>4.52±0.45</td>
<td>115.66±17.93</td>
<td>87.91±4.01</td>
</tr>
<tr>
<td>HD</td>
<td>200</td>
<td>4.00±0.77</td>
<td>125.42±19.30</td>
<td>93.97±7.72</td>
</tr>
</tbody>
</table>

NS=normal saline, Veh=hyperlipidemia, Control=Simvastatin, (4 mg/kg/d), LD=low dose (50 mg/kg/d), MD=middle dose (100 mg/kg/d), HD=high dose (200 mg/kg/d)

*P* values: <0.01, compared with *a* NS; *b* Veh
important factor inducing atherosclerosis and cardiovascular diseases\textsuperscript{21}. HDL transports cholesterol from tissue and blood to liver for catabolism, and helps to avoid atherosclerosis\textsuperscript{22}. The biflavonoid from \textit{C. oleifera} can inhibit the contents of TC and TG, and increase the level of HDL so as to prevent atherosclerosis.

The biflavonoid from \textit{C. oleifera} can eliminate free radicals \textit{in vivo}, which can be evaluated by MDA, SOD and GSH-Px activities. MDA is lipid peroxide produced by cells at the imbalance of oxidative and antioxidative system, which can combine phospholipid, protein and nucleic acid to form stable and insoluble substance, and induce cell damage. SOD and GSH-Px are important antioxidative enzymes, which can protect integrity of cell membrane structure and function, and eliminate free radicals. The antioxidant can improve antioxidative level \textit{in vivo} and prevent cells from oxidative injury through diminishing free radicals and MDA content, or strengthening SOD and GSH-Px activities. The present results show that the biflavonoid from the shells of \textit{C. oleifera} has strong capacity of eliminating free radicals in blood, so that it can prevent cells from the injury induced by free radicals.

Hyperlipidemia is correlated with antioxidative capacity\textsuperscript{23}. Oxidative stress links hyperlipidemia with the pathogenesis of atherosclerosis\textsuperscript{24}. When free radical produced by oxidative stress exceeds the eliminating capacity of antioxidant system, excessive free radical results in oxidation, denaturalization and deposition of blood lipid. The results of the present study that high-fat diets increased free radical production \textit{in vivo}, are in agreement with Dobrian \textit{et al}\textsuperscript{25}. Now that the metabolism of dietary cholesterol occurs in hepatocytes, where large amount of reactive oxygen species are generated\textsuperscript{26}, it is understandable that a high-fat diet leads to the elevation of free radical and lipid peroxides\textsuperscript{27}. Therefore free radical is a key factor on atherosclerosis. Current drugs in most common use for hyperlipidemia are statins including simvastatin, lovastatin, etc., but long use of them has a lot of side effects such as liver injury, striated muscle dissolution, etc.\textsuperscript{28}. Biflavonoid is natural edible product from \textit{C. oleifera} without side effects of statins, moreover it achieves the hypolipidemic effect by purging internal free radical and enhancing antioxidative ability, it is prospective for prevention and therapy of hyperlipidemia and atherosclerosis.

**Conclusion**

The biflavonoid with bimolecular kaempferol structure has been found in the shells of \textit{C. oleifera}. The separation procedure includes reflux extraction, hydrolysis and crystallization, which is simple and proper for industrial production. The biflavonoid can control mice weight and liver coefficient, decrease the blood lipid, and improve antioxidative levels \textit{in vivo}. It is useful for preventing high fat diet induced hyperlipidemia. The hypolipidemic mechanism is related to the elimination of free radicals and improvement of antioxidative ability.

**Conflict of Interest**

The authors declare that there are no conflicts of interest.

**Acknowledgement**

The authors thank the staff in the laboratory center of South China University of Technology for spectroscopic data analysis. The financial support for
this work from the NSFC (No. 81173646) is also acknowledged.

References