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The antioxidative effect of icariin in human erythrocytes against free-radical-induced haemolysis

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Abstract

Icariin (2-(4′-methoxyl phenyl)-3-rhamnosido-5-hydroxyl-7-glucosido-8-(3′-methyl-2-butylenyl)-4-chromanone) is the major component in Herba Epimedii used in traditional Chinese medicine for the treatment of atherosclerosis. This work focuses on the antioxidative effect of icariin on free-radical-induced haemolysis of human erythrocytes, in which the initial free radical derives from the decomposition of 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH) at physiological temperature. To reveal the structure–activity relationship of icariin, the antioxidant effects of two structural analogues of icariin, acacetin (2-(4′-methoxylphenyl)-5,7-dihydroxylchromone) and norwogonin (2-phenyl-5,7,8-trihydroxylchromone), on the same experimental system were examined as well. It was found that all these chromone derivatives (Chm-OHs) dose-dependently protected human erythrocytes against free-radical-induced haemolysis. The order of antioxidative activity was norwogonin > acacetin > icariin by the analysis of the relationship between the concentration of Chm-OHs and the prolongation percentage of the lag time of haemolysis (PP%). It was also proved that the phenyl hydroxyl group attached to the chromone ring at 7-position cannot trap the free radical. On the contrary, phenyl hydroxyl groups at the 5- and 8-position in norwogonin made it a significant antioxidant in AAPH-induced haemolysis. The more hydroxyl groups attached to the chromone ring, the higher the antioxidative activity in protecting erythrocytes against free-radical-induced peroxidation.

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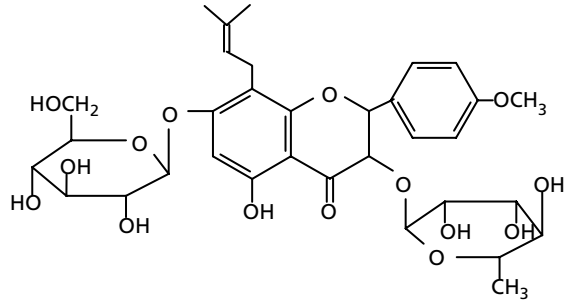
Introduction

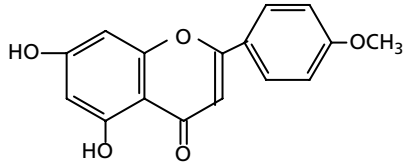
The association of free radical damage with cardiovascular disease is well documented; free-radical-induced peroxidation of polyunsaturated fatty acids in low-density lipoprotein (LDL) and erythrocyte membranes in human blood functions as the chemical pathogeny (Bland 1995). So, clinic doctors and chemists have an interest in how to protect LDL and erythrocytes against free-radical-induced peroxidation and to release man from a subhealthy status. The essential consideration of chemists is to synthesize compounds and, in particular, to extract natural ones to detect their antioxidative effect on free-radical-induced peroxidation, and reveal the structure–activity relationship (SAR) of these natural compounds for the sake of pharmacological research (Rice-Evans & Diplock 1993; Habon et al 2001). Among the natural compounds, herbs in traditional Chinese medicine have often attracted pharmacological research interest because they have proved useful in the clinical treatment of the corresponding disease for a long period. Chromone and its derivatives (Chm-OHs) are found in herbs frequently and a considerable number of reports concentrate on the methods used in the isolation of chromone from various herbs or the synthetic route of chromones analogues (Fujimoto et al 2002; Kuo et al 2002; Piao et al 2002). In addition, the inhibitory activity of Chm-OHs against free-radical-induced peroxidation, along with other pharmacological functions, has been widely studied (Silva et al 1998; Yagi et al 2002).

It is important to set up an in-vitro experimental model for the evaluation of the free-radical-scavenging property of an antioxidant since the in-vitro experimental system would avoid any metabolic influence on the experimental result and be suitable



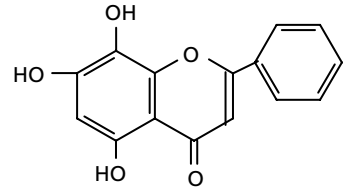
Icariin

2-(4′-methoxyphenyl)-3-rhamnosido-5-hydroxy-7-glucosido-8-(3′-methyl-2-butylenyl)-4-chromanone



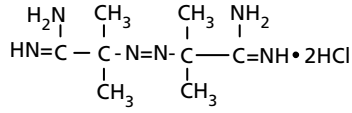
Acacetin

2-(4′-methoxyphenyl)-5,7-dihydroxychromone



Norwogonin

2-phenyl-5,7,8-trihydroxychromone



AAPH

2,2′-azobis(2-amidinopropane hydrochloride)

Figure 1 The structure and nomenclature of icariin, acacetin, norwogonin and free radical initiator AAPH.

for the exploration of SAR. Free-radical-induced haemolysis of human erythrocytes provides a convenient in-vitro experimental model (Niki et al 1988; Gieseg et al 2001; Zou et al 2001). This is because the erythrocyte membrane contains abundant polyunsaturated fatty acids that are very susceptible to peroxidation by free radicals generated concentration-dependently from the decomposition, at 37°C, of a water-soluble azo compound, 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH), resulting in haemolysis. Therefore, we can follow the process of free-radical-induced peroxidation directly by determination of the haemoglobin concentration outside the erythrocytes.

We chose the above experimental model to evaluate the antioxidative activity of icariin (2-(4′-methoxylphenyl)-3-rhamnosido-5-hydroxyl-7-glucosido-8-(3′-methyl-2-butylenyl)-4-chromanone), the major ingredient of Herba Epimedii used for the treatment of cardiovascular disease in traditional Chinese medicine. Moreover, to clarify the SAR of icariin, the antioxidant effects of two structural analogues, acacetin (2-(4′-methoxylphenyl)-5,7-dihydroxyl chromone) and norwogonin (2-phenyl-5,7,8-trihydroxyl-chromone), were also determined. The structures of Chm-OHs, along with the free radical initiator, AAPH, are shown in Figure 1.

Materials and Methods

Materials

2,2′-Azobis(2-amidinopropane dihydrochloride) (AAPH) was purchased from Aldrich and dissolved in phosphate-buffered saline (PBS) (containing (in mM): NaCl 150, Na_2HPO_4 8.1, NaH_2PO_4 1.9, pH 7.4) (Zavodnik et al 1999)). Icariin, acacetin and norwogonin were purchased from the Institute of Pharmaceutical and Biological Reagents (Beijing, China) and dissolved in dimethyl sulfoxide (DMSO). Human erythrocytes were provided by the Red Cross Center for Blood (Changchun, China), who were authorized legally to be responsible for collecting blood from healthy subjects and provided blood products for clinical and scientific usage in our city. Erythrocytes were washed three times with PBS to remove the plasma. Then, the erythrocytes were centrifuged at 1700 *g* for

exactly 10 min to obtain compact erythrocytes for experimental use (Zavodnik et al 1999).

Experimental procedure

The haemolysis experimental procedure was similar to that described in references (Niki et al 1988; Kuang et al 1994; Liu et al 2002; Zou et al 2001). In brief, AAPH–PBS solution (30 mM as the final concentration) was added to a suspension of erythrocytes in PBS (3.0%, v/v) in which the DMSO solution of one kind of Chm-OH was added in advance to a certain concentration and the suspension was incubated at 37°C. Samples were taken from the above incubation mixture and centrifuged at 1700 *g* for 5 min to remove the erythrocytes and obtain the supernatant. The percentage of haemolysis was determined by measuring the absorbance of the supernatant at 540 nm (A_{540}) and compared with that of complete haemolysis in the absence of Chm-OH. The haemolytic process was followed by withdrawing samples from the above incubation mixture at appropriate time intervals. Finally, the inhibitory concentration for 50% inhibition, IC50, of icariin, acacetin and norwogonin, respectively, was obtained graphically (Vinson et al 1995; Liu et al 2002). To avoid the influence of DMSO on the haemolysis, it is worth noting that the final volume of DMSO in all the experiments was 1.0% (v/v), and the same amount of DMSO was added to the control experiment as well.

Statistical analysis

Every experiment was repeated at least three times to obtain reproducible data within 10% experimental error. Data points shown in the figures were the average value of three independent measurements, in which the deviation was not indicated for figure clarity. Statistical analysis of the effect of concentration on the inhibitory percentage was performed by one-way analysis of variance using Origin 6.0 professional Software. The relationship between the concentration of Chm-OHs and the prolongation percentage (PP%) was carried out by linear regression. A significance level of $P < 0.01$ denoted significance in all cases.

Results

The inhibitory concentration for 50% inhibition (IC50) of haemolysis of icariin, acacetin and norwogonin

To compare the antioxidative activity of icariin, acacetin and norwogonin, the IC50 value was determined. Vinson et al (1995) reported a simple method for obtaining the IC50 graphically to compare IC50s under the same experimental conditions, that is, a certain incubation period should be selected to compare the haemolysis percentage. The haemolysis of a suspension of erythrocytes (3.0% v/v in PBS) containing icariin, acacetin and norwogonin, respectively, with five concentration intervals was initiated by addition of AAPH to reach a final concentration of 30 mM at 37.0°C. In this case, we chose 200 min as the incubation period, and recorded the corresponding haemolysis percentage since the haemolysis percentage at this incubation period point was identified clearly (see Figure 3, vide post). Then, the correlation of the concentration of Chm-OHs with the corresponding inhibitory percentage (100% – haemolysis percentage), is illustrated in Figure 2, in which the cross points between various lines and the horizontal dotted line indicate the IC50 values (Liu et al 2002). Thus, the IC50 of icariin was 35.8 μM, similar to that of acacetin (35.6 μM) and the IC50 of norwogonin was 28.5 μM, the smallest among these Chm-OHs.

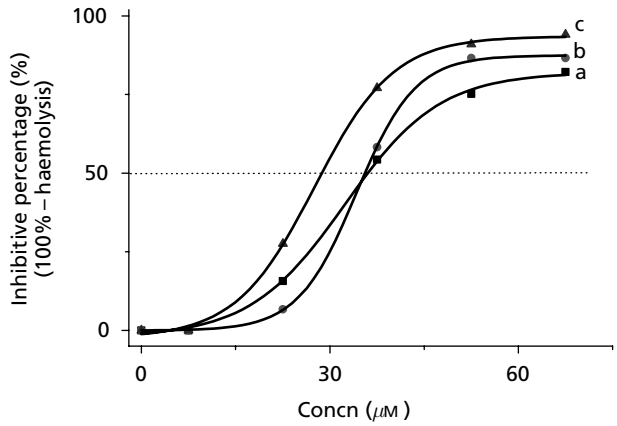


Figure 2 The relationship between the concentration of icariin (a), acacetin (b), norwogonin (c) and inhibitory percentage (100% – haemolysis percentage) at 200 min. The cross point between the lines and the dot line (50% inhibition) indicates the IC50.

The dosage-dependent antioxidative activity of icariin, acacetin and norwogonin

Figure 3 shows the haemolysis process of 3.0% (v/v in PBS) human erythrocytes suspension induced by 30 mM AAPH, with the final concentration of icariin, acacetin and norwogonin added to the above incubation mixture varying from 7.50 μM to 67.5 μM, respectively.

The line a, the control experiment in the absence of Chm-OHs, indicated that the haemolysis still showed a lag effect. The lag time of haemolysis, t_{lag}, revealed that the endogenous antioxidants (AH) in the erythrocyte (i.e. α-tocopherol, catalase, superoxide dismutase and glutathione) can scavenge the initiating or propagating radicals to protect the erythrocytes against free-radical-induced haemolysis (Niki et al 1988; Kuang et al 1994; Zou et al 2001). Haemolysis increased rapidly after depletion of all the endogenous antioxidants. The addition of icariin, acacetin and norwogonin at various concentrations prolonged the t_{lag} to different extents. The t_{lag} values in the absence and presence of Chm-OHs at various concentrations are listed in Table 1.

The extent of t_{lag} prolongation brought about by the addition of Chm-OHs can be expressed quantitatively by prolongation percentage, PP%, according to equation 1 (Yu et al 1999) and is also listed in Table 1.

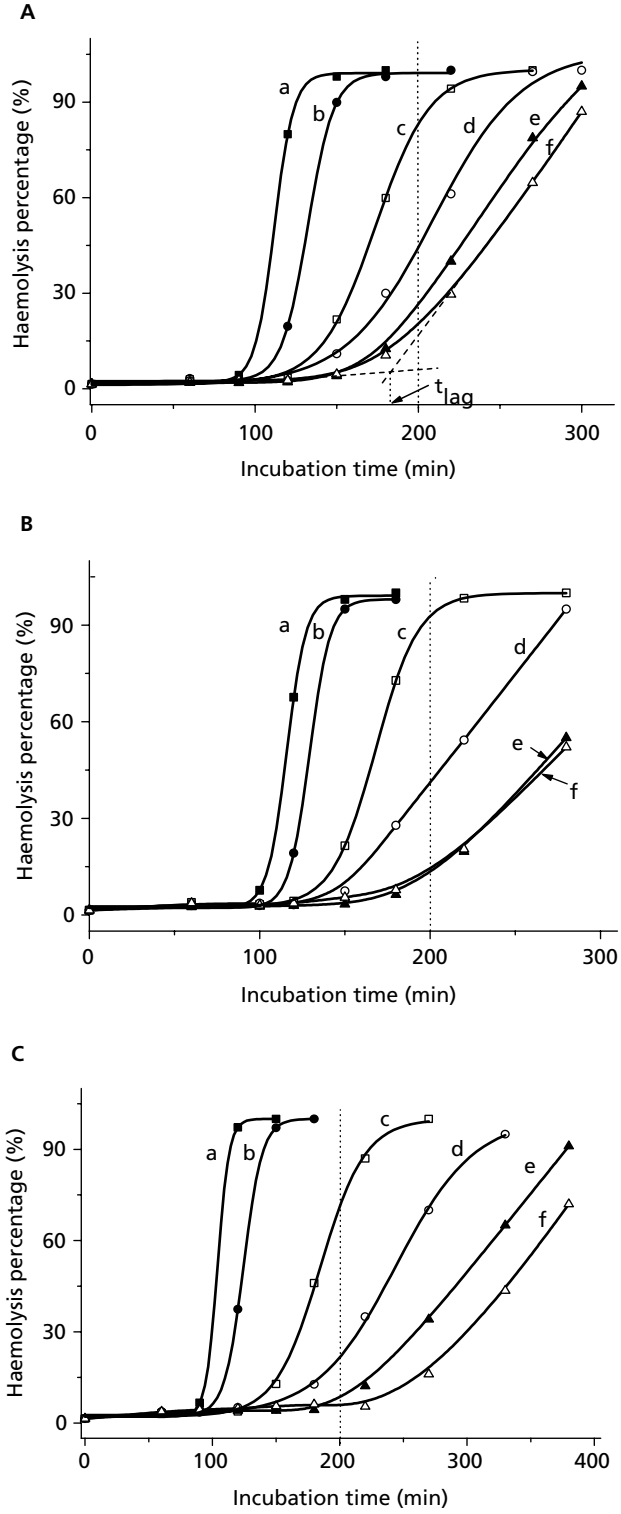


Figure 3 Haemolysis curves of human erythrocytes (3% suspension in PBS, pH 7.4) initiated by AAPH (30 mM) at 37°C in the presence of the chromone derivatives (Chm-OH) icariin (A), acacetin (B) and norwogonin (C). a, Native erythrocytes; b, a + 7.50 μM Chm-OH; c, a + 22.5 μM Chm-OH; d, a + 37.5 μM Chm-OH; e, a + 52.5 μM Chm-OH; f, a + 67.5 μM Chm-OH.

$$PP\% = \{[t_{lag}(\text{Chm-OH}) - t_{lag}(0)]/t_{lag}(0)\} \times 100 \quad (1)$$

where t_{lag}(Chm-OH) refers to the lag time in the presence of Chm-OHs, while $t_{lag}(0)$, as the control, stands for the lag time generated by endogenous antioxidants. The addition of icariin, acacetin and norwogonin can prolong the t_{lag} with an increase in the concentration, resulting in an increase in PP%. The quantitative relationship between PP% and the concentration can be obtained by linear regression and expressed by equation 2.

$$PP\% = AC + B \quad (2)$$

where C refers to the concentration of icariin, acacetin and norwogonin, the coefficient A (concentration sensitivity) reveals the influence of the increasing concentration on the PP% and B is the constant in the equation. For Chm-OHs, the quantitative correlation of Chm-OHs concentration, C, with PP% is expressed concretely as the following equations (the number in the parenthesis is the correlation coefficient of the equation):

$$\text{icariin: } PP\% = 1.22C + 5.45 \ (0.9898) \quad (3)$$

$$\text{acacetin: } PP\% = 1.34C - 1.11 \ (0.9760) \quad (4)$$

$$\text{norwogonin: } PP\% = 2.46C - 1.04 \ (0.9981) \quad (5)$$

Hence, the high correlation coefficients in the above equations demonstrates that icariin, acacetin and norwogonin are dosage-dependent antioxidants that can protect human erythrocytes against free-radical-induced haemolysis. Norwogonin is the best antioxidant since the concentration sensitivity (A in equation 2) of norwogonin is 2.46, the largest one among these Chm-OHs.

Discussion

Many research works have dealt with the isolation and structural characterization of chromones (Feng et al 2002; Lopez-Martin et al 2002; Mbah et al 2002), and most scientific attention has been paid to the pharmacology of the chromones (Gieseg et al 2001; Semba et al 2002; Yagi et al 2002; Roma et al 2003). Study on the structure–activity relationship (SAR) of chromones will reveal by which site the chromones play their antioxidant role in free-radical-induced peroxidation and will provide useful information on the design of chromone-analogue antioxidants. This work mainly focuses on discussion of the SAR. The active site of an antioxidant is the phenyl hydroxy group. The reasons why we selected acacetin and norwogonin as antioxidants in this work are due to their being structural analogues of icariin. The comparison of antioxidative activity of icariin with its structural analogues will reveal the SAR of icariin.

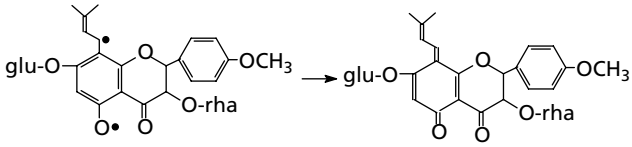
Icariin contains two phenyl hydroxy groups, at the 5- and 7-positions. The latter hydroxy group is etherified by

Table 1 Lag time of haemolysis, t_{lag}, in the presence of various chromone derivatives

Concn of chromone (μM)	Icariin		Acacetin		Norwogonin	
	t_{lag} (min)	PP(%)[a]	t_{lag} (min)	PP(%)[a]	t_{lag} (min)	PP(%)[a]
0.00	100 ± 3.0	0.0	103 ± 2.0	0.0	96 ± 1.5	0.0
7.50	117 ± 3.5	17.0	117 ± 3.0	13.6	109 ± 3.3	13.5
22.5	133 ± 4.0	33.0	123 ± 3.7	19.4	151 ± 4.5	57.3
37.5	158 ± 4.7	58.0	150 ± 4.5	45.6	187 ± 5.0	94.8
52.5	170 ± 5.1	70.0	187 ± 5.6	81.6	213 ± 6.0	121.8
67.5	183 ± 5.5	83.0	190 ± 5.7	84.4	256 ± 7.7	166.7

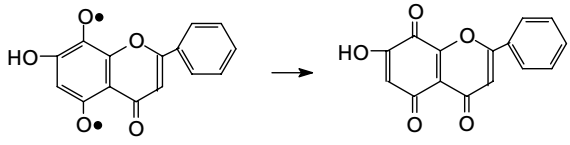
[a]$PP(\%) = \{[t_{lag}(\text{Chm-OH}) - t_{lag}(0)]/t_{lag}(0)\} \times 100$, where t_{lag}(Chm-OH) is the lag time of haemolysis in the presence of Chm-OHs at various concentrations, and $t_{lag}(0)$ is the lag time of haemolysis generated by endogenous antioxidants. Statistical analysis was performed using a one-way analysis of variance by Origin 6.0 Professional Software, and a significance level of $P < 0.01$ vs 0 μM chromone denoted significance in all cases.

glucose and cannot trap free radicals. The hydroxy group at the 7-position in acacetin is a free phenyl hydroxy group. However, the similar IC50 values for icariin and acacetin (35.8 μM and 35.6 μM, respectively) reveals that the free hydroxy group at the 7-position of acacetin does not increase its antioxidative activity over that of icariin, demonstrating that the hydroxy group at the 7-position of chromone cannot trap free radicals. Only the hydroxy group at the 5-position of chromone can inhibit the free radical. It can be found that there is a special position in icariin, 3′-methyl-2-butylenyl at the 8-position, which is the common position of benzyl and allyl groups. The hydrogen atom at this benzyl and allyl position can be easily abstracted by free radicals to form a stable free radical. If the hydroxy group at the 5-position has already formed a free radical, these two radicals within the same molecule can convert to a non-radical product by the following equation:



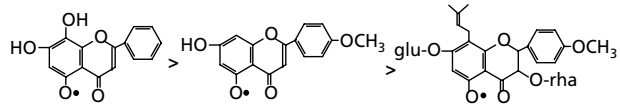
(6)

The IC50 of another structural analogue, norwogonin (28.5 μM), is lower than that of icariin and acacetin, indicating that the ability to form the free radical at the benzyl and allyl position is lower than that at the hydroxy position. So, the free radical of the two hydroxy groups at the *ortho*-position in norwogonin can more easily form non-radical products, following equation 7.

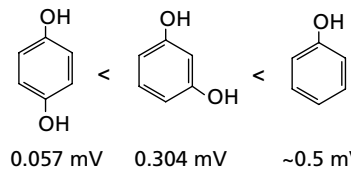


(7)

The linear relationship between the Chm-OH concentration and PP%, as equations 3–5 show, indicates that Chm-OHs are dosage-dependent antioxidants. Moreover, the coefficient of equation 5 (2.46) is higher than that in equation 4 (1.34) and equation 3 (1.22), revealing that the antioxidative effect of norwogonin increases more remarkably with an increase in concentration than the other two chromones. So, the order of antioxidative activity is norwogonin > acacetin > icariin. This demonstrates that the radical generated from norwogonin is more stable than those from the other ones; the order of stability of the corresponding free radical is:



This result can be easily understood by looking at the oxidative potential of the structural analogues hydroquinone, resorcinol and phenol (Denisov & Khudyakov 1987), the order of oxidative potential of which is:



The low oxidative potential makes the compound easily oxidized. Norwogonin can be oxidized to form a free radical more easily due to it possessing a hydroquinone structure species. However, the hydroxy group attached to the chromone ring in icariin makes it the most difficult among Chm-OHs to form a free radical. The resorcinol species contained in acacetin makes it more difficult to be oxidized than norwogonin, but easier than icariin.

Conclusion

In short, all these chromone derivatives can protect human erythrocytes against free-radical-induced haemolysis

remarkably. The order of antioxidative activity is norwogonin > acacetin > icariin by the analysis of the relationship between the concentration of Chm-OHs and the prolongation percentage of the lag time of haemolysis. It is also proved that the phenyl hydroxyl group attached to the chromone ring at the 7-position cannot trap free radicals. On the contrary, phenyl hydroxyl groups at the 5- and 8-positions in norwogonin make it a significant antioxidant in AAPH-induced haemolysis. The more hydroxyl groups attached to the chromone ring, the higher the antioxidative activity in protecting erythrocytes against free-radical-induced peroxidation.

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