Icariin: A Special Antioxidant To Protect Linoleic Acid against Free-Radical-Induced Peroxidation in Micelles

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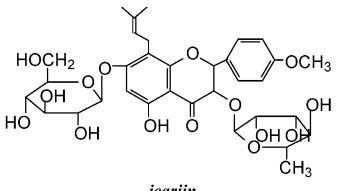
The objective in this work is to determine the antioxidant capacity and effectiveness of icariin (2-(4′-methoxylphenyl)-3-rhamnosido-5-hydroxyl-7-glucosido-8-(3′-methyl-2-butylenyl)-4-chromanone), the major component in *herba epimedii* being used widely in traditional Chinese medicine for the treatment of artherosclerosis and neuropathy, in which 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced peroxidation of linoleic acid (LH) in sodium dodecyl sulfate (SDS) acts as the experimental system. By containing an intramolecular hydrogen bond, icariin protects LH against AAPH-induced peroxidation of LH only in SDS, an anionic micelle. The number of trapping peroxyl radicals ($LOO^{\bullet}$), *n*, by icariin is just 0.0167 whereas α-tocopherol (TOH) and L-ascorbyl-6-laurate (VC-12) are 2.14 and 1.25, respectively, with reference to the *n* of 6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2.00. This is also related to how the intramolecular hydrogen bond enhances the bond dissociation enthalpy (BDE) of O−H in icariin. However, calculation of the inhibition rate constant, k_{inh}, a kinetic parameter to describe the reaction between the antioxidant and $LOO^{\bullet}$, results in a k_{inh} of icariin at about one magnitude larger than those of Trolox, TOH, and VC-12. This fact reveals that, by the view of kinetics, icariin is an antioxidant with much higher effectiveness. In addition, the antioxidant capacities of icariin used together with other antioxidants have been determined and the results indicate that the *n* of icariin decreases markedly while the *n* values of Trolox and TOH increase, even if the *n* of icariin is a negative value in the presence of VC-12. Furthermore, an analysis of k_{inh} in this case reveals that the $k_{inh}^{icariin}$ increases nearly one magnitude with the decrease of k_{inh}^{Trolox} and no remarkable change occurs for k_{inh}^{TOH}. The negative value of $k_{inh}^{icariin}$ in the presence of VC-12 can be regarded as the icariin functions as a prooxidant that can be rectified by VC-12 effectively. These findings implicate that the evaluation of antioxidant activity should not only focus on an *n* value, a thermodynamic possibility, but k_{inh} and the charge property of the micelle should be also taken into account. To some extent, the latter factors are more important than the thermodynamic possibility.

Introduction

The exploration on health relevant to oxidation induced by reactive oxygen species (ROS) and reactive nitrogen species (RNS)[1] and the investigation on the supplementation of antioxidants to maintain health[2,3] arouse much attention from scientific research interests with respect to the discovery and validation of the activity of antioxidants from either natural compounds[4] or synthesized ones[5] and the explanation of the antioxidant mechanism in view of chemical kinetics[6] or the foundation of novel methods for evaluation of antioxidant capacity.[7] In this regard, natural compounds with the background of medicinal herb exhibit superior benefits for two reasons: (1) natural antioxidants provide a complicated structure, of which synthesis is difficult to achieve for the structure−activity relationship (SAR) analysis of the antioxidant[8] and (2) the research of clinic used medicinal herb will provide many revelations regarding the antioxidant mechanism. The above research will benefit the further exploitation of medicinal herb.

With *herba epimedii* being used widely in traditional Chinese medicine for the treatment of artherosclerosis and neuropathy, antioxidant activity of its major component, icariin (2-(4′-methoxylphenyl)-3-rhamnosido-5-hydroxyl-7-glucosido-8-(3′-methyl-2-butylenyl)-4-chromanone), is the major objective in this work.



icariin
(2-(4'-methoxylphenyl)-3-rhamnosido-5-hydroxyl-7-glucosido-8-(3'-methyl-2-butylenyl)-4-chromanone)

The central structure of icariin is flavonoid, an important kind of natural antioxidant.[9] Meanwhile, the antioxidant activity of ginsenoside can be enhanced by the contained rhamnoside moiety.[10] Icariin, as a flavonoid involving a rhamnoside moiety, has been verified to protect human erythrocytes against free-radical-induced hemolysis.[11] On the basis of the above research background of icariin, it is necessary to explore its antioxidant mechanism chemically to reveal its structure−activity relation-

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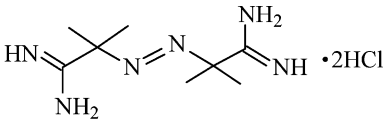
ship (SAR) and microenvironment−activity relationship (MAR) in detail.

Linoleic acid (LH) dissolved in micelles is selected to be the experimental system to imitate biological surroundings[12] owing to the fact that the free-radical-induced peroxidation of LH in micelles has been proven to follow the same classical law as that in homogeneous solutions.[13] Thus, all the chemical kinetic formulas correlated to the free-radical reaction in solutions are available for kinetic deduction in micelles, which the free-radical reaction is followed by oxygen uptake determination.[14] The free-radical source is supplied by the decomposition of a water-soluble azo compound, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH, R−N═N−R), at 37 °C,[15]

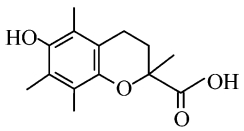


AAPH (R-N=N-R)
2,2'-azobis(2-amidinopropane) dihydrochloride

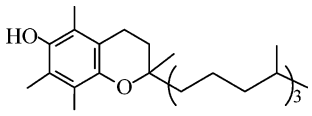
which the free-radical generation rate, R_g, can be controlled by the concentration of AAPH.[16]

$$R_g = (1.4 \pm 0.2) \times 10^{-6}[\text{AAPH}]\ \text{s}^{-1} \quad (1)$$

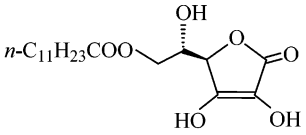
Presented here is a study on the measurement of antioxidant capacity of icariin in AAPH-induced peroxidation of LH in sodium dodecyl sulfate (SDS) micelles, with icariin used alone or in combination with 6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), α-tocopherol (TOH), and L-ascorbyl-6-laurate (VC-12), respectively.



Trolox
6-hydroxyl-2,5,7,8- tetramethyl chroman-2-carboxylic acid



TOH
α-tocopherol



VC-12
L-ascorbyl-6-laurate

Results and Discussion

Kinetic Deduction of AAPH-Induced Peroxidation Based on the Oxygen Uptake Determination. The procedure of AAPH-induced peroxidation of LH can be expressed as the following equations[14]

Initiation:

$$\text{R}-\text{N}=\text{N}-\text{R} \xrightarrow{k_d} 2e\text{R}^\bullet + \text{N}_2 + (1-e)\text{R}-\text{R} \quad (2)$$

$$\text{R}^\bullet + \text{O}_2 \xrightarrow{\text{fast}} \text{ROO}^\bullet \quad (3)$$

$$\text{ROO}^\bullet + \text{LH} \rightarrow \text{ROOH} + \text{L}^\bullet \quad (4)$$

Propagation:

$$\text{L}^\bullet + \text{O}_2 \xrightarrow{\text{fast}} \text{LOO}^\bullet \quad (5)$$

$$\text{LOO}^\bullet + \text{LH} \xrightarrow{k_p} \text{LOOH} + \text{L}^\bullet \quad (6)$$

Termination:

$$\text{LOO}^\bullet + \text{LOO}^\bullet \xrightarrow{2k_t} \text{nonradical product (NRP)} \quad (7)$$

where k_d, k_p, and k_t are the rate constants for decomposition of the initiator, chain propagation, and termination, respectively, and e designates the fraction of the initiator that is effective to initiate the peroxidation due to the cage effect of the solvent.[16] With an antioxidant (AH) adding to the above reaction system, AH traps the peroxyl radical ($\text{LOO}^\bullet$) to generate an antioxidant radical, $\text{A}^\bullet$, as eq 8 shows. If the $\text{A}^\bullet$ is so stable that couples rapidly with the $\text{LOO}^\bullet$ to form the nonradical product (NRP), LOOA, as eq 9 shows, then the peroxidation would be inhibited effectively.

$$\text{LOO}^\bullet + \text{AH} \xrightarrow{k_{inh}} \text{LOOH} + \text{L}^\bullet \quad (8)$$

$$\text{LOO}^\bullet + \text{A}^\bullet \xrightarrow{\text{fast}} \text{LOOA} \quad (9)$$

The above reaction procedure can be followed by the determination of the variety of the oxygen concentration in the process of the reaction as Figure 1 shows.

The peroxidation of LH is initiated at point B by the addition of AAPH and inhibited by the addition of antioxidant at point C. The treatment of reactions 2−9 by the steady-state hypothesis yields the inhibition rate, R_{inh}, expression as eq 10, where R_i

$$R_{inh} = -\text{d}[\text{O}_2]/\text{d}t = k_{inh}n[\text{AH}][\text{LOO}^\bullet] = (k_pR_i[\text{LH}])/(nk_{inh}[\text{AH}]) \quad (10)$$

denotes the real rate at which the free radical initiates the propagation. The stoichiometric factor, n, refers to the number of $\text{LOO}^\bullet$ molecules trapped by every AH molecule,[17] and furthermore, n can be expressed as[18]

$$n = (R_it_{inh})/[\text{AH}] \quad (11)$$

where t_{inh} stands for the inhibition period of an antioxidant, namely, the durative time of the CD segment in Figure 1. In fact, n is always applied to assess the antioxidant capacity.[17,19] After depletion of all the antioxidant at point D, the oxygen consumption rate increases rapidly and the reaction rate in the period of propagation, R_p, the slope of the DE segment in Figure 1, is given by

$$R_p = -\text{d}[\text{O}_2]/\text{d}t = (k_p/(2k_t)^{0.5})R_i^{0.5}[\text{LH}] \quad (12)$$

where $k_p/(2k_t)^{0.5}$ is referred to as the oxidizability of the substrate, representing the susceptibility of LH to undergo peroxidation. Finally, the kinetic chain length, kcl, that defines the cycle number of chain propagation in the period of inhibition, kcl_{inh}, and propagation, kcl_p, is given by eq 13.

$$\text{kcl}_{inh} = R_{inh}/R_i$$

and

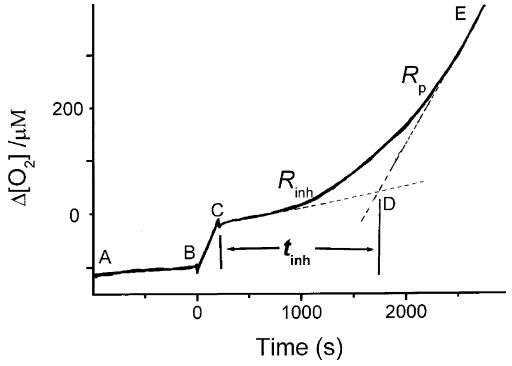


Figure 1. Take a real oxygen uptake curve, for example, to indicate the determination of t_{inh}, R_{inh}, and R_p. LH (10.9 mM in SDS/PBS) sealed in the pool exhausted oxygen from point A to B, then 100 μL of AAPH (400 mM) was injected at point B to initiate peroxidation of LH whose oxygen exhaustion was rapid from point B to C (the final concentration of AAPH in the pool was 20 mM). Then 10 μL of VC-12 (1.33 mM) was injected at point C (the final concentration of VC-12 in the pool was 6.65 μM). After all the VC-12 was depleted, the oxygen exhaustion increased. The slope during point C to point D was assigned to R_{inh}, and the slope during point D to point E was R_p. The duration period from point C to point D was an inhibition period, t_{inh}. An operation skill in the oxygen uptake experiment was that the antioxidant was mixed with LH solution before AAPH was injected for the sake of saving oxygen in the sealed pool. Thus, the exhaustion of oxygen in the period BC was omitted, and in fact, AAPH was injected at point C. Moreover, to contain enough oxygen in the sealed pool for completing the whole reaction, the LH/antioxidant solution was saturated by oxygen in advance to increase $[O_2]$ before the pool sealed, and the vertical axis herein indicated the variety of $[O_2]$ rather than $[O_2]$ itself.

$$\mathrm{kcl}_p = R_p/R_i \tag{13}$$

Therefore, the kinetic detail of a free-radical-related reaction can be described by n, k_{inh}, oxidizability, and kcl.

Antioxidant Capacity of Icariin and Other Antioxidants. Above all, the kinetic discussion on a free-radical-related reaction is based on knowing R_i, which can be obtained from the slope, n/R_i, of eq 14, with an equivalent style to eq 11. So,

$$t_{inh} = (n/R_i)[\mathrm{AH}] \tag{14}$$

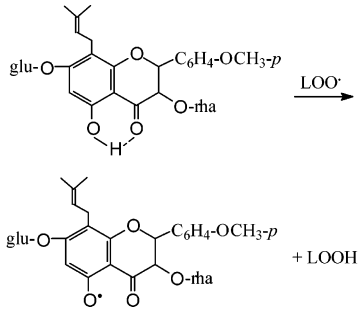
the relationship between t_{inh} and [AH] should be set up on a standard antioxidant whose n is known. In general, Trolox acts as the standard reference antioxidant and its n is suggested to be 2.[7,16] The t_{inh} values in the presence of various concentrations of Trolox are collected in Table 1, and the equation of $t_{inh} \sim$ [Trolox] is listed in Table 2.

So far, the R_i can be calculated as 7.70×10^{-9} M s^{-1} according to eq 14. In the case of [AAPH] = 20 mM, the rate of radical generation from the decomposition of AAPH, R_g, is 2.8×10^{-8} M s^{-1}, implying that not all the free radicals derived from AAPH can result in the radical propagation. This is because only the free radical transferring from the aqueous to micelle phase can really initiate radical propagation, thus, the ratio between R_i and R_g, $\epsilon = R_i/R_g$, is designated as the phase-transfer efficiency.[16] Herein, $\epsilon = 0.275$ indicates that only 27.5% radical generated from AAPH actually initiates free-radical propagation.

Table 1 also involves t_{inh} in the presence of various concentrations of other antioxidants, and moreover, the relationships between t_{inh} and the concentration of other antioxidants are found and listed in Table 2. On the basis of known R_i, the n values of other antioxidants can be obtained by eq 15 and are

$$n = R_i \times (\text{the coefficient in the equation of } t_{inh} \sim [\mathrm{AH}]) \tag{15}$$

listed in Table 2 as well. The fact that the n value of TOH is similar to that of Trolox can be ascribed to their similar structure and indicates that the packaging in the SDS micelle does not hinder the ability of TOH to trap LOO$^\bullet$, as eq 8 shows. Whereas the n value of VC-12, a lipophilic derivative of vitamin C,[20,21] is lower than that for TOH. As for icariin, a much lower n value implies that it seems to be a weak antioxidant. This can be understood easily because an intramolecular hydrogen bond formed between the hydrogen atom in −OH and the oxygen atom in C═O (see eq 16) increases the bond dissociation enthalpy (BDE) of O−H. For example, the BDE of O−H in phenol is 96.71 kcal/mol, whereas it increases to 99.10 kcal/mol while a −CHO attaches to the ortho-position of −OH.[22] As a result, the abstraction of the hydrogen atom in −OH by LOO$^\bullet$ as shown in eq 16 is more difficult than in other antioxidants, that is, the BDE of O−H in TOH is only 78.1 kcal/mol.[17]



(16)

The analysis of n, a thermodynamics parameter, however, just provides an aspect for identifying antioxidant capacity. The factual antioxidant behavior should be revealed by an inhibition rate constant, k_{inh}, a kinetic parameter. When k_p is taken as 100,[23] the k_{inh} values of all these antioxidants are calculated according to eq 10 on the basis of R_i and n and collected in Table 1. The k_{inh} values of Trolox, TOH, and VC-12 are in agreement with the reported data.[19,21] Surprisingly, the k_{inh} of icariin is much higher than those of the other antioxidants, indicating that icariin plays the most effective role in suppressing radical propagation as eqs 8 and 9 show. The charge property of the microenvironment in which icariin locates should be taken into account when explaining its antioxidant behavior. Icariin, a lipophilic antioxidant, must be dissolved in a SDS micelle, and the initiating radical, ROO$^\bullet$, a positively charged species, would be attracted to the surface of the anionic surfactants, such as $-SO_3^-$. Thus, ROO$^\bullet$ would react preferably with icariin molecules that have the polar −OH group pointed toward the polar side of the micelle as eq 17 shows.

This deduction can be proven by our experiment, that is, icariin does not exhibit antioxidant effectiveness while dissolved in Triton X-100, a neutral micelle, and cetyltrimethylammonium bromide (CTAB), a cationic micelle. This is because the neutral or cationic surfactants do not benefit from attracting ROO$^\bullet$ and, therefore, icariin in these micelles cannot act as an antioxidant.

The discussions on k_{inh} in the literature generally focus on the comparison of the k_{inh} magnitude,[24] in which the value is obtained experimentally under only one appointed concentration.[19,21] As for the investigation on the relationship between k_{inh} and antioxidant concentration in this work, another interesting phenomenon is found, as outlined as in Figure 2, that k_{inh} of either icariin or other antioxidants decreases with an increase in their concentrations.

TABLE 1: Inhibition of AAPH-Induced Peroxidation of Linoleic Acid by Icariin or Other Antioxidants[a]

antioxidants	concentration (μM)	R_p (10^{-8} M s^{-1})	R_{inh} (10^{-8} M s^{-1})	t_{inh} (s)	$k_p/(2k_t)^{0.5}$	k_{inh} (10^4 M^{-1} s^{-1})	kcl_p	kcl_{inh}
Trolox	1.53	23.7	7.78	894	0.248	3.52	30.7	10.1
	3.06	22.2	6.77	1343	0.233	2.02	28.9	8.8
	4.59	21.4	5.46	1681	0.224	1.67	27.8	7.1
	6.12	19.7	5.03	2074	0.206	1.36	25.6	6.5
	7.65	18.9	4.07	2516	0.198	1.34	24.5	5.3
TOH	2.65	22.0	7.80	1091	0.231	1.89	28.6	10.1
	5.30	19.8	5.57	1714	0.207	1.33	25.7	7.2
	7.95	17.9	5.31	2743	0.188	0.927	23.3	6.9
	10.6	17.4	5.25	3299	0.182	0.704	22.6	6.8
	13.2	15.0	5.21	3974	0.157	0.568	19.5	6.8
VC-12	2.18	23.4	9.44	767	0.245	3.25	30.4	12.3
	4.09	19.7	6.96	1112	0.206	2.35	25.5	9.0
	6.65	18.7	6.40	1474	0.196	1.57	24.3	8.3
	8.86	17.1	5.99	1908	0.179	1.26	22.2	7.8
	10.9	16.8	5.24	2182	0.176	1.46	21.8	6.8
icariin	10.8	22.4	8.68	641	0.235	53.7	29.1	11.3
	21.5	19.4	8.03	674	0.203	29.0	25.2	10.4
	43.0	18.9	6.90	761	0.198	16.9	24.5	9.0
	86.1	15.8	6.61	875	0.166	8.82	20.6	8.6
	172	15.6	5.87	994	0.164	4.96	20.3	7.6

[a] [LH] = 10.9 mM, [AAPH] = 20 mM, $R_i = 7.70 \times 10^{-9}$ M s^{-1}, $R_g = 2.8 \times 10^{-8}$ M s^{-1}, $\epsilon = R_i/R_g = 0.275$.

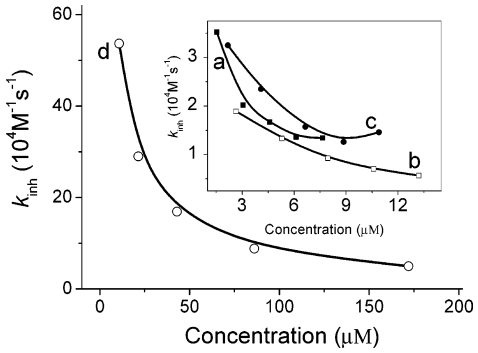
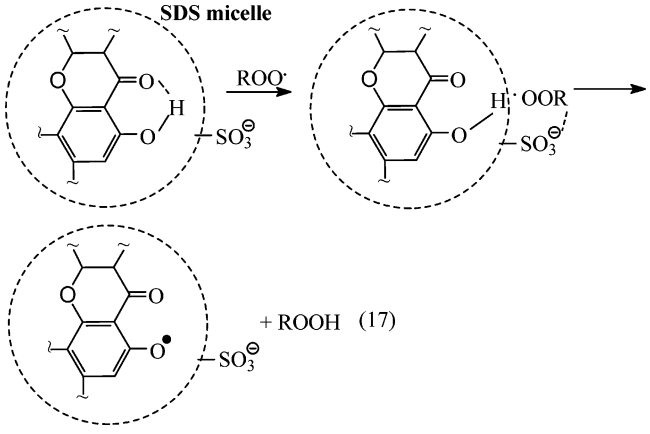


Figure 2. Relationship between k_{inh} and the concentration of Trolox (a), TOH (b), VC-12 (c), and icariin (d).



To the knowledge of physical chemistry, the rate constant of a reaction is independent of the variety of the reactant concentration. Obviously, the variety of k_{inh} is not consistent with this rule. To review the kinetic deduction involving eqs 2−9, one can find that obtaining eq 10, which is applied to calculate k_{inh}, is, in fact, based on an assumption that an antioxidant only reacts with LOO$^\bullet$, as eq 8 shows. If eq 10 is converted to its equivalent style, then we obtain eq 18

$$k_{inh} = (k_p R_i[LH])/(nR_{inh}[AH]) \quad (18)$$

To calculate k_{inh} after measuring R_{inh} and obtaining R_i and n with known [LH] and [AH], it can be found that k_{inh} is inversely proportional to [AH]. So, the decrease of k_{inh} with the increase of [AH] can be understood reasonably. But if the reaction of ROO$^\bullet$ with AH (eq 19)[15]

$$ROO^\bullet + AH \rightarrow ROOH + A^\bullet \quad (19)$$

is taken into account, the present chemical kinetic model should be improved and discussed in further work.

TABLE 2: Equations of t_{inh} ~ Concentration and the Stoichiometric Factor, *n*, of Every Antioxidant

antioxidant	equation of t_{inh} ~ concentration	*n*
Trolox	$t_{inh} = 259.8$[Trolox] + 508.9	2.00
TOH	$t_{inh} = 277.8$[TOH] + 356.9	2.14
VC-12	$t_{inh} = 162.8$[VC-12] + 423.6	1.25
icariin	$t_{inh} = 2.17$[icariin] + 644.1	0.0167

With respect to the oxidizability of LH as listed as in Table 1, the increase of antioxidant concentrations reduces the $k_p/(2k_t)^{0.5}$ gradually, demonstrating that, the higher the concentrations of either icariin or other antioxidants, the more markedly the LH is protected by antioxidants. As for kcl, it can be found that the addition of various antioxidants shortens the cycles of radical propagation significantly.

Antioxidant Capacity of Icariin used in Combination with Other Antioxidants. In the case of various concentrations of Trolox, TOH, and VC-12 added to icariin with different concentrations, t_{inh}, R_{inh}, and R_p are detected and collected in Table 3.

By comparing t_{inh} with the corresponding value in Table 1, one can find that the t_{inh} value of icariin used together with other antioxidants is quite similar to that of other antioxidants used alone. It seems that icariin does not play any role in the combinative usage with other antioxidants. Icariin, however, as the above-mentioned discussion demonstrates, indeed protects LH against AAPH-induced peroxidation. So, the t_{inh} values in Table 3 should still be regarded as the common contribution from other antioxidants and icariin, and as listed as in Table 4, the t_{inh} values should be expressed by the multiple linear regressions relevant to the concentration of both icariin and other

TABLE 3: Inhibition of AAPH-induced Peroxidation of Linoleic Acid by Icariin Used Together with Other Antioxidants[a]

concentration (μM)		R_p	R_{inh}	t_{inh}		k_{inh}^{other}	$k_{inh}^{icariin}$		
Trolox	icariin	(10^{-4} M s^{-8})	(10^{-8} M s^{-1})	(s)	$k_p/(2k_t)^{0.5}$	(10^4 M^{-1} s^{-1})	(10^4 M^{-1} s^{-1})	kcl_p	kcl_{inh}
1.53	10.8	22.8	8.58	904	0.239	2.89	389	29.6	11.2
3.06	21.5	22.7	8.20	1296	0.238	1.51	204	29.5	10.6
4.59	43.0	22.0	6.65	1786	0.230	1.24	126	28.6	8.6
6.12	86.1	21.3	6.51	2239	0.223	0.951	64.2	27.7	8.5
7.65	172	17.1	4.68	2689	0.179	1.06	44.6	22.2	6.1
concentration (μM)		R_p	R_{inh}	t_{inh}		k_{inh}^{other}	$k_{inh}^{icariin}$		
TOH	icariin	(10^{-8} M s^{-1})	(10^{-8} M s^{-1})	(s)	$k_p/(2k_t)^{0.5}$	(10^4 M^{-1} s^{-1})	(10^4 M^{-1} s^{-1})	kcl_p	kcl_{inh}
2.65	10.8	19.7	6.51	1130	0.206	2.35	99.6	25.6	8.5
5.30	21.5	18.8	5.85	2034	0.197	1.31	55.4	24.4	7.6
7.95	43.0	18.3	5.76	2693	0.191	0.888	28.2	23.7	7.5
10.6	86.1	17.8	5.50	3341	0.187	0.698	14.8	23.2	7.1
13.2	172	13.4	5.48	4315	0.141	0.560	7.40	17.5	7.1
concentration (μM)		R_p	R_{inh}	t_{inh}		k_{inh}^{other}	$k_{inh}^{icariin}$		
VC-12	icariin	(10^{-8} M s^{-1})	(10^{-8} M s^{-1})	(s)	$k_p/(2k_t)^{0.5}$	(10^4 M^{-1} s^{-1})	(10^4 M^{-1} s^{-1})	kcl_p	kcl_{inh}
2.18	10.8	18.5	7.82	848	0.194	3.53	−97.2	24.0	10.2
4.09	21.5	18.0	7.09	1156	0.188	2.07	−53.6	23.4	9.2
6.65	43.0	17.6	6.02	1501	0.184	1.50	−31.6	22.8	7.8
8.86	86.1	17.5	5.69	2030	0.184	1.19	−16.7	22.8	7.4
10.9	172	16.8	5.18	2167	0.176	1.06	−9.18	21.8	6.7

[a] See footnote in Table 1.

TABLE 4: Equations of t_{inh} ~ Concentration and the Stoichiometric Factor, *n*, of Icariin Used in Combination with Other Antioxidants

equation of t_{inh} ~ concentration	n_{other}	$n_{icariin}$
$t_{inh} = 287.4$[Trolox] + 0.30[icariin] + 443.4	2.21	0.0023
$t_{inh} = 267.2$[TOH] + 1.56[icariin] + 475.7	2.06	0.0120
$t_{inh} = 181.2$[VC-12] − 1.33[icariin] + 444.5	1.39	−0.0102

antioxidants as eq 20 shows. Thus, the number of trapping

$$t_{inh} = (n_{other}/R_i)[\text{other antioxidants}] + (n_{icariin}/R_i)[\text{icariin}] \quad (20)$$

peroxyl radicals of icariin used together with other antioxidants, $n_{icariin}$, and the number of peroxyl radicals trapped by other antioxidants in this case, n_{other}, can be calculated from the coefficients in eq 20. Compared with the corresponding *n* in Table 2, except $n_{icariin}$ and n_{TOH} which decrease slightly, $n_{icariin}$ decreases predominantly while used in combination with Trolox and even is a negative value while used together with VC-12, whereas n_{Trolox} and n_{VC-12} increase. This fact reveals that the antioxidant capacity of either icariin or other antioxidants varies remarkably in combinative usage, especially if icariin plays a prooxidative role in the presence of VC-12.

It has been pointed out that the analysis of antioxidant capacity just provides an antioxidant possibility rather than factual antioxidant behavior described by k_{inh}. Therefore, the k_{inh} values of icariin in the presence of other antioxidants, along with the k_{inh} values of other antioxidants in this case, have been calculated according to eq 18 by using the corresponding n_{other} and $n_{icariin}$ and listed in Table 3. To clarify the variety of k_{inh} of icariin used alone and together with other antioxidants, Figures 3 and 4 outline the k_{inh} of icariin in the absence and presence of Trolox and TOH, respectively, in which the insets in the figures indicate the k_{inh} change of Trolox and TOH.

As can be found from Figure 3, with the decrease of k_{inh} of Trolox, the k_{inh} values of icariin at every concentration point increase significantly. But the increase of k_{inh} of icariin in the presence of TOH, as Figure 4 shows, is not as large as that in the presence of Trolox, meanwhile, the variety of k_{inh} of TOH is not remarkable in this case. The antioxidant function of Trolox is regarded to be same as that of TOH because they have the same active group, −OH, for trapping free radicals except that the former is a water-soluble antioxidant and the latter is a lipophilic one. The difference of the k_{inh} variety between Trolox and TOH and the influences of Trolox and TOH on the k_{inh} of icariin reveal the effect of the distributive status of these two antioxidants on the antioxidative property of icariin. This can be understood easily by eq 21.

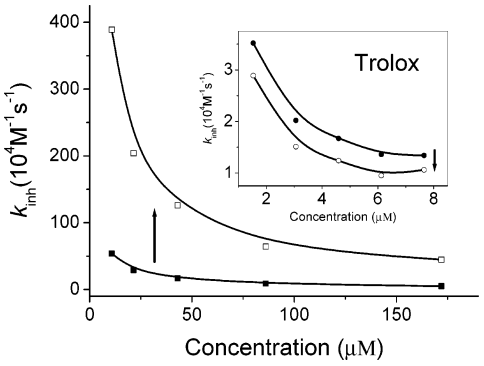


Figure 3. k_{inh} of icariin used alone (■) and together with Trolox (□); the inset chart shows the k_{inh} of Trolox used alone (●) and together with icariin (○).

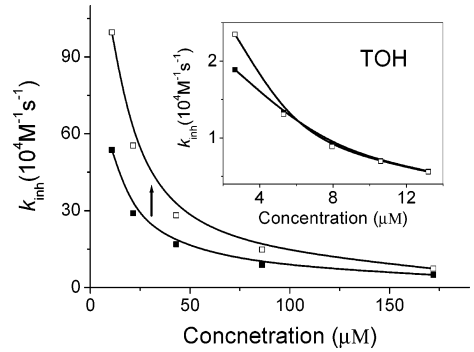
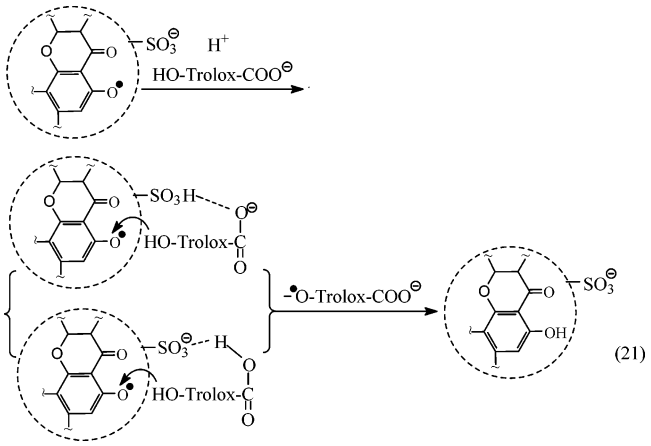


Figure 4. k_{inh} of icariin used alone (■) and together with TOH (□); the inset chart shows the k_{inh} of TOH used alone (●) and together with icariin (□).



Trolox may ionize at pH = 7.0, so the H^+ from the ionization of Trolox can combine with $-SO_3^-$ on the surface of SDS, also return to the $-COO^-$ of Trolox since Trolox is a weak acid, leading to the tight interaction between the micelles containing the icariin radical and the Trolox anion. The icariin radical is repaired conveniently at the SDS surface by the donation of the hydrogen atom from the −OH in Trolox. This recycle procedure is similar to the radical of TOH within a human erythrocytes membrane repaired by intracellular ascorbic acid at the surface of the membrane.[25] But the opportunity for the TOH packaged in SDS micelles to contact the micelle containing an icariin radical is less than Trolox, which distributes in the aqueous phase and transfers freely. Consequently, the variety of the k_{inh} of TOH is also not as remarkable as the variety of the k_{inh} of Trolox.

Figure 5 illustrates the k_{inh} of icariin in the absence or presence of VC-12, and the inset of the figure outlines the k_{inh} change of VC-12.

The negative value of k_{inh} of icariin in the presence of VC-12 can be regarded as the rate constant of the reverse reaction, that is, LH is peroxidized induced by the icariin radical as eq 22 shows

$$\mathrm{LH} + \mathrm{icariin}^{\bullet} + \mathrm{O_2} \xrightarrow{-k_{inh}} \mathrm{LOO}^{\bullet} + \mathrm{icariin} \qquad (22)$$

and the radical of icariin is derived from being oxidized by the VC-12 radical as eq 23 shows. Additional support for the above

$$\mathrm{VC\text{-}12}^{\bullet} + \mathrm{icariin} \rightarrow \mathrm{VC\text{-}12} + \mathrm{icariin}^{\bullet} \qquad (23)$$

deduction is from a study revealing that, by supplementation with a large dosage, ascorbic acid can play a prooxidative role to oxidize the species of DNA in vivo[26] and VC can accelerate the oxygen uptake in this experimental system, indicating VC acts as a prooxidant in this case.[27] Besides VC-12 functions as an antioxidant to trap the peroxyl radical, the VC-12 radical, although packaged in SDS micelles, can also act as a prooxidant to abstract the hydrogen atom of icariin. Consequently, the icariin radical oxidizes LH and acts as a prooxidant in this case.

Conclusion

In summary, owing to the fact that it contains an intramolecular hydrogen bond, icariin is a such a special antioxidant that it protects linoleic acid against AAPH-induced peroxidation just in SDS micelles. Although the *n* value of icariin is lower than Trolox, TOH, and VC-12, its k_{inh} is higher than that of the aforementioned three antioxidants by about one magnitude, demonstrating that *n*, as a thermodynamics value, just implies

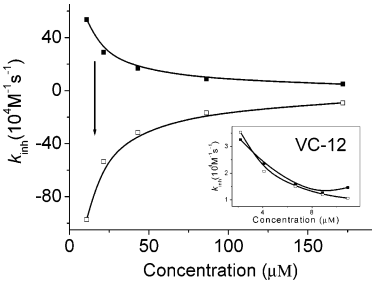


Figure 5. k_{inh} of icariin used alone (■) and together with VC-12 (□); the inset chart shows the k_{inh} of VC-12 used alone (■) and together with icariin (□).

the possibility of antioxidant capacity, whereas k_{inh}, as a kinetic parameter, reveals the antioxidant behavior in detail. The variety of k_{inh} relevant to the antioxidant concentration implicates that the reaction between the antioxidant and the initiating radical should be involved and the current chemical kinetic model should be renovated. In addition, either the thermodynamics factor or the kinetic parameter and microenvironment chargeproperty should be taken into account when evaluating antioxidant activity. To some extent, the latter factors are more important than the former one.

Experimental Section

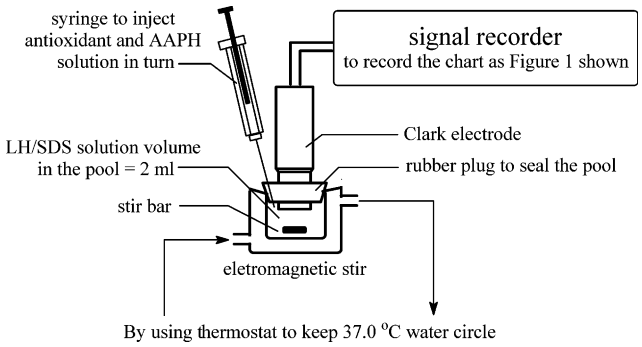
SDS, CTAB, and vitamin C, analytical grade agents, were purchased from Sino-western Chemical Ltd., Beijing, China, and icariin was from the Institute of Pharmaceutical and Biological Reagent, Beijing, China. 2,2′-Azobis(2-amidinopropane dihydrochloride) (AAPH), α-tocopherol (TOH), Trolox, Triton X-100, and linoleic acid (LH) were purchased from Aldrich or Fluka and used as received.

AAPH was dissolved directly in PBS (PBS, 5 mM Na_2HPO_4, 5 mM NaH_2PO_4, 0.2 mM EDTA, pH = 7.0) and 20 mM was the final concentration used in this work. SDS, CTAB, and Triton X-100 were dissolved in PBS to reach each critical micelle concentration (CMC), 100 mM, 20 mM, and 20 mM, respectively, and agitated ultrasonically to form the micelle solution. VC-12 was synthesized following the method reported in the literatures.[20] As for the antioxidants, Trolox was dissolved in PBS directly and α-tocopherol and VC-12 were dissolved in the same micelles as the one in which LH was dissolved. However, icariin was so special that it could not be dissolved in PBS directly, although two sugar moieties are contained in its molecular structure. But icariin can be dissolved in micelles easily, so, it was used as a micelles solution in the experiment.

The process of AAPH-induced peroxidation of LH was followed in situ by a SP-3 oxygen uptake apparatus equipped with a Clark electrode that was sensitive to the variety of oxygen concentrations as low as 10^{-8} M (Shanghai Institute of Phytobiology, Chinese Academy of Sciences) (see Scheme 1).

In brief, the LH micelle solution was put into a pool with a 37.0 °C water circle by thermostat and stirred for 5 min to reach a saturated concentration of oxygen in the air (0.219 μmol/mL). Then the pool was sealed by the Clark electrode and now the volume of the LH micelle solution in the pool was 2 mL. As shown as in Figure 1,[18] the oxygen uptake rate was slow in the period of AB, then increased in BC since the AAPH solution was injected by a syringe at point B to initiate the peroxidation of LH (eqs 2−7). The oxygen uptake rate decreased after point C due to the fact that the antioxidant solution was injected at point C (eqs 8 and 9) and restored quickly after all the antioxidant was exhausted after point D. The slopes of the CD and DE segments were designated as R_{inh} and R_p, and the

SCHEME 1: Equipment Used for the Oxygen Uptake Experiment



duration period between the added and exhausted antioxidant was referred as the inhibition period, t_{inh}.

Every experiment was repeated at least three times with the standard deviation within 10%, and the data presented in the tables were the average values from the above three independent measurements. The equation of $t_{inh} \sim$ [AH] was carried out statistically by one-way ANOVA by Origin Professional software, in which $P < 0.001$ indicated a significant difference.

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