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Investigation on the antioxidation of the flavonoids based on the photoinduced chemiluminescence of lucigenin by a new Zinc(II) phthalocyanine compound

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Abstract

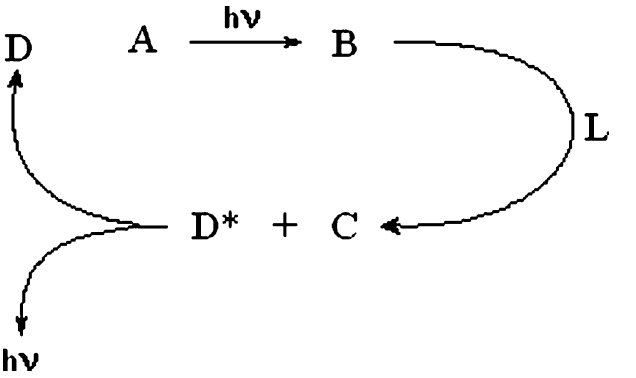
A zinc(II) phthalocyanine compound, tetra-α-(2,2,4-tirmethyl-3-pentoxy) Phthalocyanine Zinc ($ZnPc(OR)_4$) was synthesized in this paper and this zinc (II) phthalocyanine compound was used as the photosensitizer in the photoinduced chemiluminescence (PCL) of lucigenin in N,N-dimethylformamide (DMF). The photoexcited $ZnPc(OR)_4$ would produce singlet molecular oxygen ($^1O_2$), which would further react with DMF to form corresponding DMF radicals, such as $\cdot CH_3$ and $\cdot CH_2N(CH_3)CHO$, or corresponding alkylperoxyl radicals. Then the carbon centred radical would react with lucigenin to initiate the chemiluminescence. These results would provide useful data to establish a method for evaluation of the ability of $^1O_2$ generation of phthalocyanine. It was also found in this paper that the flavonoids could effectively inhibit this PCL system, which parallelled very well to flavonoids' radical-scavenging capacity. The mechanism of this PCL system and the relationship between the molecular structure of flavonoids and their radical-scavenging activity are also discussed in detail in this paper.

Keywords: *Photochemiluminescence, lucigenin, N,N-dimethylformamide, flavonoids, radical-scavenging activity*

Introduction

Light emission from molecules excited by a chemical reaction is called chemiluminescence (CL). The CL generated by a photochemical reaction is called photo-induced chemiluminescence (PCL), therefore, two processes are involved in a PCL.

Compared to the convenient CL, PCL has some advantages: (1) the potochemical reaction is dependent on the wavelength of the induced light, which is favourable to improve the selectivity of the PCL; (2) without introducing any impurity into the system the system will not be diluted, which avoids the problems caused by addition of some chemicals; and (3) many



Scheme 1.

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photochemical reactions occur based on the free radical and have high reaction rate, which is favourable to perform the online analysis.

It is well known that two types of mechanism are involved in a photosensitive reaction, but the most common way to generate the $^1O_2$ is 'Type II' photoreaction occurring between oxygen and photosensitizer. The excited photosensitizer species (triplet state) is formed under a specific light radiation. These species transfer the absorbed energy to ground triplet molecular oxygen ($^3O_2$) to generate $^1O_2$. However, it is very difficult to identify and observe the short-lived $^1O_2$ directly in biological systems. CL has become a useful method for detection of free radical and the reactive oxygen species (ROS), such as $^1O_2$ and O_2^-, can be detected by using some special CL probe. At present, the usually used CL probes for detection of ROS is luminol, lucigenin and cypridina luciferin analogue (CLA) [1–3].

Zinc(II) phthalocyanine compounds have high quantum yields to produce singlet oxygen, which lead to their usage as photosensitizers in photodynamic therapy or washing powders as photobleaches [4,5]. However, unsubstituted phthalocyanines are known to have significant π–π interaction between neighbouring molecules so that they have strong tendency to form aggregates, which causes their low solubility in common solvents, thus greatly limiting their further applications. In this paper, we report the synthesis of zinc(II) phthalocyanine derivatives substituted by 2,2,4-tirmethyl-3-pentoxy. This substituted phthalocyanine shows the higher solubility in common organic solvent than that of unsubstituted phthalocyanines. It was found that photoexcited Zn(II) phthalocyanine complex produced singlet oxygen ($^1O_2$) in air-saturated solutions. Subsequently, the $^1O_2$ would react with dimethylformamide (DMF) to form corresponding DMF radicals (carbon centred radicals: $\cdot CH_3$ and $\cdot CH_2N(CH_3)$ CHO or corresponding alkylperoxyl radicals. These carbon centred radicals further reacted with lucigenin to initiate the CL. The CL intensity is related to the amount of generated radical or $^1O_2$ after irradiation.

Another aim of this work is to study the antioxidant ability of the flavonic derivative, which has been tested by the PCL method. Flavonoids are a group of naturally occurring polyphenolic compounds found in fruits and vegetables [6,7]. Several papers [8–10] have indicated that these compounds have the property of inhibiting auto-oxidation reactions and scavenging of free radicals. However, the relationship between their structure and activity remains unclear. Therefore, the relationship between the structure of flavonic derivatives and their antioxidant and antiradical activity is investigated in detail.

Experimental

Chemicals

Lucigenin (N,N′-dimethyl-9,9′-diacridinium nitrate) was obtained from Sigma Chemical Company (St. Louis, MO) and used without further purification. Superoxide dismutase (SOD) and flavonoids, sodium azide (NaN_3) were obtained from Shanghai Chemical Reagent Co. (Shanghai, China). All reagents in the present experiments were of analytical grade. Double-distilled water was used throughout.

Apparatus

In this work, the measure of PCL was carried out on a BPCL ultra-weak chemiluminescence analyser controlled by a personal computer with BPCL program (Institute of Biophysics, Chinese Academy of Sciences). Reactions were initiated under the radiation of light when the infrared heat lamp, which could be filtered out with a glass filter, emitted much infrared radiation. The intensity of light is measured with a digital light meter (TES Electrical Electronic Corp.). The intensity of the infrared heat lamp was constant during the exposure, the light level was $17\ 000.0 \pm 15.1$ Lux (Lux $= 1$ lumen per square metre).

All experiments were conducted in the room temperature range of 20–23°C. Solution heating during the experiment was found to be relatively small, typically ca. 0.5°C during irradiation.

The UV-vis spectra of $ZnPc(OR)_4$ was recorded on a Lambda 800 Spectrometer (PE) and the emission spectra of the infrared heat lamp was recorded on a 970CRT fluorescence spectrometer (Shanghai, China).

Synthesis of [tetra-α-(2,2,4-tirmethyl-3-pentoxy)] Phthalocyanine Zinc

The metallophthalocyanine, [tetra-α-(2,2,4-tirmethyl-3-pentoxy)] Phthalocyanine Zinc ($ZnPc(OR)_4$), was synthesized and purified in the Institute of Functional Materials in Fuzhou University [11]. These substituted phthalocyanines exhibit high solubility in common organic solvents.

Under protection of N_2, a mixture of 3-(2,2,4-tirmethyl-3-pentoxy) phthalonitrile, anhydrous $ZnCl_2$ and 1,8-diazabicyclo[5.4.0] undec-7-ene (DBU) in pentanol was stirred and heated at 160°C for 6 h. Then the volatiles were evaporated under vacuum. The residue was extracted by toluene and followed by chromatography on a silicon gel column with toluene as eluent. Finally, the thick green eluate was evaporated to give product solid. Yield: 46%. The compound has been characterized by mass spectra and electronic spectrum, fully consistent

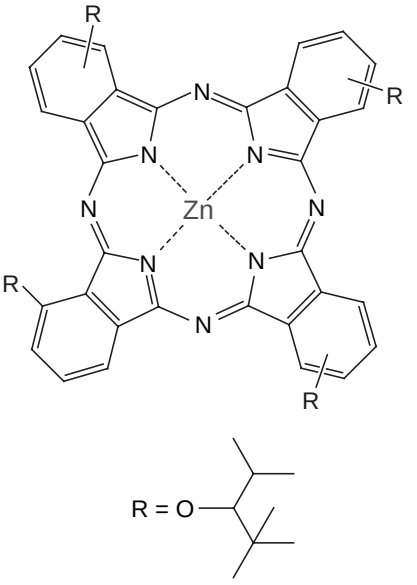


Figure 1. The molecular structure of [tetra-α-(2,2,4-tirmethyl-3-pentoxy)] Phthalocyanine Zinc.

with the assigned structures. λ_{max} (in DMF): 710 nm. MS (ESI, for $[M+H]^+$: 1090.9). The molecular structure of $ZnPc(OR)_4$ is shown in Figure 1.

Results and discussion

Quantification methods

In the presence of antioxidants, the PCL of lucigenin was inhibited. Figure 2 shows the light emission kinetics of the phthalocyanine-lucigenin system with addition of flavonoids. As a parameter of the radical scavenging activity for a compound, the suppression area was obtained by subtraction of the emission area obtained in the presence of anti-radicals from that

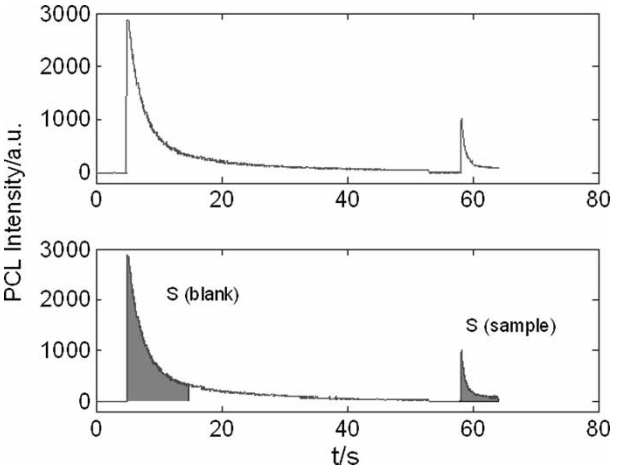


Figure 2. Light emission kinetics of the phthalocyanine-lucigenin system with addition of flavonoids. Chemiluminescence was integrated over the initial 10 s of each reaction. S_{blank}: Dashed integrated area corresponds to the phthalocyanine-lucigenin system. S_{sample}: Dashed integrated area corresponds to the phthalocyanine-lucigenin system with addition of flavonoids.

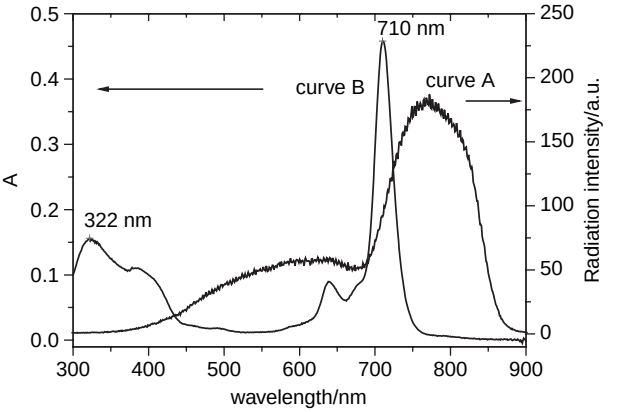


Figure 3. The spectral distribution of the infrared heat lamp (A) and the absorption spectra of the [tetra-α-(2,2,4-tirmethyl-3-pentyloxy) Phthalocyanine Zinc (B).

obtained in the absence of anti-radicals. The anti-radical activity is defined as follow,

$$\text{Antiradical activity } (\%) = S_{supp}/S_{blank} \times 100\% = (S_{blank} - S_{sample})/S_{blank} \times 100\% \quad (1)$$

Emission characteristics and the kinetics of PCL

It was found that phthalocyanine exhibited enough efficiency to initiate the luminescence of lucigenin by the red-light (see Figure 2, curve A) and the wavelength of this red-light was in the range of the Q-Band absorption of phthalocyanine (see Figure 3, curve B). One of the most important characteristics of the CL reaction is its kinetic profile. Figure 2 shows the typical CL intensity vs time profile. As can be seen from this figure, the CL signal decayed slowly and the decreasing curve can be described by exponential law with τ_e about 5 s.

Another important characteristic of the CL reaction is its emission spectrums. Figure 4 shows the main emission characteristic of lucigenin, which was obtained by eight pieces of filters. The result shows

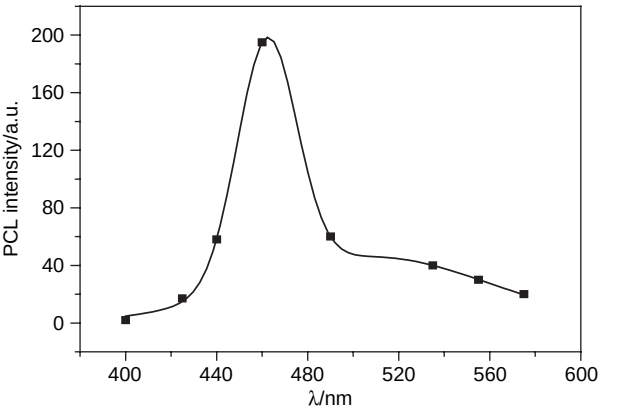


Figure 4. Chemiluminescent spectrum of the $ZnPc(OR)_4$-lucigenin system. [Lucigenin] = 3.5 nmol/L, $[ZnPc(OR)_4]$ = 10 μmol/L.

that the maximum emission wavelength was 460 nm. We found that PCL and the direct CL by H_2O_2 in alkaline solution were well matched. The emitter of lucigenin in DMF was still N-methylacridone (NMA), the oxidized product of lucigenin.

Spectroscopic properties of phthalocyanines and generation of singlet oxygen

For practical applications of phthalocyanines, it is important to study the photosensitizing effectiveness of phthalocyanines whether they are highly aggregated or not. It was known that the aggregations of molecules would take up the electronic energy and convert it into vibrational motion. The production of singlet oxygen is reduced drastically in general when aggregations are formed, owing to the rapid decay of aggregators' singlet excited states by internal conversion to the ground state. The aggregation effect depends on the substituents and the solvents. The introduction of either long chains or bulky substituents to the periphery of the macrocycle should prevent the aggregation. In this work we used the $ZnPc(OR)_4$ for the photosensitized oxidation of lucigenin. It can be found that, in DMF solution of $ZnPc(OR)_4$, the clear and sharp monomer band at ~710 nm is observed and no band corresponding to aggregator is observed (see Figure 3, curve B).

Discussion on the reaction pathway of photosensitizer

It was found that the PCL was enhanced in the presence of O_2 and reduced with the aeration of Argon. So we investigated the production of active oxygen radicals. It is known that the photosensitizer maybe react with a substrate in two different pathways, namely pathway Type I and Type II [12]. The pathway involving the electron transfer, in which a triplet state photosensitiser reacts firstly with a substrate or molecular oxygen to yield superoxide anion radical (such as $O_2^{-\cdot}$), is termed Type I. While, in the Type II pathway, the triplet state of photosensitiser reacts firstly with molecular oxygen to yield $^1O_2$.

In our experiment, NaN_3 (0.05 mol/L), which was considered as a chemical scavenger for $^1O_2$, was added to the solutions containing lucigenin and phthalocyanine, which resulted in strong inhibition of the PCL (see Figure 5). On the other hand, we found that the PCL of lucigenin was almost not influenced by SOD, which indicates that O_2^- is not a reactant on the pathway to lead light emission. These results suggested that phthalocyanine mostly elicited the formation of $^1O_2$, rather than the superoxide radical anions when it was exposed to radiation. Hence, it can be concluded that the Type II mechanism is the dominating route under these conditions.

However, lucigenin is not specific for $^1O_2$. In fact, lucigenin-based CL is considered to be selectively sensitive to the superoxide anion and is not derived

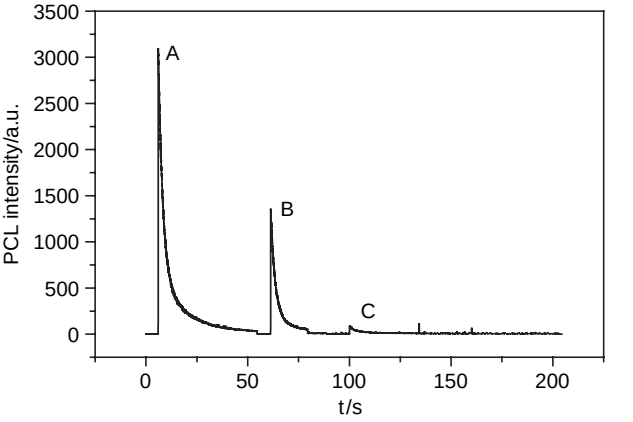


Figure 5. Effect of NaN_3 on PCL. (A) [lucigenin] = 3.5 nmol/L; (B) [lucigenin] = 3.5 nmol/L, $[NaN_3] = 10^{-6}$ mol/L; (C) [lucigenin] = 3.5 nmol/L, $[NaN_3] = 5.0 \times 10^{-6}$ mol/L.

from other reactive oxygen species, such as H_2O_2, singlet oxygen or hydroxyl radicals [13–15], which are all generated during oxidation processes. We proposed an additional new CL mechanism, which is different from the CL mechanism based on the $^1O_2$ and/or O_2^-. In DMF solution, the oxidized species may be related to a product of DMF radicals (carbon centred radicals: $\cdot CH_3$ and $\cdot CH_2N(CH_3)CHO$) [16,17], which are responsible for the chemical reactions in solution. These carbon centred radicals react with oxygen in solutions to form corresponding peroxidation alkylperoxyl radicals ($ROO^{\bullet}$-species). The mechanism of this PCL system can be shown as in Scheme 2.

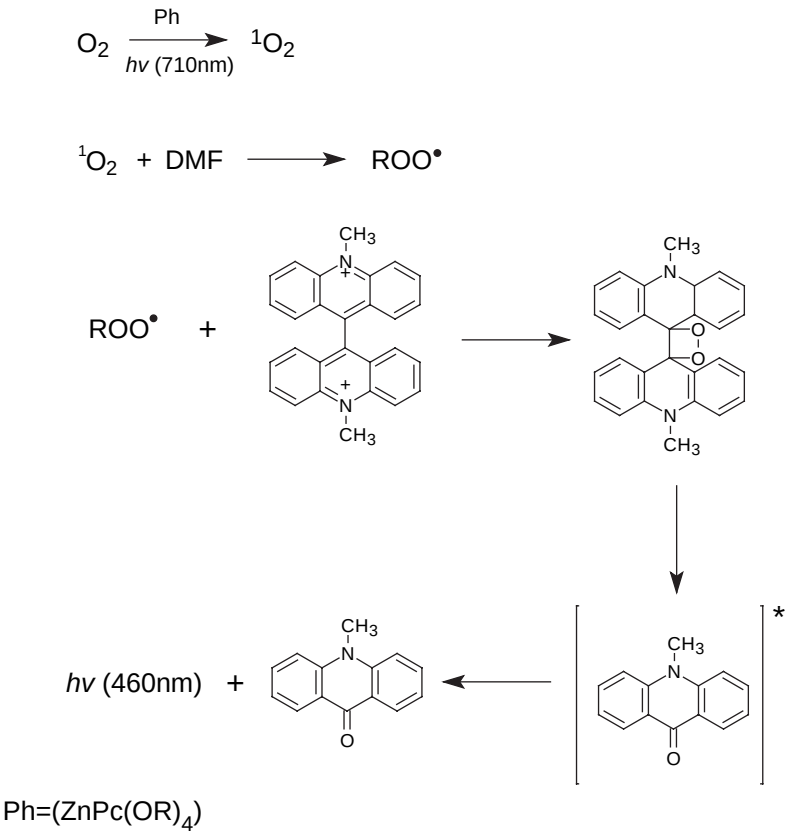
Optimization of the CL reaction conditions

As the speed of the CL reaction was very fast, in the subsequent study the solution (air saturated) was excited with the infrared heat lamp for 10 s and then transferred into the analyser immediately.

Several solvents, such as DMF, acetonitrile, acetone, methanol and formamide, have been tried as the media for this CL reaction and the results show that DMF gave the highest CL intensity and, hence, was chosen as the optimum reaction media. The enhancement of CL in the DMF is probably due to the more efficient reaction of DMF with $^1O_2$ radicals to form corresponding DMF radicals (carbon centred radicals: $\cdot CH_3$ and $\cdot CH_2N(CH_3)CHO$) or corresponding alkylperoxyl radicals, which initiates the reaction with lucigenin.

The effect of phthalocyanine concentration on the CL intensity is illustrated in Figure 6. It can be seen from Figure 6 that the CL intensity is increased with increasing of phthalocyanine concentration up to 1×10^{-5} mol/L and then reaches maximum and constant. Therefore, 1×10^{-5} mol/L phthalocyanine was used in subsequent experiments.

Under the optimum conditions, the calibration curve was obtained for lucigenin by using a series of



standard solutions. The CL intensity was found to be linear with the concentration of lucigenin in the range of 0.1 ~ 10 nmol/L. The detection limit (defined by IUPAC, $C_{LOD} = 3\ S_b/m$, where S_b is the standard deviation of blank and m is the slope of the calibration graph) is found to be 0.073 nmol/L. The relative standard deviation (RSD) for 5 nmol/L lucigenin was 4.9% based on five replicated measurements.

Detection of anti-radical activity of flavonoids

Flavonoids have anti-tumour, anti-viral and antibacterial activities and their anti-radical and antioxidative properties have been studied extensively. Natural flavonoids are very effective in reducing CL. We roughly considered that the inhibition could be likely related to the protective effect of flavonoids: scavenging of radical. PCL intensity was decreased in the presence of these radical scavengers and the reductions were linearly proportional to the concentration of scavengers. Using the above optimal conditions for this CL measurement system, the intra-assay coefficients of variation of the same 12 samples measured at one time were no more than 7%. Therefore, quantification of the ROO-scavenging activity of each flavonoid would allow assessment of the total antioxidant potency. The present study indicated that the radical scavenging potentials of the flavonoids differed greatly from one to another.

Figure 7 and Table 1 summarize the radical-scavenging activity of various flavonoids and it is found that most of these compounds show remarkable radical-scavenging activity. The anti-radical activity within a wide range was calculated as a percentage of CL suppressing rate. From Figure 7 and Table 1, we also know that the anti-radical ability of flavonoids is dependent on molecular structure. The number of free hydroxyl groups of the flavonoid molecule, the presence of an ortho-hydroxylation on the B-ring and a C_2-C_3 double bond in the C-ring are possibly the key effect factors on the antioxidant and anti-radical activities.

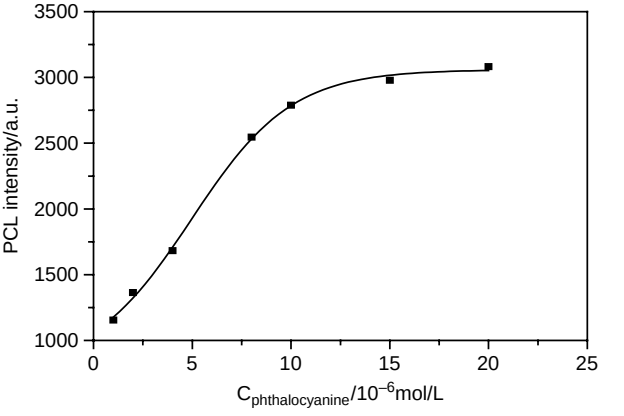


Figure 6. Effect of $ZnPc(OR)_4$ concentration on PCL. [Lucigenin] = 3.5 nmol/L, T_p = 10 s.

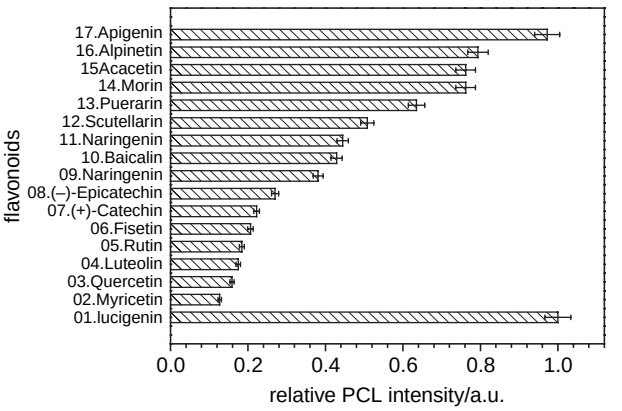


Figure 7. Photoinduced chemiluminescence of the phthalocyanine-lucigenin system in the absence and presence of flavonoids as a quenching agent. Roo-scavenging activity is expressed as the means ± SD of three determinations. All bar graphs depict averages and the associated error bars represent standard deviations. [Lucigenin] = 3.5 nmol/L, [$ZnPc(OR)_4$] = 10 μmol/L, [Flavoids] = 5.0 μmol/L, Tp = 10 s.

Importance of the phenolic hydroxyl on the B-ring

Table 1 shows that Myricetin, Quercetin, Luteolin, Rutin, Fisetin, (+)-Catechin and (−)-Epicatechin all demonstrate their obvious inhibition ability for this PCL. Taking Myricetin, Quercetin and Morin for example (the molecular structures are shown in Figure 8), these three compounds have the same molecular structure on A and C-ring, while Myricetin demonstrates the strongest radical-scavenging activity due to the presence of three phenolic hydroxyl groups on its B-ring. Both Quercetin and Morin have two phenolic hydroxyl groups on their B-ring; however, the radical-scavenging activity of Quercetin is much higher than that of Morin, this is because the former has the ortho-dihydroxyl groups on its B-ring. Therefore, it can be concluded that the number and position of phenolic hydroxyl groups on the B-ring of flavonoids plays a key role in a radical scavenging activity.

The high potential of phenolic compounds to scavenge radical may be explained by their ability of donating a hydrogen atom from their phenolic hydroxyl group as described in Equation (2).

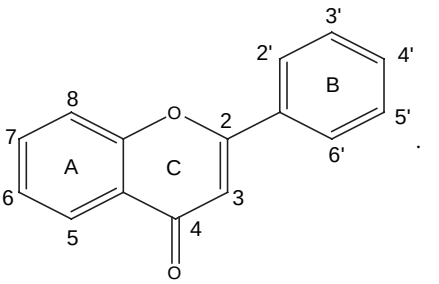
$$ROO^{\cdot} + ArOH \rightarrow ROOH + ArO^{\cdot} \quad (2)$$

This kind of reaction gives the phenoxyl radical ($ArO^{\bullet}$), which subsequently undergoes a change to a resonance structure by redistributing unpaired electron on the aromatic ring. Thus, $ArO^{\bullet}$ exhibit much lower reactivity as compared with $ROO^{\bullet}$. Quercetin has the ortho-dihydroxyl groups on its B-ring, so its anti-radical activity is higher than that of Morin.

Effects of A-ring and B-ring phenolic hydroxyl

It can be known from Table 1 that the ability of Baicalin and Scutellarin for scavenging the $^1O_2$ is much lower than that of the seven above-mentioned compounds, which indicates that the radical-scaven-

Table 1. Comparison of the different flavonoids' radical-scavenging capacity.



No	Compound	Substituent	Ortho-OH	Suppressing rate (%)
1	Myricetin	3,5,7,3′,4′,5′-hexahydroxy	B-ring	87.2
2	Quercetin	3,5,7,3′,4′-pentahydroxy	B-ring	83.9
3	Luteolin	5,7,3′,4′-tetrahydroxy	B-ring	82.3
4	Rutin	5,7,3′,4′-tetrahydroxy	B-ring	81.3
5	Fisetin	3,7,3′,4′-tetrahydroxy	B-ring	79.1
6	(+)-Catechin	3,5,7,3′,4′-pentahydroxy	B-ring	77.5
7	(−)-Epicatechin	3,5,7,3′,4′-pentahydroxy	B-ring	72.8
8	Naringenin	5,7,4′-trihydroxy	/	61.7
9	Baicalin	5,6-dihydroxy	A-ring	56.9
10	Naringin	5,4′-dihydroxy	/	55.1
11	Scutellarin	5,6,4′-trihydroxy	A-ring	49.0
12	Puerarin	7,4′-dihydroxy	/	36.2
13	Morin	3,5,7,2′,4′-pentahydroxy	/	23.5
14	Acacetin	5,7-dihydroxy, 4′-OCH_3	/	23.4
15	Alpinetin	7-OH, 5-OCH_3	/	20.2
16	Apigenin	5,7,4′ -trihydroxy	/	6.20

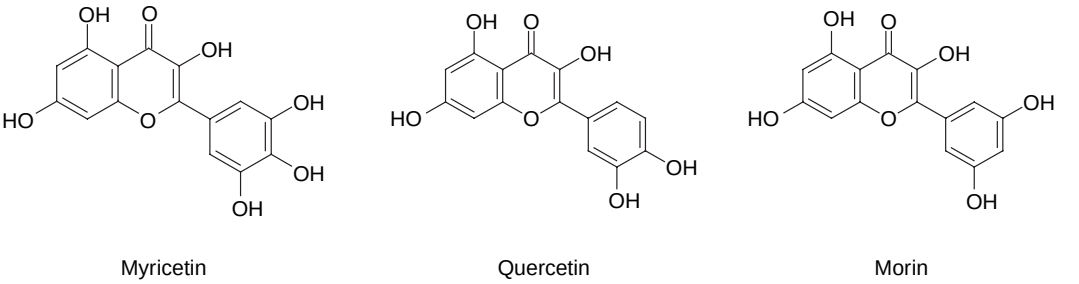


Figure 8. Molecular structure of Myricetin, Quercetin and Morin.

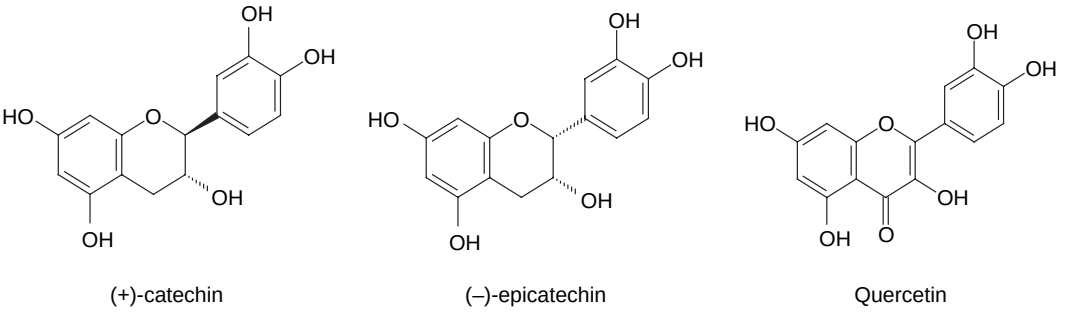


Figure 9. Molecular structure of (+)-Catechin, (−)-Epicatechin and Quercetin.

ging activity of the ortho-dihydroxyl groups on A-ring is much lower than that of ortho-dihydroxyl groups on B-ring. The flavonoid compounds, such as Alpinetin, Acacetin and Puerarin, with only one or without any hydroxyl group on B-ring usually exhibit weaker activity for scavenging $^1O_2$.

The reason for the phenolic hydroxyl on B-ring having higher anti-radical activity than that of phenolic hydroxyl on A-ring may be explained as the A-ring is conjugated well with the C-ring, while the property of the C-ring makes the O–H bond energy of the hydroxyl group on A-ring stronger, which is unfavourable for dissociation of hydrogen, so the radical-scavenging activity of hydroxyl group on A-ring is decreased.

Effect of stereoscopic structure

The stereoscopic structure between the C-3 group and the B-ring of flavonoids (see Figure 9) makes a contribution to radical scavenging activity. For instance, quercetin showed higher radical-scavenging activity than that of (+)-catechin and (−)-epicatechin.

Effect of the double bond between C_2 and C_3

Regarding the effect of the double bond between C_2 and C_3, some people thought that the conjugation between the A-ring and B-ring would be enhanced due to the presence of the double bond between C_2 and C_3, which would stabilize the phenoxy radical. On the other hand, the double bond between C_2 and C_3 was an electron withdrawn group, which would decrease the activity of the hydrogen of the phenolic hydroxyl and further decrease the radical-scavenging activity. We compared the PCL suppressing rate of Naringenin and Apigenin (see Figure 10) and found that the ability of the former to scavenge radicals was much stronger than that of the latter. Therefore, we can conclude that flavonoids with a singlet bond at C_2–C_3 have high anti-radical activity.

Conclusions

Based on the lucigenin mediated CL, the objectives of the present study were: (1) to develop an alternative, routinely applicable method for the measurement of the trace $^1O_2$ or free radicals generated by phthalocyanines; and(2) to research structure activity relations for the scavenging free radical potentials of different types of flavonoids.

Compared with electron spin resonance (ESR), the CL method needs a smaller quantity of test compound and is of higher sensitivity, more simple and less expensive. However, it is difficult to precisely quantify and detect free radicals directly. Further

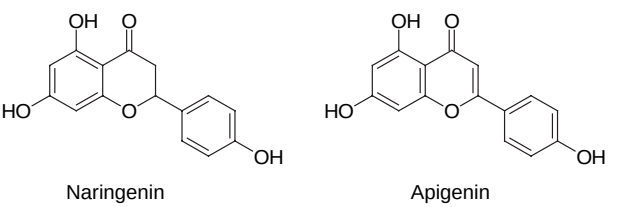


Figure 10. Molecular structure of Naringenin and Apigenin.

studies concerning the mechanism are presently in progress in our laboratory.

Acknowledgements

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