Food Chemistry 115 (2009) 380–386

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

High throughput chemiluminescence platform for evaluating antioxidative activity of total flavonoid glycosides from plant extracts

Hong Yao [a,b], Bin Wu [a], Yiyu Cheng [a,*], Haibin Qu [a,*]

[a] Pharmaceutical Informatics Institute, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, China
[b] Department of Pharmaceutical Analysis, Faculty of Pharmacy, Fujian Medical University, Fuzhou 350004, China

ARTICLE INFO

Article history:
Received 14 July 2008
Received in revised form 27 September 2008
Accepted 30 November 2008

Keywords:
Luminol
Chemiluminescence
Antioxidant activity
Total flavonoid glycosides
High throughput
DPPH·
H_2O_2

ABSTRACT

A high throughput chemiluminescence (CL) platform for rapidly evaluating antioxidant activity of total flavonoid glycosides from plant extracts was developed originally based on their inhibition effects on the CL reaction of 1,1-dipheny-2-picrylhydrazyl (DPPH·)–luminol or H_2O_2–luminol system. With the method, total flavonoid glycosides extracts were screened through detecting the inhibited CL intensity, which was reversely correlative to antioxidant activity of the studied plant extracts. A complete analysis could be performed rapidly in a 96-well plate format, giving a very high throughput of 96 samples in about 10 min including sampling and detection. Owing to its automatic, rapid, sensitive and high throughput characteristics, the presented platform has been used to evaluate the antioxidant activity of pure compounds and eleven total flavonoid glycosides extracts from crude drugs (*Radix Scutellariae*, *Rhizoma Belamcandae*, *Fructus Aurantii Immaturus*, *Caulis Lonicerae*, *Flos Carthami*, *Pollen Typhae*, *Folium Apocyni Veneti*, *Flos Lonicerae*, *Radix Puerariae*, *Fructus Aurantii*, *Flos Chrysanthemi*).

1. Introduction

It is well known that free radicals and/or active oxygen species play important roles in the development of some diseases, such as the aging process, heart disease and cancer (Finkel & Holbrook, 2000). It is significant to look for efficient ways to decrease or depress yielding of free radicals in body for health. Thus, finding some natural antioxidative compounds, which could be isolated from herbal medicine and efficiently clear free radicals, has attracted increasing attention.

As extracting herbal medicine to obtain the antioxidant compounds, it remains a key problem that how to simply and rapidly ascertain the studied plants containing antioxidative compounds. For this goal, many methods, including free radicals and active oxygen scavenging in vitro, have been developed for evaluating the antioxidant activity of studied plant extracts (Antolovich, Prenzler, Patsalides, McDonald, & Robards, 2002; Termentzi, Kefalas, & Kokkalou, 2006). Generally, most of these methods require some suitable substrates so that antioxidative effectiveness may be evaluated by measuring the inhibition extent of oxidation between the substrate and the radicals or active oxygen species with the studied plant extracts. Alternatively, the ability to scavenge specific radicals or active oxygen species may be targeted as, for example, hydroxyl radical ($OH^{\cdot}$) (Jung, Heo, & Wang, 2008; Parejo et al., 2002), superoxide radical ($O_2^{\cdot-}$, $OOR^{\cdot}$) (Magalhães, Segundo, Reis, & Lima, 2008; Tsukagoshi, Saito, & Nakajima, 2008), nitric oxide radical ($NO^{\cdot}$) (Silva, Malva, & Dias, 2008), DPPH· (Barros et al., 2008; Karagöler, Erdağ, Emek, & Uygun, 2008) or Hydrogen peroxide (H_2O_2) (Ak & Gülçin, 2008; Coyle, Philips, Morrisroe, Chancellor, & Yoshimura, 2008; Spiteller, Özen, Smelcerovic, Zuehlke, & Mimica-Dukić, 2008) and detected by combination of special analysis techniques including spectrophotometry (Kulys & Bratkovskaja, 2007), electrochemistry (Milardovic, Kereković, Derrico, & Rumenjak, 2007; Shpigun, Arharova, Brainina, & Ivanova, 2006), chemiluminescence (Narasimhan, Govindarajan, Vijayakumar, & Mehrotra, 2006; Taubert et al., 2003), flow injection analysis (Wang, Wang, & Yang, 2007), fluorescence (Aldini et al., 2006; Woraratphoka, Intarapichet, & Indrapichate, 2007), capillary electrophoresis (Sha, Yin, Zhang, He, & Yang, 2007), high performance liquid chromatography (Baltrušaitytė, Venskutonis, & Čeksterytė, 2007) and high performance liquid chromatography/mass spectroscopy (Chen et al., 2008), etc. However, all these methods adopt an assay mode of sample by one after another, which is very time-consuming and display inferior throughput. In addition, many of these methods suffer serious interference from reaction reagents or solution background, which require some additional sample pretreatment procedures, such as filtration or centrifugation. Therefore, there is an improved demand for the development of simple and rapid methods with high throughput property to evaluate the antioxidant activity of plants extracts.

* Corresponding author. Tel./fax: +86 571 88208428.
E-mail addresses: Chengyy@zju.edu.cn (Y. Cheng), quhb@zju.edu.cn (H. Qu).

0308-8146/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2008.11.100

In this study, a novel and high throughput chemiluminescence (CL) platform for rapidly evaluating antioxidative activity was constructed successfully based on the screened materials' inhibition effects on the luminol CL system with the typical free radical or active oxygen species, DPPH· or H2O2 as the CL provocation agent. As our best knowledge, it is the first time to report the DPPH·–luminol system and its application, so we optimise the reaction conditions of the DPPH·–luminol CL. Using this platform (with the DPPH·–luminol or H_2O_2–luminol CL system), a complete assay can be performed rapidly in a 96-well plate format including manual sampling, automatic addition of the CL reaction reagents and detection, giving a high throughput of 96 samples in about 10 min. Compared with the previous methods, this platform requires low consumption of agents and automatic operation and especially, possesses a high throughput property of sample assay, which can be competent enough for the antioxidant screening of a large-scale plant samples.

Using the presented CL platform, we screened eleven total flavonoid glycosides extracts from *Radix Scutellariae*, *Rhizoma Belamcandae*, *Fructus Aurantii Immaturus*, *Caulis Lonicerae*, *Flos Carthami*, *Pollen Typhae*, *Folium Apocyni Veneti*, *Flos Lonicerae*, *Radix Puerariae*, *Fructus Aurantii*, *Flos Chrysanthemi* to assess their antioxidant activity and search for antioxidants. Although these plant species have been extensively studied for their chemical components, little information about their flavonoid glycosides content or antioxidative and antiradical scavenging activity has been hitherto reported. The results were compared with those obtained with two reference substances: baicalin, an antioxidant of natural origin; ascorbic acid, one of the most widely used antioxidants employed in the food and pharmaceutical industries.

2. Experimental

2.1. Plant materials and chemicals

All the eleven Chinese crude drugs, *Radix Scutellariae* (RS), *Rhizoma Belamcandae* (RB), *Fructus Aurantii Immaturus* (FAI), *Caulis Lonicerae* (CLE), *Flos Carthami* (FC), *Pollen Typhae* (PT), *Folium Apocyni Veneti* (FAV), *Flos Lonicerae* (FL), *Radix Puerariae* (RP), *Fructus Aurantii* (FA) and *Flos Chrysanthemi* (FCI) were purchased from the Chinese crude drug branch office of Xiaoshan medicine Ltd., Com. (Hangzhou, China).

Luminol was obtained from the Institute of Analytical Science of Xi'an Jiaotong University (Xi'an, China). DPPH· was purchased from Sigma–aldrich Co. (St. Louis, MO, USA). 30% H_2O_2 solution was obtained from the Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Baicalin (BN) and L-ascorbic acid (AA) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Analytical reagent-grade chemicals and double-distilled water were used to prepare all solutions.

The 10 mg ml^{-1} standard stock solutions of Baicalin and AA were freshly prepared by diluting 100 mg of them in 10 ml methanol and water, respectively. The standard solutions were stored in a refrigerator and protected from light. Dilutions of these standards were prepared by appropriate dilution with water. A 0.01 M luminol stock solution was prepared by dissolving luminol in 0.01 M sodium hydroxide solution. The working solution of luminol was prepared by directly diluting this stock solution to 1.0×10^{-4} M with 0.01 M sodium hydroxide solution. A 0.01 M DPPH· stock solution was freshly prepared by dissolving 39 mg DPPH· solid in methanol and diluting with methanol to 10 ml. The working solution of DPPH· was freshly prepared by diluting this stock solution to 1.0×10^{-5} M with double-distilled water. 0.1% H_2O_2 solution was freshly prepared by appropriately diluting 30% H_2O_2 with double-distilled water.

2.2. Extraction of total flavonoid glycosides and sample preparation

Before extraction, all the cut crude drugs were crushed and through a 20-eye sieve to obtain the homologous size of powders. The powders obtained were firstly soaked in chloroform for 30 min, followed by reflux extraction in a 90 °C water bath with about 10 times volume of chloroform for 1 h. After the extraction procedure repeated twice, the residues were separated from the chloroform extracts by filtration with absorbent gauze. The residues were then refluxed and extracted in the 90 °C water bath with about 10 times volume of 70% ethanol solution (v/v) for twice (1 h per time). The ethanol extracts were obtained by filtration with filter paper and concentration with decompression equipment. The extracts were adsorbed statically on a D101 macroporous resin column for 30 min. The elution was firstly carried out with distilled water for 30 min, followed by elution of 50% ethanol (v/v) for 1 h. Finally, the elutes of 50% ethanol (v/v) were collected, concentrated and lyophilised in turn to prepare the total flavonoid glycosides solids. Before used, the total flavonoid glycosides extracts obtained were grinded into fine powder with a mortar and stored at a dry box away from light.

About 1 mg ml^{-1} sample stock solutions of total flavonoid glycosides from the 11 crude drugs were prepared by dissolving 10 mg of the respective extract powder above obtained with 10 ml methanol. Sample solution of each plant extract with a series of concentrations (0.5, 1.0, 10, 50 and 100 μg ml^{-1}) was prepared by appropriate dilution of the each sample stock solution with methanol.

2.3. HPLC conditions

Chromatographic validation of the extracts was performed on a reversed-phase Agilent SB-C18 column (250 mm × 4.6 mm i.d.) by the HPLC system (Agilent 1100, American) equipped with the quaternary pump and DAD detector. The mobile phase consisted of solvent A and solvent B with gradient programme (0–30 min, 10–30% A; 30–45 min, 30–55% A; 45–55 min, 55–60% A; 55–65 min, 60–90% A; 65–70 min, 90–95% A) using a 0.02% acetonitrile solution of formic acid as solution A and a 0.02% aqueous solution of formic acid as solution B. The flow rate was maintained at 1.0 ml min^{-1} and the injection volume was 10 μl. The DAD detection was carried out at 280 nm. The column temperature was kept at 30 °C.

2.4. CL apparatus

An infinite F200 microplate reader instrument (Tecan Group Ltd., Switzerland) was used for CL study. The setup of the CL system for antioxidant determination is shown in Fig. 1. The piston injection pump 1 was used to inject DPPH· or H_2O_2 (oxidant) solution in the storage bottle 1 at an injection rate of 300 μl s^{-1} into a well of the 96-well plate (greiner black plate), which had been pre-added a extract solution containing total flavonoid glycosides. The luminol solution was then added with another pump 2 in the same well at 300 μl s^{-1}. After that, the 96-well plate is promptly moved by the movable plate carrier to adjust the well added the luminol solution to be just below the detection head. Along with mixing of the extract solution, the oxidant (DPPH· or H_2O_2) solution and the luminol solution, CL was produced resulting from the oxidation of the oxidant and the luminol, which could be inhibited by the studied extract. The CL was simultaneously transmitted with the optical fibre and collected with the photomultiplier tube (PMT) of the ultra-weak CL analyser equipped with the Magellan data acquisition treatment interface (Tecan Group Ltd., Switzerland, Switzerland). The signal was recorded using an IBM-compatible computer. The manipulation of the injection pump and movable

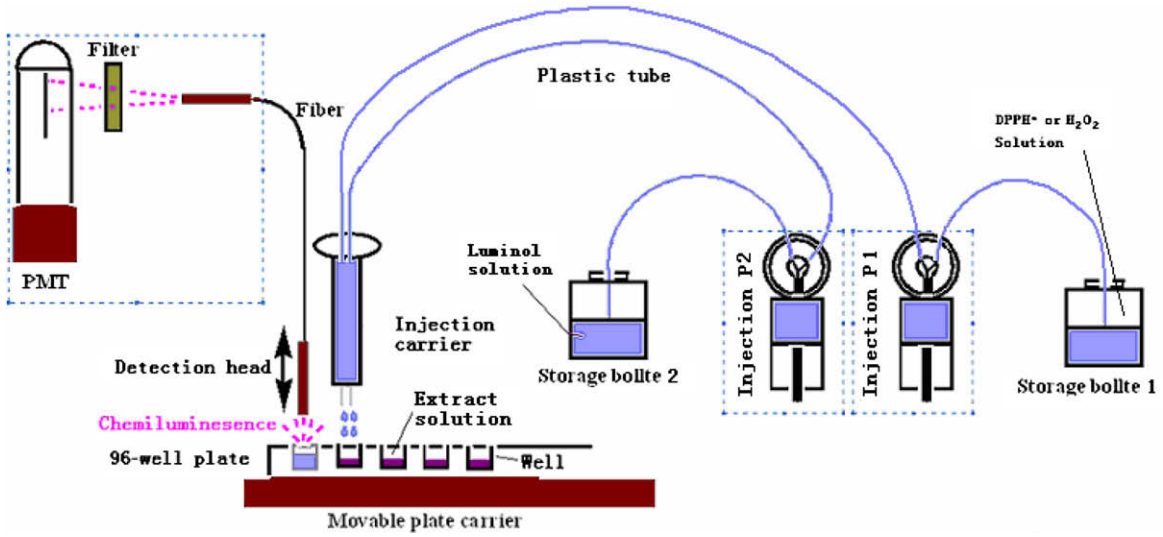

Fig. 1. Schematic diagram of CL platform for the determination of antioxidant activity.

plate carrier, date acquisition and treatment were performed with a Magellan software running under Windows XP.

2.5. DPPH· scavenging activity

Sample solutions of each plant extract with a series of concentrations (0.1, 0.5, 1.0, 10, 50, 100 and 1000 μg ml^{-1}) were measured. With a 96-well microplate, all sample solutions (10 μl per sample) were added in different wells in order with an eppendorf transferpettor. After that, the 96-well microplate was placed on the movable plate carrier in the infinite F200 instrument. 100 μl DPPH· (1.0×10^{-5} M) was then automatically injected into a well pre-added the extract solution, followed by the automatic addition of 100 μl luminol solution (1.0×10^{-5} M). After waiting for 1 s, CL intensity (I) was automatically detected with a 0.1 s integration time and recorded with the Magellan software. The CL of the control (using 10 μl methanol instead of the extract sample solution) was performed at the same conditions. The CL of the background was recorded using 10 μl methanol and 100 μl double-distilled water instead of the extract sample and DPPH· solutions, respectively. All the wells were examined automatically and one by one by the instrument in about 10 min. In order to obtain good accuracy, the test of DPPH· scavenging activity for all sample solutions, the controls and the background were repeated three times. The inhibition percentage of DPPH·–luminol CL by the extract solution was calculated according to the formula:

$$\text{Inhibition}(\%) = [(I_{\text{control}} - I_{\text{sample}})/(I_{\text{control}} - I_b)] \times 100\%$$

where I_{sample}, I_{control} and I_{b} were the CL intensities for the extract solutions, the controls and the background, respectively. The Inhibition rate was plotted against the sample extract concentration, and a logarithmic regression curve was established in order to calculate the IC_{50} (inhibitory concentration 50), which is the amount of sample necessary to decrease by 50% the CL intensity from the control. The results are expressed as antiradical efficiency.

2.6. H_2O_2 scavenging activity

The test of H_2O_2 scavenging activity is similar to that of DPPH· scavenging activity. All sample solutions (10 μl per sample) were pre-added in different wells in order with an eppendorf transferpettor and the 96-well microplate was placed on the movable plate carrier in the infinite F200 instrument. 100 μl H_2O_2 (0.1%) was then automatically and in order injected into all the wells on a 96-well plate. After standing for 5 min, 100 μl luminol solution (1.0×10^{-5} M) was injected into one well to produce CL (Here, preliminary test confirmed that the CL with the standing time of 5–10 min was stable and easily detected. Thus, the standing time was selected to be 5 min). The CL intensity (I) was automatically detected with a 0.1 s integration time and recorded with the Magellan software. The CL test of each well was undergone one by one and all the wells in the same microplate could be examined in about 5 min. The CL of the control (using 10 μl methanol instead of extract sample solution) was performed at the same conditions. Also, the CL of the background was recorded using 10 μl methanol and 100 μl double-distilled water instead of the extract sample and H_2O_2, respectively. The test of H_2O_2 scavenging activity for all sample solutions, the controls and the background were repeated three times. The inhibition percentage of H_2O_2–luminol CL by the extract solution was calculated according to the formula:

$$\text{Inhibition}(\%) = [(I'_{\text{control}} - I'_{\text{sample}})/(I'_{\text{control}} - I_b)] \times 100\%$$

where I_{sample}', I_{control}' and I_{b} were the CL intensities for the extract solutions, the controls and the background, respectively. The Inhibition percentage was plotted against the sample extract concentration, and a logarithmic regression curve was established in order to calculate the IC_{50} (inhibitory concentration 50), which is the amount of sample necessary to decrease by 50% the CL intensity from the control. The results are expressed as antioxidative efficiency.

3. Results

3.1. Extraction of total flavonoid glycosides

As it is known, many plants simultaneously contains flavonoids and flavonoid glycosides compounds, which could possess high efficient antioxidative activity. When extracting the crude drugs to obtain total flavonoid glycosides, it is necessary to isolate and get rid of the flavonoids from the extracts of the total flavonoid glycosides. Referring to literature (Naczk & Shahidi, 2004; Stalikas, 2007), flavonoids can be extracted from herbal medicines with chloroform as extraction solvent by which flavonoid glycosides seldom can be extracted. However, flavonoid glycosides can always be extracted with polar solvents, such as some mixture solvents, methanol–water and ethanol–water. The flavonoid glycosides can

also be purified by using macroporous resin chromatography techniques. Based on these facts, it should be able to obtain the total flavonoid glycosides containing negligible flavonoids by using the extraction and purification procedures above mentioned in the Section 2.2. In preliminary tests, the crude drug, *Radix Scutellariae* was selected to optimise the experimental conditions for extraction and purification, including the time, frequency and the volumes of chloroform or 70% ethanol refluxing, and the constituents of elution solvent for the macroporous resin chromatography. The final lyophilised powders, the total flavonoid glycosides extracts, were identified by high performance liquid chromatography. The peak of standard baicalin is at 27.8 min (Fig. 1 Supporting information) and the profiles for the 70% ethanol extract (Fig. 2b) and the 50% ethanol elute (Fig. 2c) also appear the baicalin peak at 27.8 min and 27.9 min, respectively and some unknown peaks before the baicalin peak, which could be attributed to the other flavonoid glycosides. There is no peak of the baicalin is observed in the HPLC profile of chloroform extract (Fig. 2a), but some peaks occur with retention times beyond 30 min, which could be partly attributed to flavonoid aglycones. These results show that the extraction and purification procedures in the Section 2.2 can well serve the obtaining of total flavonoid glycosides. In addition, the durability test of the isolation procedure for the other crude drugs was further confirmed by selecting *Flos Lonicerae* and *Radix Puerariae* as trial objects. The final HPLC profiles of the total flavonoid glycosides extracts for the two crude drugs were shown in Figs. 2 and 3 Supporting information.

3.2. DPPH·–luminol CL system conditions

Since it is the first time to report the DPPH·–luminol CL system, the experiment conditions were examined. The concentration of DPPH· had a great effect on the CL intensity of DPPH·–luminol reaction with the proposed CL platform. When keeping the luminol concentration at 1.0×10^{-4} M in 0.01 M NaOH solution and using 10 μl distilled water as the determined sample, the CL intensity (I) was continuously increasing with increasing the concentration of DPPH· over the range 1.0×10^{-6}–1.0×10^{-5} M. Above 1.0×10^{-5} M, the CL intensity (I) decreased gradually (*Fig.* 4 Supporting information). Thus, 1.0×10^{-5} M DPPH· was selected for subsequent studies.

The influence of 5.0×10^{-6}–1.0×10^{-3} M luminol in 0.01 M NaOH on the CL reaction was examined using 10 μl distilled water as the determined sample when the concentration of DPPH· was fixed at 1.0×10^{-5} M. The maximum CL intensity was obtained when the concentration of luminol was 1.0×10^{-4} M, hence 1.0×10^{-4} M luminol in 0.01 M NaOH was selected for further studies (*Fig.* 5 Supporting information). Similarly, the medium NaOH of luminol solution was optimised at 0.01 M.

In addition, Injection rates of luminol and DPPH· solutions between 50 μl s^{-1} and 300 μl s^{-1} were studied under the above conditions. The optimal injection rates for the two solutions were 300 μl s^{-1}, respectively. The selected injection volume for the both solutions was 100 μL and the integration time for CL determination

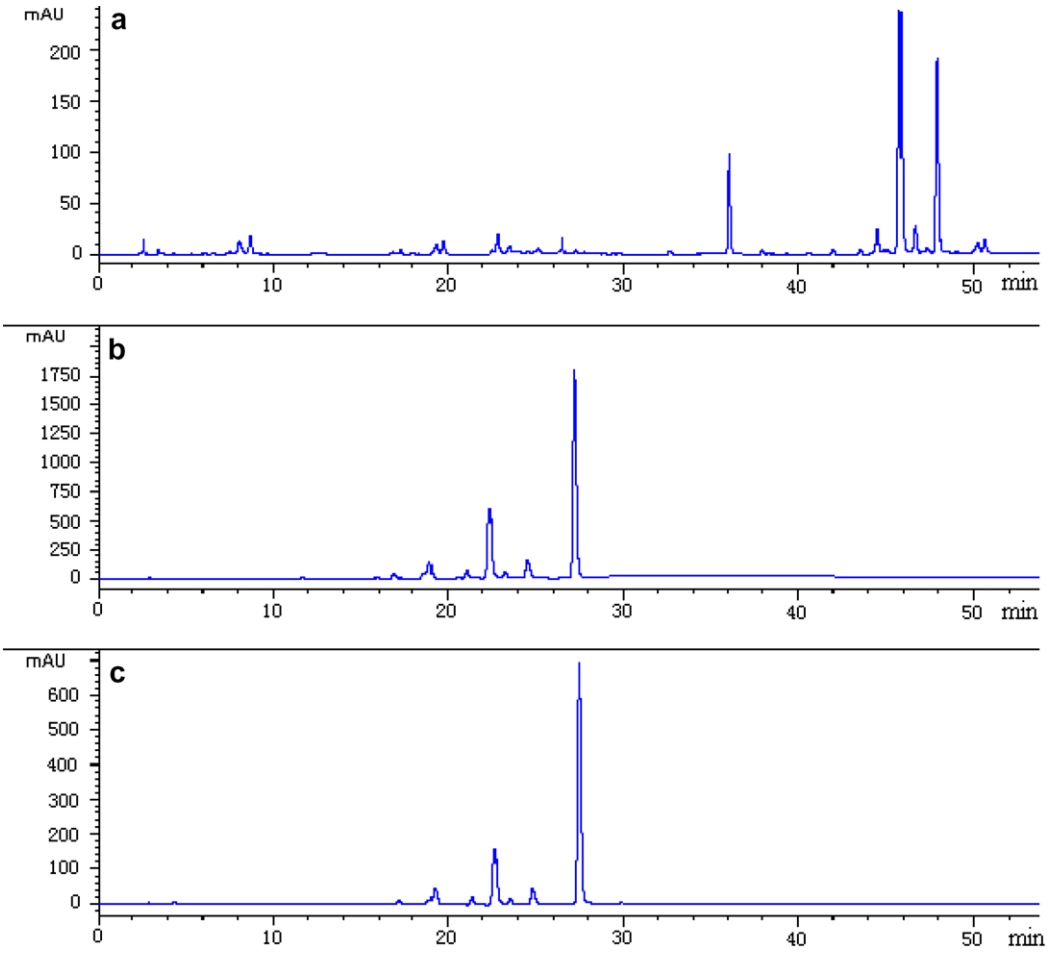

Fig. 2. HPLC profiles of (a) chloroform extract, (b) 70% ethanol extract and (c) 50% ethanol elute. Crude drug: *Radix Scutellariae*.

Table 1
DPPH·–luminol CL inhibition rate of the total flavonoid glycosides extracts and standard antioxidants.[A,B]

Plant resources or standard antioxidants	The concentrations of extracts or standard antioxidants (μg ml^{-1})						
	0.1	0.5	1	10	50	100	1000
	DPPH·–luminol CL inhibition rate (%)						
AA	/	/	/	7.83 ± 4.10^{m}	97.24 ± 4.10^{c}	99.93 ± 0.06^{a}	99.97 ± 0.03^{a}
BN	19.38 ± 1.35^{l}	55.55 ± 2.19^{i}	85.27 ± 2.19^{f}	93.73 ± 0.33^{e}	98.35 ± 0.70^{b}	99.97 ± 0.14^{a}	99.98 ± 0.53^{a}
RS	17.94 ± 2.61^{l}	53.27 ± 1.38^{i}	82.43 ± 3.55^{f}	93.13 ± 1.74^{e}	98.09 ± 0.56^{b}	99.47 ± 0.34^{a}	99.98 ± 0.72^{a}
RB	/	8.80 ± 5.93^{m}	28.18 ± 1.41^{k}	63.96 ± 27.43^{h}	98.16 ± 0.31^{b}	99.41 ± 0.30^{a}	99.97 ± 0.43^{a}
FAI	/	/	7.40 ± 5.97^{m}	59.55 ± 0.37^{h}	94.97 ± 1.84^{d}	99.81 ± 0.03^{a}	99.94 ± 0.03^{a}
CLE	/	3.90 ± 0.33^{n}	13.18 ± 0.17^{l}	22.53 ± 0.93^{k}	51.33 ± 1.81^{i}	72.48 ± 0.76^{g}	99.41 ± 0.11^{a}
FC	/	27.13 ± 5.66^{k}	59.11 ± 2.60^{h}	95.80 ± 3.44^{d}	99.92 ± 0.01^{a}	99.95 ± 0.06^{a}	99.98 ± 0.01^{a}
PT	/	9.40 ± 2.62^{m}	24.92 ± 11.51^{k}	66.01 ± 17.63^{h}	98.43 ± 0.94^{b}	99.48 ± 0.31^{a}	99.41 ± 0.50^{a}
FAV	/	27.47 ± 1.58^{k}	37.30 ± 2.31^{j}	97.47 ± 1.19^{c}	99.79 ± 0.15^{a}	99.71 ± 0.30^{a}	99.74 ± 0.20^{a}
FL	/	30.02 ± 0.47^{j}	43.10 ± 2.42^{i}	97.11 ± 1.16^{c}	99.79 ± 0.17^{a}	99.72 ± 0.30^{a}	99.72 ± 0.31^{a}
RP	/	48.49 ± 12.30^{i}	52.27 ± 7.17^{i}	91.30 ± 0.17^{e}	99.91 ± 0.06^{a}	99.94 ± 0.07^{a}	99.96 ± 0.01^{a}
FA	/	13.24 ± 4.38^{l}	14.50 ± 2.65^{l}	53.92 ± 3.33^{i}	92.32 ± 0.63^{e}	99.60 ± 0.03^{a}	99.91 ± 0.06^{a}
FCI	/	39.67 ± 6.20^{j}	47.11 ± 2.54^{i}	99.45 ± 0.31^{a}	99.95 ± 0.17^{a}	99.83 ± 0.24^{a}	99.97 ± 0.03^{a}

[A] Values are the average of three replicates ± SD; "/" represents a value near zero (below 0.1%).
[B] Values in the same column with different superscripts are significantly ($p \leqslant 0.05$) different.

was 0.1 s because they produced high CL intensity (*Fig.* 6 Supporting information).

3.3. DPPH· scavenging activity

A rapid estimation of radical scavenging ability by using DPPH· in vitro could save much laboratory work and provide valuable information about screened samples, giving a basis for further isolation procedures. The radical (DPPH·) scavenging activities of the total flavonoid glycosides extracts (a series of concentrations) were calculated as DPPH·–luminol CL inhibition rate (%). The results were compared with those of two standard antioxidants, AA and BN. As shown in Table 1, all the DPPH· CL inhibition rates (%) for the eleven extracts and two standard compounds appear the positive dose-dependent relationships. Except for the extract from *Caulis Lonicerae* (*CLE*), 100 μg ml^{-1} of the other extracts or the standard antioxidants had similar and excellent radical scavenging activities (beyond 99.4% of DPPH·–luminol CL inhibition rate, $p > 0.05$). At 10 μg ml^{-1}, the CL inhibition rates of Baicalin and the total flavonoid glycosides extracts of *Radix Scutellariae* (*RS*), *Flos Carthami* (*FC*), *Folium Apocyni Veneti* (*FAV*), *Flos Lonicerae* (*FL*), *Radix Puerariaev* (*RP*) *and Flos Chrysanthemi* (*FCI*) were about 93.73%, 93.13%, 95.80%, 97.47%, 97.11%, 91.30% and 99.45%, respectively. When at 1 μg ml^{-1}, the CL inhibition rate can still reach about 82.43% for the *RS* extract, followed by about 59.11% and 52.27% for *FC* and *RP* extracts, respectively. At 0.5 μg ml^{-1} level, the extract of *RS* compared with the other extracts possessed the best radical scavenging activity (about 53.27% inhibition rate). Among the eleven extracts, the total flavonoid glycosides of *CLE* appeared the worst radical (DPPH·) scavenging activity.

From Table 1, the half-inhibition concentrations (IC_{50}) of the eleven extracts and standard antioxidants on DPPH·–luminol CL were calculated and shown in Fig. 3. The standard antioxidant BN possessed a lowest IC_{50} value, 0.37 ± 0.03 μg ml^{-1}, followed by 0.45 ± 0.06, 0.46 ± 0.07, 0.60 ± 0.07, 0.63 ± 0.05, 0.72 ± 0.03 and 0.73 ± 0.02 μg ml^{-1} for the flavonoid glycosides extracts from *RS*, *FCI*, *RP*, *FL*, *FAV* and *FC*, respectively. The IC_{50} values of the extracts from *RB*, *PT*, *FA* and *FAT* were within the range of 2–6 μg ml^{-1}. The flavonoid glycosides extract of *CLE* showed a worst IC_{50} values, 17.63 ± 0.33 μg ml^{-1}. However, the standard antioxidant, AA also had a worse IC_{50} values, 13.57 ± 2.42 μg ml^{-1}.

According to these results above, the radical scavenging activity of total flavonoid glycosides could be suggested as: *RS extract* > *FCI extract* > *RP extract* > *FL extract* > *FAV extract* > *FC extract* > *RB extract* > *PT extract* > *FA extract* > *FAT extract* > *CLE extract*.

3.4. H_2O_2 scavenging activity

H_2O_2 scavenging ability was widely used to evaluate the antioxidative activity of plant extracts. The most common applied method is to determine the inhibition rate of plant extracts studied on H_2O_2–luminol CL reaction. In this paper, the presented CL platform also used a H_2O_2–luminol CL system. Similar to the supposed DPPH·–luminol CL system, the conditions for H_2O_2–luminol CL system were optimised and the optimal ones were described as in Section 2.5.

With the H_2O_2–luminol CL platform, the antioxidative (scavenging H_2O_2) activities of the eleven extracts and BN were examined. As shown in Table 2, all the CL inhibition rates (%) appear the positive dose-dependent relationships, which are similar to those in Table 1. At every concentration level from 0.5 to 1000 μg ml^{-1}, the total flavonoid glycosides extract of *RS* possessed the highest CL inhibition rate compared with the other ten extracts. At 1000 μg ml^{-1}, the total flavonoid glycosides extracts from *RS*, *FC*, *FAV*, *FL* and *FCI* showed the CL inhibition rates beyond 90%. At 100 and 50 μg ml^{-1}, still, the five extracts showed good H_2O_2 scavenging activity (the CL inhibition rates at the two concentrations were beyond 86% and 77%, respectively). At 1 μg ml^{-1}, only did the extracts from *RS* showed considerable H_2O_2 scavenging activity

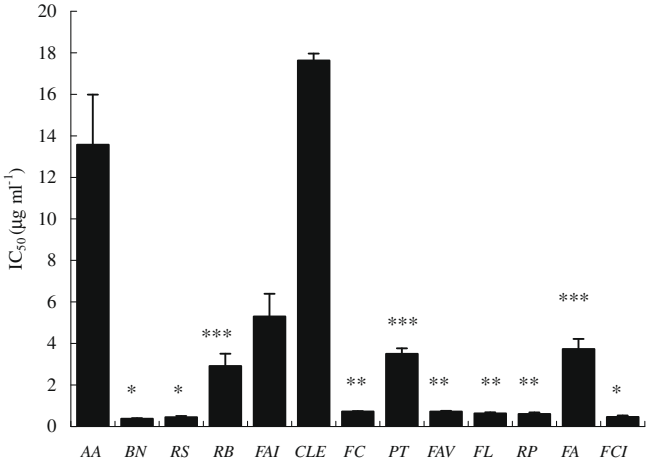

Fig. 3. IC_{50} of total flavonoid glycosides extracts on DPPH·–luminol CL (average ± SD, $n = 3$). Values in the column with same superscripts are no statistical different ($p \geqslant 0.05$).

Table 2
H_2O_2–luminol CL inhibition rate of the total flavonoid glycosides extracts and standard antioxidants.[A,B]

Plant resources or standard antioxidants	The concentrations of extracts or standard antioxidants (μg ml^{-1})					
	0.5	1	10	50	100	1000
	H_2O_2–luminol CL inhibition rate (%)[a]					
BN	23.02 ± 3.88^{i}	64.07 ± 7.28^{f}	72.71 ± 1.89^{f}	92.02 ± 1.20^{c}	95.88 ± 0.66^{b}	98.03 ± 0.16^{a}
RS	25.02 ± 1.79^{i}	67.52 ± 4.36^{f}	71.59 ± 3.44^{f}	93.37 ± 2.54^{c}	95.12 ± 0.43^{b}	97.95 ± 1.08^{a}
RB	/	/	23.32 ± 0.49^{i}	28.63 ± 7.91^{i}	64.21 ± 4.94^{f}	87.70 ± 1.35^{c}
FAI	/	13.37 ± 3.65^{j}	18.11 ± 4.02^{i}	50.79 ± 6.12^{g}	65.99 ± 1.52^{f}	86.41 ± 6.19^{c}
CLE	/	/	/	6.78 ± 0.99^{k}	50.67 ± 4.22^{g}	75.56 ± 0.17^{e}
FC	/	23.09 ± 10.81^{i}	52.25 ± 1.80^{g}	77.93 ± 4.43^{e}	87.67 ± 1.73^{c}	92.91 ± 5.01^{c}
PT	/	40.82 ± 7.28^{h}	43.42 ± 4.98^{f}	66.38 ± 3.51^{f}	68.48 ± 2.27^{f}	83.35 ± 0.05^{d}
FAV	/	46.72 ± 6.20^{h}	58.63 ± 1.26^{g}	77.10 ± 1.45^{e}	87.39 ± 0.29^{c}	93.99 ± 2.53^{c}
FL	/	32.42 ± 3.21^{i}	52.04 ± 6.84^{g}	78.11 ± 1.33^{e}	86.99 ± 0.72^{c}	92.94 ± 3.36^{c}
RP	/	/	/	21.81 ± 3.75^{i}	34.36 ± 2.80^{i}	62.51 ± 0.51^{f}
FA	/	26.36 ± 2.84^{i}	44.33 ± 0.60^{h}	57.13 ± 4.43^{g}	63.55 ± 3.79^{f}	88.42 ± 1.76^{c}
FCI	/	14.94 ± 10.08^{j}	49.58 ± 6.23^{g}	84.32 ± 1.61^{d}	90.73 ± 4.10^{c}	92.94 ± 2.87^{c}

[A] Values are the average of three replicates ± SD; "/" represents a value near zero (below 0.1%).
[B] Values in the same column with different superscripts are significantly ($p \leqslant 0.05$) different.

(vs. those from the other extracts at the same concentration level, $p > 0.05$).

IC_{50} of the eleven extracts and BN on H_2O_2–luminol CL were calculated and shown in Fig. 4. BN had a good IC_{50} values, 1.66 ± 0.76 μg ml^{-1}. The *RS* extract possessed a lowest IC_{50} value, 1.40 ± 0.76 μg ml^{-1}, followed by 1.85 ± 1.73, 2.45 ± 1.14, 5.65 ± 0.99 and 5.71 ± 0.72 μg ml^{-1} for the flavonoid glycosides extracts from *FAV*, *FL*, *PT* and *FCI*, respectively. IC_{50} values of the extracts from *FA*, *FC*, *RB* and *FAI* were within the range of 10–100 μg ml^{-1}. The total flavonoid glycosides extracts of *CLE* and *RP* showed inferior IC_{50} values, 229.93 ± 34.27 μg ml^{-1} and 187.81 ± 27.79 μg ml^{-1}, respectively. Combined these results with the CL inhibition rates, the H_2O_2 scavenging activities of total flavonoid glycosides extracts could be suggested as: *RS extract* > *FAV extract* > *FL extract* > *FCI extract* > *PT extract* > *FC extract* > *FA extract* > *RP extract* > *RB extract* > *FAT extract* > *CLE extract*.

4. Discussion

According to the screening results of the presented CL platform, although the orders of DPPH· and H_2O_2 scavenging activities for the eleven total glycosides extracts were a little disagreed, it can still find that the crude drugs, *RS*, *FC*, *PT*, *FAV*, *FL*, *FA* and *FCI* could contain a considerable amount of flavonoid glycosides. Especially, the extracts from RS, FC could include bioactive flavonoid glycosides, which are able to scavenge radical and active oxygen species high efficiently. These results could guide the further isolation work to obtain efficient antioxidative compounds from *RS*, *FC*, *PT*, *FAV*, *FL*, *FA* and *FCI*. However, the largest difference between DPPH· and H_2O_2 scavenging examinations using the presented CL platform were observed for the *RP* extract. In DPPH·–luminol CL test, *RP* was an excellent inhibitor, but extremely converse in H_2O_2–luminol CL test. The reasons could be that the *RP* extract included, besides flavonoid glycosides, the other compounds, which could greatly enhance the H_2O_2–luminol CL and antagonize the flavonoid glycosides. Actually, similar results can also be found in previous reports with some reported reasons (Parejo et al., 2002): (1) antioxidant properties of single compounds within a group can vary remarkably so that the same levels of extracts do not necessarily correspond to the same antioxidant responses; (2) the different methods used to determine the antioxidant activity are based on different mechanisms of reaction so that they often give different results; and (3) extracts are very complex mixtures of many different compounds with distinct polarity as well as antioxidant and prooxidant properties, sometimes showing synergic actions. Thus, although the presented CL platform is rapid, sensitive and high throughput, it could have a possibility to provide false positive or negative result in antioxidant screening. In consequence, extracts showing poor antioxidant properties with the presented CL platform should not be discarded as poor sources of antioxidants without having been tested with other methods and compared with different standards.

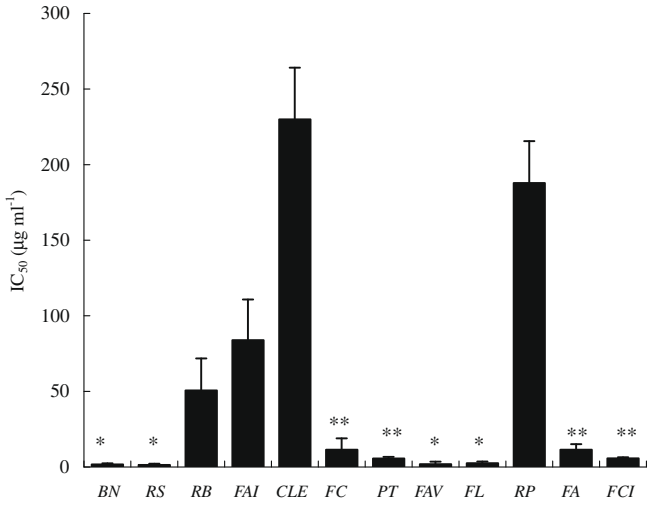

Fig. 4. IC_{50} of total flavonoid glycosides extracts on H_2O_2–luminol CL (average ± SD, $n = 3$). Values in the column with same superscripts are no statistical different ($p \geqslant 0.05$).

Noticeably, ascorbic acid can greatly enhance the CL intensity of H_2O_2–luminol system. The reason could be that H_2O_2 oxidise ascorbic acid to produce the ascorbyl radical, which may act as redox mediator accelerating luminol oxidation by formation of excited luminol radical that is a resource of emitting CL. Ascorbic acid could inhibit the DPPH·–luminol CL, but the inhibition was very inferior to the same concentration of baicalin. These results identified that ascorbic acid was not suitable to be used as a reference substance for the presented CL platform. In our work, the other reference substance was baicalin, which belongs to flavonoid glycosides and showed excellent antioxidative activity with the presented CL platform. With the platform, 0.1 μg ml^{-1} baicalin could even be detected with the inhibition rates of 7.7 ± 2.12% and 19.38 ± 1.75% for H_2O_2–luminol and DPPH·–luminol systems, respectively. Obviously, baicalin is a quite suitable positive control, because it has similar chemical and physicochemical properties to those of the samples to be studied.

5. Conclusion

A novel chemiluminescent platform for rapidly evaluating antioxidative activity of total flavonoid glycosides from different plant extracts was developed originally based on their inhibition effects on DPPH·–luminol and H_2O_2–luminol. A complete analysis could be performed rapidly in a 96-well plate format, giving a very high throughput of 96 samples in about 10 min including sampling and detection. Compared with the previous methods, this platform requires low consumption of agents and automatic operation, shows sensitive detection ability and especially, possesses a high throughput property of sample assay and the surprising dynamic ranges of 0.5–1000 μg ml^{-1} (using DPPH·–luminol CL) and 1–1000 μg ml^{-1} (using H_2O_2–luminol CL) for evaluating antioxidant activity of flavonoid glycosides. Owing to these intriguing advantages, the presented CL platform were successfully used to screen the antioxidant activity of the total flavonoid glycosides extracts from the eleven crude drugs studied, which provided a further study clue that it is possible to find the high efficient antioxidative compounds from the plant resources, *RS*, *FC*, *FAV*, *FL*, *FA* and *FCI*.

Acknowledgements

We thank for Prof. Yuhai Tang of Xi'an Jiaotong University for gifting Luminol reagent to us. The work was financially supported by Programme for New Century Excellent Talents in University (NCET-06-0515) and the National Basic Research Programme of China (2005CB523402)

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2008.11.100.

References

Ak, T., & Gülçin, İ. (2008). Antioxidant and radical scavenging properties of curcumin. *Chemico-Biological Interactions, 174*, 27–37.

Aldini, G., Piccoli, A., Beretta, G., Morazzoni, P., Riva, A., Marinello, C., et al. (2006). Antioxidant activity of polyphenols from solid olive residues of c.v. Coratina. *Fitoterapia, 77*, 121–128.

Antolovich, M., Prenzler, P. D., Patsalides, E., McDonald, S., & Robards, K. (2002). Methods for testing antioxidant activity. *Analyst, 127*, 183–198.

Baltrušaitytė, V., Venskutonis, P. R., & Čeksterytė, V. (2007). Radical scavenging activity of different floral origin honey and beebread phenolic extracts. *Food Chemistry, 101*, 502–514.

Barros, L., Falcão, S., Baptista, P., Freire, C., Vilas-Boas, M., & Ferreira, I. C. F. R. (2008). Antioxidant activity of *Agaricus* sp. Mushrooms by chemical, biochemical and electrochemical assays. *Food Chemistry, 111*, 61–66.

Chen, Y., Li, S., Lin, X., Luo, H., Li, G., & Yao, H. (2008). On-line screening and identification of radical scavenging compounds extracted from *Flos Lonicerae* by LC-DAD–TOF-MS. Chromatographia. doi:10.1365/s10337-008-0695-2.

Coyle, C. H., Philips, B. J., Morrisroe, S. N., Chancellor, M. B., & Yoshimura, N. (2008). Antioxidant effects of green tea and its polyphenols on bladder cells. *Life Sciences, 83*, 12–18.

Finkel, T., & Holbrook, N. J. (2000). Oxidants, oxidative stress and biology of aging. *Nature, 408*, 239–247.

Jung, M. J., Heo, S.-I., & Wang, M.-H. (2008). Free radical scavenging and total phenolic contents from methanolic extracts of *Ulmus davidiana*. *Food Chemistry, 108*, 482–487.

Karagöler, A. A., Erdağ, B., Emek, Y. Ç., & Uygun, D. A. (2008). Antioxidant activity and proline content of leaf extracts from *Dorystoechas hastate*. *Food Chemistry, 111*, 400–407.

Kulys, J., & Bratkovskaja, I. (2007). Antioxidants determination with laccase. *Talanta, 72*, 526–531.

Magalhães, L. M., Segundo, M. A., Reis, S., & Lima, J. L. F. C. (2008). Methodological aspects about in vitro evaluation of antioxidant properties. *Analytica Chimica Acta, 613*, 1–19.

Milardovic, S., Kereković, I., Derrico, R., & Rumenjak, V. (2007). A novel method for flow injection analysis of total antioxidant capacity using enzymatically produced $ABTS^{\cdot+}$ and biamperometric detector containing interdigitated electrode. *Talanta, 71*, 213–220.

Naczk, M., & Shahidi, F. (2004). Extraction and analysis of phenolics in food. *Journal of Chromatography A, 1054*, 95–111.

Narasimhan, S., Govindarajan, R., Vijayakumar, M., & Mehrotra, S. (2006). Free radical scavenging potential of *Chlorophytum tuberosum* baker. *Journal of Ethnopharmacology, 104*, 423–425.

Parejo, I., Viladomat, F., Bastida, J., Rosas-Romero, A., Flerlage, N., Burillo, J. S., et al. (2002). Comparison between the radical scavenging activity and antioxidant activity of six distilled and nondistilled mediterranean herbs and aromatic plants. *Journal of Agricultural and Food Chemistry, 50*, 6882–6890.

Sha, B.-B., Yin, X.-B., Zhang, X.-H., He, X.-W., & Yang, W.-L. (2007). Capillary electrophoresis coupled with electrochemical detection using porous etched joint for determination of antioxidants. *Journal of Chromatography A, 11167*, 109–115.

Shpigun, L. K., Arharova, M. A., Brainina, K. Z., & Ivanova, A. V. (2006). Flow injection potentiometric determination of total antioxidant activity of plant extracts. *Analytica Chimica Acta, 573–574*, 419–426.

Silva, B. A., Malva, J. O., & Dias, A. C. P. (2008). St. John's Wort (*Hypericum perforatum*) extracts and isolated phenolic compounds are effective antioxidants in several in vitro models of oxidative stress. *Food Chemistry, 110*, 611–619.

Spiteller, M., Özen, T., Smelcerovic, A., Zuehlke, S., & Mimica-Dukić, N. (2008). Phenolic constituents and the in vitro antioxidant activity of the flowers of *Hypericum venustum*. *Fitoterapia, 79*, 191–193.

Stalikas, C. D. (2007). Extraction, separation, and detection methods for phenolic acids and flavonoids. *Journal of Separation Science, 30*, 3268–3295.

Taubert, D., Breitenbach, T., Lazar, A., Censarek, P., Harlfinger, S., Berkels, R., et al. (2003). Reaction rate constants of superoxide scavenging by plant antioxidants. *Free Radical Biology and Medicine, 35*, 1599–1607.

Termentzi, A., Kefalas, P., & Kokkalou, E. (2006). Antioxidant activities of various extracts and fractions of *Sorbus domestica* fruits at different maturity stages. *Food Chemistry, 98*, 599–608.

Tsukagoshi, K., Saito, T., & Nakajima, R. (2008). Analysis of antioxidants by microchip capillary electrophoresis with chemiluminescence detection based on luminol reaction. *Talanta*. doi:10.1016/j.talanta.2008.03.026.

Wang, X., Wang, J., & Yang, N. (2007). Chemiluminescent determination of chlorogenic acid in fruits. *Food Chemistry, 102*, 422–426.

Woraratphoka, J., Intarapichet, K. O., & Indrapichate, K. (2007). Phenolic compounds and antioxidative properties of selected wines from the northeast of Thailand. *Food Chemistry, 104*, 1485–1490.