

Full Length Research Paper

Determination of scavenging effect of Chinese medicinal herbs on hydroxyl radical using a new chemiluminescence system

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A novel flow injection chemiluminescence (FI-CL) method was developed for determination of scavenging effect of Chinese medicinal herbs on hydroxyl radical ($\cdot\text{OH}$). It was shown that a strong chemiluminescence (CL) signal was observed when methylene blue (MB) was mixed with Fenton reagent in an acidic medium. The CL intensity was decreased significantly when the extract of Chinese medicinal herb was added to the reaction system and partially scavenged the hydroxyl radicals in the solution. The extent of decrease in the CL intensity had a good stoichiometrical relationship with herb concentration. Based on this, we developed a new method for the determination of scavenging effect of Chinese medicinal herbs on hydroxyl radical using a flow injection chemiluminescence (FI-CL) technique. The proposed method was successfully applied to the evaluations of hydroxyl radical scavenging capacity of 18 kinds of Chinese medicinal herbs. The results showed that *Rhus chinensis* Mill has the highest antioxidant activity.

Key words: Flow injection, chemiluminescence, hydroxyl radical, Chinese medicinal herbs.

INTRODUCTION

Hydroxyl radical ($\cdot\text{OH}$) is by far the most damaging free radical; its action can have devastating effects within the body. Ageing, cancer, radiation damage and phagocytosis are associated with it (Cheng et al., 2002; Knight, 1995). Therefore, the investigation of natural antioxidants and their capacity of scavenging hydroxyl radicals have always attracted attentions. Many Chinese medicinal herbs contain chemical active composition such as polyphenols, flavonoids, saponins and polysaccharides (Chen et al., 2006), therefore, they are the potential resource of natural antioxidants which have many significant advantages over the synthetic antioxidants. It had been found that the effectiveness of Chinese herbal medicines is closely associated with their antioxidant capacity (Pan et al., 2004; Yang et al., 2006). In fact, some anti-free radical active substances have been screened out from the Chinese medicinal herbs (Cai et al., 2004). Thus, it is important for medicine and

food industries to develop a simple and sensitive method for quantitative evaluation of hydroxyl radical scavenging ability of plants. Several analytical methods such as electron spin trap (Espinoza et al., 2009), high-performance liquid chromatography (HPLC) (Pezo et al., 2008), capillary electrophoresis (CE) (Kati et al., 2009), ultraviolet spectrophotometry (UV) (Chen et al., 2008), spectrophotometry (Yang et al., 2006) and fluorescence (Qu et al., 2004) have been utilized for this purpose. However, both HPLC, CE and fluorescence methods involve laborious processes or time-consuming procedures; while the UV and spectrophotometry methods suffer from lack of high sensitivity.

The chemiluminescence (CL) method was widely employed in the analysis of pharmaceutical compounds due to its simplicity, ease of manipulation, and high sensitivity (Kricka, 2003); it therefore could be an effective tool in determination of the scavenging effect of Chinese medicinal herbs on hydroxyl radical.

Methylene blue, a fluorescent dye usually adopted in spectroscopic analysis (Sheng et al., 2008; Zhang et al., 2005) was chosen as the chemiluminescent reagent in

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our new CL system. Fenton reaction is a typical reaction of generating hydroxyl radical and often used to evaluate the hydroxyl radical-scavenging capacity of plant active constituents (Julio et al., 2009; Ivan et al., 2009).

It was observed that a strong CL emission signal occurred when methylene blue reacted with the hydroxyl radicals generated from Fenton reagent in an acidic medium. The water extract of Chinese medicinal herb was found to be able to inhibit the CL intensity by partially scavenging the hydroxyl radicals in the solution. The extent of CL intensity reduction exhibited a good stoichiometric relationship with the herbs concentration. Based on this, we established a new flow injection chemiluminescence (FI-CL) method for the evaluation of hydroxyl radical-scavenging capacity of Chinese medicinal herbs.

EXPERIMENTAL

Reagents and solutions

All the chemicals used were of analytical grade and deionized water was used throughout the experiments. A stock solution of 2.0×10^{-2} mol/L methylene blue (Shenyang Reagent Plant) was prepared by dissolving 0.7478 g methylene blue in 5 ml of deionized water and diluting to 100 ml. Hydrogen peroxide (Guang Zhou Xinjian Fine Chemical Plant) was stored in a fridge at 4°C, and diluted to a suitable concentration just before usage. A stock solution of 1.5×10^{-3} mol/L ferrous iron (Fe^{2+}) was prepared daily by dissolving 0.1471 g ferrous ammonium sulfate 6-Hydrate (Guangdong Shantou Xilong Chemical Co., Ltd.) in 50 ml of sulfuric acid solution (0.1 mol/L) and diluting to 250 ml with deionized water. The sulfuric acid stock solution (0.1 mol/L) was prepared by diluting 2.72 ml of 98% concentrated sulfuric acid (Shanghai Shiyi Chemicals Reagent Co., Ltd.) to 500 ml with deionized water.

Sample preparation

18 commonly used Chinese medicinal herbs, with major active components varying from phenolic acids to fatty acids, were carefully selected and purchased from local traditional Chinese medicine shop for this study. The scientific names, family, used parts, and representative components of the herbs are listed in Table 1.

Considering that Chinese medicinal herbs are traditionally boiled in water to produce a soup for patients to drink, therefore herb samples were prepared using boiling water extraction method. 1.0 g smashed plant was soaked with 80 ml of deionized water in a conical flask for 20 min and then extracted at 100°C for 40 min, the extract was filtered and the residue was washed with 5 ml hot water twice; the washings and the filtrate were mixed together and diluted to 100 ml with deionized water.

Apparatus

The CL measurements were performed using a FI-CL analyzer (IFFL-E, Xi'an Remex Analysis Instrument Co., Ltd. China). This analyzer consisted of two peristaltic pumps and a six-way injection valve equipped with a 75 μl sample loop (Figure 1). A PTFE tubing (0.8 mm i.d.) was used to connect all components in the flow system. The flow-cell was a coil of glass tubing (1 mm i.d., total length 100 mm), positioned in front of the detection window of a

sensitive photomultiplier tube (PMT) which was operated at a negative high pressure of 750 V. The CL signal collected by the PMT was recorded with a computer employing CL analysis software.

All ultrasonic oscillations of reagents were performed using an ultrasonic cleaner (KQ-2200E, Kunshan Ultrasonic Instrument Co., Ltd.).

Procedure

As shown in the schematic diagram of experimental manifold (Figure 1), the solutions of Fe^{2+} and sample (or deionized water) were mixed by a 3-way mixing valve and propelled by the peristaltic pump (P_1) into the flow-cell as carrier stream at the flow rate of 4.7 ml/min; H_2O_2 and methylene blue solutions were merged together by another 3-way mixing valve and propelled by the peristaltic pump (P_2) at the flow rate of 7.0 ml/min through a 10 cm tubing into a six-way valve which was then injected 75 μl of the mixed solution into the carrier stream. The light output from the flow-cell was detected by a photomultiplier tube (PMT).

The scavenging rate of a Chinese medicinal herb was calculated based on the decrement of the CL emission intensity (ΔI) according to the following equation:

$$\text{Scavenging rate (s)} = [(I_0 - I_s) / I_0] \times 100\% \quad (1)$$

Here, I_0 and I_s are the CL emission intensities in the absence and presence of Chinese medicinal herb, respectively.

RESULTS AND DISCUSSION

Kinetic curve of CL reaction

The kinetic characteristic curve of the reactive system is shown in Figure 2. The CL signal generated from the mixed solution of methylene blue and Fenton reagent in an acidic medium reached its maximum intensity at 3.5 s and then extinguished immediately within 3 s thereafter (Figure 2a), indicating that the luminescence reaction was a rapid reaction. In the presence of *Rubia cordifolia* L. extract, a relatively weaker CL signal with the same CL emission time and similar peak shape was obtained (Figure 2b).

Selection of the experimental manifold and apparatus parameter

Various types of the flow injection (FI) manifolds were investigated; the results showed that the maximal CL emission signal was obtained when using the manifold depicted in Figure 1; therefore, this manifold was employed in this study.

The flow rate is important to FI-CL detection. If the flow rate is too high or too low, a suitable CL emission signal can not be obtained. The effects of flow rate on the CL intensity were examined in the range of 3.5 to 7.5 ml/min. It was shown that the CL intensity was kept at a constant with the increase in the flow rate of the carrier stream delivered by P_1 , while it was significantly enhanced with

Table 1. Chinese medicinal herbs used in this study.

Plant	Chinese name	Scientific name	Family	Part used	Major representative components
1	Wu Bei Zi	<i>Rhus chinensis</i> Mill.	Anacardiaceae	Gall	Gallotannin, gallic acid
2	He Zi	<i>Terminalia chebula</i> Retz.	Combretaceae	Fruit	Ellagitannins, gallic acid
3	Lian Qiao	<i>Forsythia suspense</i> (Thunb.) Vahl.	Oleaceae	Fruit	Forsythigenol, forsythin, rutin
4	Jin Yin Hua	<i>Lonicera japonica</i> Thunb.	Caprifoliaceae	Floral bud	Chlorgenic acid, luteolin, luteolin-7-glucoside
5	Yue Ji Hua	<i>Rosa chinensis</i> Jacq.	Rosaceae	Flower	Quercetin, catechin, anthocyanins, gallic acid, tannins
6	Sheng Di	<i>Rehmannia glutinosa</i> Libosch.	Scrophulariaceae	Root	Ferulic acid, caffeic acid
7	Gou Qi Zi	<i>Lycium barbarum</i> L.	Solanaceae	Fruit	Coumarins (scopoletin)
8	Huang Qin	<i>Scutellaria baicalensis</i> Georgi	Labiatae	Root	Baicalein, baicalin, chrysin, wogonin
9	Dang Gui	<i>Angelica sinensis</i> (Oliv.) Diels	Umbelliferae	Root	Vanillin, p-cresol, ferulic acid
10	Ye Ju Hua	<i>Chrysanthemum indicum</i> L.	Asteraceae	Inflorescence	Acaciin, luteolin, flavone glycosides, coumarins
11	Gan Cao	<i>Glycyrrhiza uralensis</i> Fisch.	Leguminosae	Root	Glycyrrhizin, liquiritin, liquirigenin
12	Yin Xing Ye	<i>Ginkgo biloba</i> L.	Ginkgoaceae	Leaves	Keampferol, luteolin, myricetin, quercetin, isorhamnetin, syringetin
13	Huang Qi	<i>Astragalus mongholicus</i> Bge.	Leguminosae	Root	Astragalinal, calycosin, formononetin
14	He Shou Wu	<i>Polygonum multiflorum</i> Thunb.	Polygalaceae	Root	Chrysophanol, emodin, rhein, physcion, tannins, resveratrol
15	Wu Wei Zi	<i>Schisandra chinensis</i> (Turcz.) Baill.	Magnoliaceae	Fruit	Schizandrins, schizatherins, wulignan
16	Qian Cao	<i>Rubia cordifolia</i> L.	Rubiaceae	Root	Purpurin, alizarin, munjistin
17	Jin Qian Cao	<i>Lysima chiachristinae</i> Hance	Primulaceae	Whole plant	Isorhamnetin, quercetin, kaempferol
18	Huo Ma Ren	<i>Cannabis sativa</i> L.	Moraceae	Seeds	Oleic acid, linoleic acid, linolenic acid

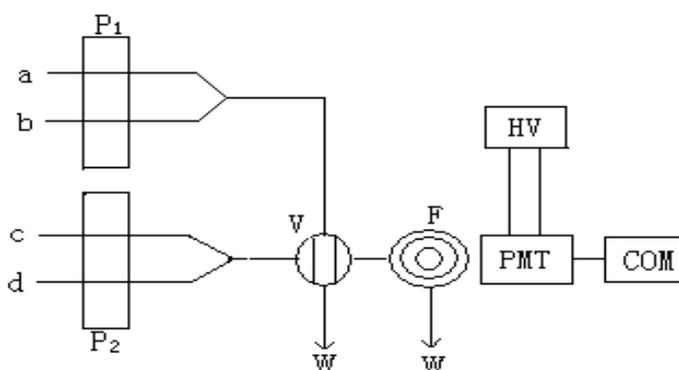


Figure 1. Schematic diagram of the flow injection chemiluminescence analysis system. a, Fe^{2+} and H_2SO_4 solution; b, sample solution or H_2O ; c, H_2O_2 solution; d, methylene blue; P_1 , P_2 , peristaltic pump; V, 6 way injection valve; F, flow cell; W, waste solution; PMT, photomultiplier tube; HV, negative high voltage; COM, computer.

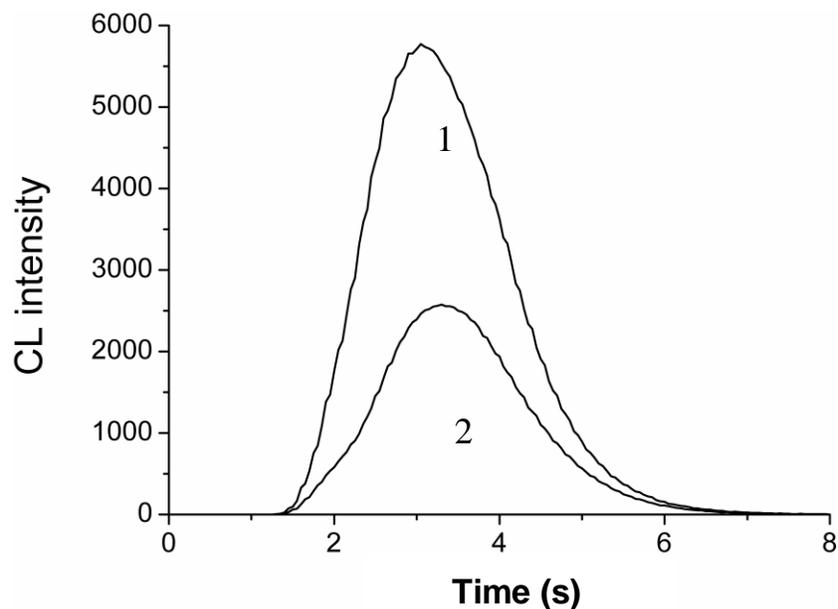


Figure 2. Kinetic curve of chemiluminescence 1. $1.5 \times 10^{-3} \text{ mol}\cdot\text{L}^{-1} \text{ Fe}^{2+}$ + 0.5% H_2O_2 + $3.2 \times 10^{-5} \text{ mol}\cdot\text{L}^{-1}$ methylene blue; 2. 1 + *R. cordifolia* L. extract.

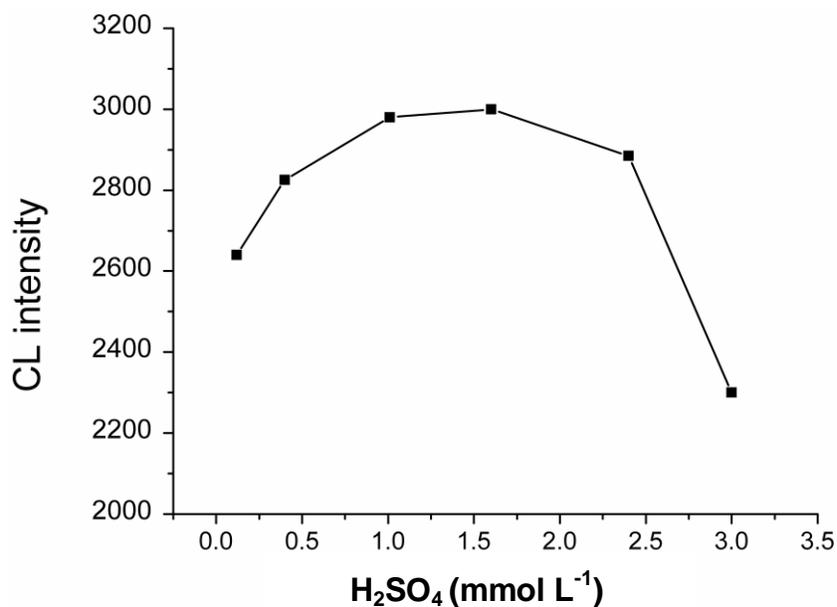


Figure 3. The effect of H_2SO_4 concentration on CL intensity.

the increase in the flow rate of H_2O_2 and methylene blue mixed solution propelled by P_2 . As a result of a compromise between reagent consumption and CL intensity, a flow rate of 4.7 ml/min for the carrier stream and a flow rate of 7.0 ml/min for H_2O_2 and methylene blue mixed solution were adopted respectively in our experiments.

Effect of sulfuric acid concentration

The effect of the concentration of sulfuric acid used for preparing Fe^{2+} solution on the CL intensity was investigated in the range of 1.0×10^{-4} to $5.0 \times 10^{-3} \text{ mol/L}$. The results (Figure 3) showed that the CL intensity increased with the increase of sulfuric acid in the solution,

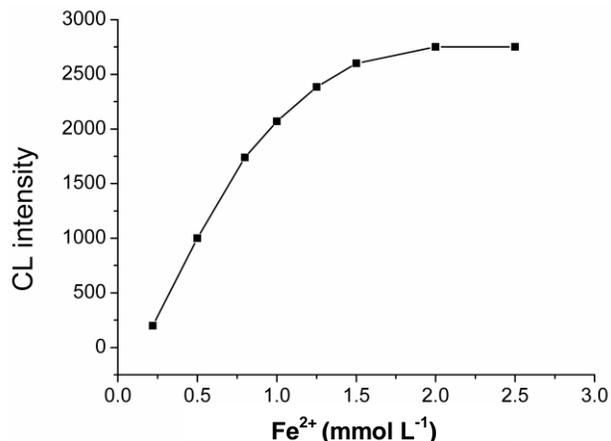


Figure 4. The effect of Fe²⁺ concentration on CL intensity.

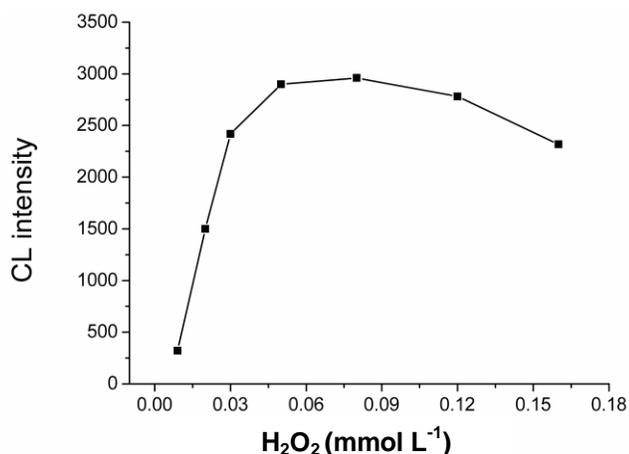


Figure 5. The effect of H₂O₂ concentration on CL intensity.

and reached a maximal point at 1.0×10^{-3} mol/L. Thus, 1.0×10^{-3} mol/L sulfuric acid was used in the CL reaction system.

Effect of Fe²⁺ concentration

Ferrous iron (Fe²⁺), which functions as the catalyst in the Fenton reagent, is closely associated with the yield of hydroxyl radicals. The influence of Fe²⁺ concentration on the CL intensity was studied in the range of 2.0×10^{-4} to 2.5×10^{-3} mol/L. The result (Figure 4) showed that the CL intensity reached its maximal point when the concentration of Fe²⁺ was 1.5×10^{-3} mol/L. Thus, 1.5×10^{-3} mol/L Fe²⁺ was adopted in our CL system.

Effect of hydrogen peroxide concentration

Hydrogen peroxide is the main source of hydroxyl radical;

its concentration could exert a comparative strong impact on the CL intensity. The effect of H₂O₂ concentration was investigated in the range of 0.02 to 0.08 mol/L. The results (Figure 5) demonstrated that the CL intensity increased sharply with increasing concentration of H₂O₂ and then decreased when the H₂O₂ concentration reached 0.05 mol/L. The probable reason responsible for the decrease in CL intensity was that the excessive hydrogen peroxide reacted directly with hydroxyl radicals resulting in the reduction of hydroxyl radical concentration in the solution, and indirectly inhibited the reaction of hydroxyl radical with methylene blue (Shen et al., 2007). In this study, the H₂O₂ concentration of 0.05 mol/L was used for the CL reaction.

Effect of methylene blue concentration

The effect of methylene blue concentration was investigated in the range of 8.0×10^{-6} to 8.0×10^{-5} mol/L. It was shown that (Figure 6) the CL intensity increased sharply when the methylene blue concentration ranged from 8.0×10^{-6} to 3.2×10^{-5} mol/L and changed slowly after 3.2×10^{-5} mol/L. Thus, 3.2×10^{-5} mol·L⁻¹ methylene blue was chosen for the CL reaction in this research.

Validity of new CL system

Thiourea is a specific and powerful hydroxyl radical scavenger (Long et al., 2006), thus, it was employed as the reference material for the evaluation of validity of the new CL system in the determination of radical scavenging effect. A series of thiourea solutions of different concentration were prepared for the effective examination of the proposed method. The results showed that the scavenging rate increased with increasing thiourea concentration (Figure 7), indicating that thiourea concentration and hydroxyl radical scavenging rate had a significant dose-effect relationship. Therefore, this system can be used for the determination of the capacity of hydroxyl radical scavenger.

Measurement of radical scavenging capacity

10 ml of herb extract was diluted to 100 ml (200 ml for *R. chinensis* Mill) with deionized water, the scavenging rate was measured under the optimal experimental conditions, and the result was shown in Table 2. As can be seen from the table, the radical scavenging ratios of herb extracts vary from 97.7 to 25.6%, of the 18 Chinese medicinal herbs tested, the gall of *R. chinensis* Mill exhibited the highest potency in scavenging hydroxyl radical, while the seeds of *Cannabis sativa* L showed the lowest radical scavenging capacity. It is reported in literature that the radical scavenging capacity of a herb is related to its phenolic contents (Guo et al., 2008; Liu and Ng, 2000;

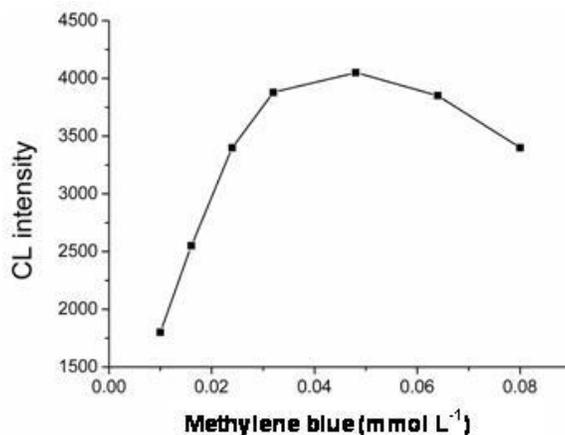


Figure 6. The effect of methylene blue concentration on CL intensity.

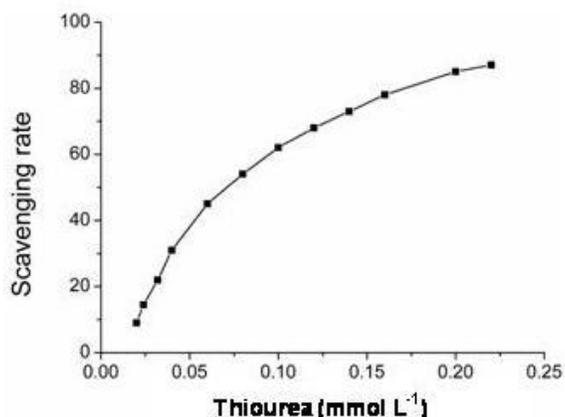


Figure 7. Effects of thiourea concentration on ·OH scavenging rate.

Wong et al., 2006). It can be seen from Table 1 that the major active components of *R. chinensis* Mill are polyphenols (gallotannin) and phenolic acids (gallic acid), the main active components of other herbs, ranking lower than *R. chinensis* Mill but still within in the top ten of the list, also include phenolic compounds such as tannins, phenolic acids, flavonoids, and simple phenols, yet the major active components of *Cannabis sativa* L are polyunsaturated fatty acids including oleic acid, linoleic acid and linolenic acid. These results indicated that phenolic compounds may contribute significantly to the radical-scavenging activities of the herbs, which is in agreement with the results of Jiang et al. (2011) and Lee et al. (2008).

Possible mechanism of the CL reaction

The possible mechanism of CL reaction of the proposed

CL system could be explained as follow: When methylene blue (MB) reacted with the hydroxyl radicals produced from Fenton reagent, a certain amount of energy was released and absorbed by the unreacted methylene blue in the solution to form the excited-state methylene blue, which emitted CL when returned to the ground state; the CL emission signal was inhibited when Chinese herb extract was added and partially scavenged the hydroxyl radicals in the CL system.

The reaction pathway can be summarized as the following:

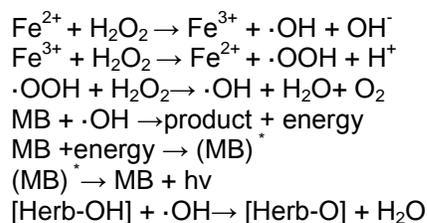


Table 2. The scavenging ratio on ·OH of extracts of 18 Chinese medicinal herbs (n = 5).

Chinese medicine herb	Scavenging rate (%)	RSD (%)
<i>R. chinensis</i> Mill.	97.7	0.21
<i>T. chebula</i> Retz.	96.8	0.10
<i>F. suspensa</i> (Thunb.) Vahl.	92.5	0.22
<i>L. japonica</i> Thunb.	91.5	0.59
<i>R. chinensis</i> Jacq.	89.5	1.6
<i>R. glutinosa</i> Libosch.	87.5	1.0
<i>L. barbarum</i> L.	86.8	1.1
<i>S. baicalensis</i> Georgi	86.7	1.7
<i>A. sinensis</i> (Oliv.) Diels	84.4	0.68
<i>C. indicum</i> L.	83.4	3.0
<i>G. uralensis</i> Fisch.	79.4	0.65
<i>G. biloba</i> L.	78.4	1.4
<i>A. mongholicus</i> Bge.	75.7	0.78
<i>P. multiflorum</i> Thunb.	74.2	1.4
<i>S. chinensis</i> (Turcz.) Baill.	62.8	4.5
<i>R. cordifolia</i> L.	58.2	4.6
<i>L. chiachristinae</i> Hance	39.1	5.2
<i>C. sativa</i> L.	25.6	3.9

Conclusion

A new flow-injection chemiluminescence method for determination of scavenging capacity of Chinese medicinal herbs to hydroxyl radicals based on the inhibition effect of herb water extract on the CL intensity in methylene blue-Fe²⁺-H₂O₂ system was established. The scavenging rates of the herbs could be measured even with a simple setup. It was believed that the reaction of methylene blue with hydroxyl radical was responsible for the CL emission in the CL system, while the degree of the reduction of the CL intensity resulted from the scavenging action of herb water extracts on hydroxyl radical had a stoichiometric relationship with herb concentration in the solution. The proposed method is not only simple and convenient, but also stable and user-friendly. It had been applied to the determination of hydroxyl radical scavenging capacity of 18 Chinese medicinal herbs with satisfactory results.

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