Classified preparation of antioxidants from Magnolia officinalis cortex and evaluation of antioxidant activity by luminol chemiluminescence assay

Running title:Classified of antioxidants and evaluation of antioxidant activity from Magnolia officinalis cortex

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Abstract

In order to systematically evaluate the antioxidant activities of Magnolia officinalis cortex, chemiluminiscence assays were applied to determinate the O^{2-}•, H_2O_2 and •OH scavenging activity of three different crude extracts, three different active constituents and two chemical compounds from Magnolia officinalis cortex in vitro. The results showed that all the samples from Magnolia officinalis cortex showed significant and dose-dependent free radical scavenging

activity. It indicated a positive correlation between the antioxidant activity of extract with the total phenols content. It was supposed that the 80% ethanolic extract contained the most efficient bioactive constituents of antioxidant. Meanwhile, the phenols play a leading role in antioxidant activity of the plant. This research demonstrated the free radical scavenging activity of alkaloids, and also provide a foundation for both the further investigation and efficient utilization of natural antioxidants of Magnolia officinalis cortex.

Keywords: Magnolia officinalis cortex; extract; antioxidant activity; free radical; chemiluminiscence.

Introduction

Magnolia officinalis cortex is a traditional medicine which is rich of pharmacologic activities [1]. It is reported that the antioxidant activity of *Magnolia officinalis cortex* could influence some other related bioactivities, such as protect effective on neuro and cerebral ischemic injury, anti-inflammatory [2,3]. However, the main deficiencies about the research on antioxidant activity of *Magnolia officinalis* cortex were below: firstly, few attentions had been paid to the other active constituents and extracts except magnolol and honokiol. Secondly, it was not know well about how to classify the antioxidant activity constituents and compare their antioxidant activity in one evaluation system. Therefore, it was difficult to evaluate the antioxidant activity holistically and systematicly base on the existing research results which would restrict the efficient comprehensive utilization of *Magnolia officinalis* cortex.

Since the characteristics of simple system, quickly response and much lower limits of detection, chemiluminesence (CL) methods have been utilized to evaluate the vitro anti-oxidant activity of antioxidant on the molecular level by detecting the free radical scavenging activity [4-7]. A CL assay of luminol for the determination of antioxidant capacity has been optimized and applied to analyses of herbal extracts [8], as the results indicated that the CL method could offer more quickly response and sensitivity than Folin–Ciocalteau and DPPH assays, as well as significantly diminished the influence of the other interfering constituents in complex food or plant extracts,

the CL method seems to be a suitable alternative method among the assays that are routinely used to screen the potential antioxidants in food or plant extracts and beverages.

In this paper, different kinds of extracts, active constituents and chemical compounds were classified and prepared. The free radical scavenging capacity of these prepared samples were measured by luminol CL assay. It would be helpful to explore the function constitution of the antioxidants of *Magnolia officinalis cortex* and promoting a potential utilization of *Magnolia officinalis cortex* extract as a natural ingredient with antioxidant function.

Results and discussion

Preparation of antioxidants from Mangnolia officinalis

Decoction and alcohol leaching were the main mode of applications of Mangnolia officinalis in traditional medicine, and the primary phytochemical substances covered polysaccharides, lignans, alkaloids [9,10]. As shown in Figure. 1, extraction and isolation scheme was designed in our researches. As a result, the primary antioxidants of Mangnolia officinalis were classified from crude to refined which conducted to systematic evaluation of antioxidant activity, these kinds of tested samples referred to as S_I: water extract; S_{II}: 60% ethanol extract; S_{III}: 80% ethanol extract; S_{IV}: polysaccharides; S_V: alkaloids; S_{VI}: lignans; S_{VII}: magnolol; S_{VIII}: honokiol.

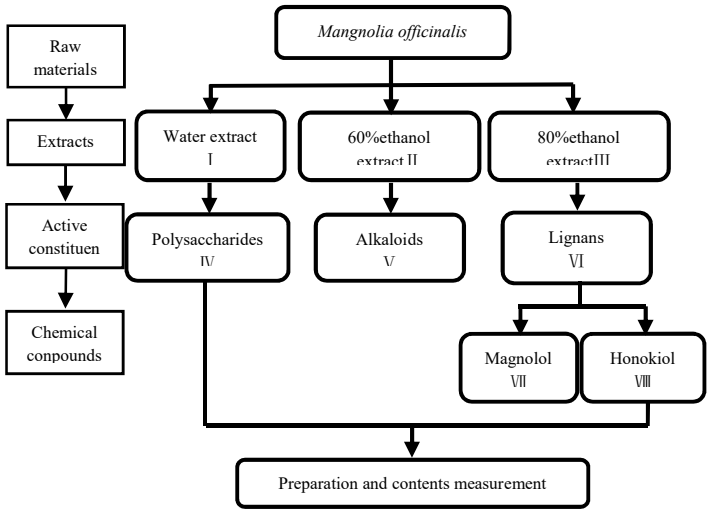
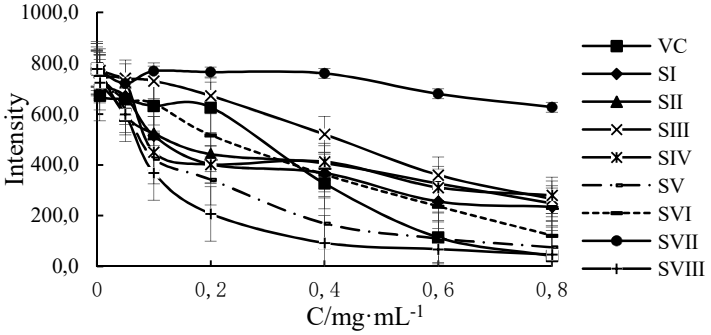


Figure 1. Extraction and isolation scheme of *Magnolia officinalis cortex's* antioxidant components.

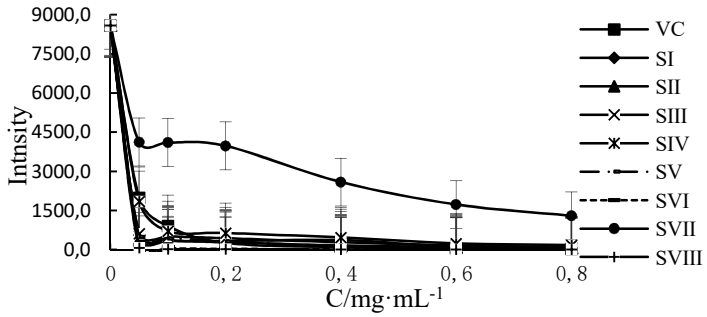
Because of the complex composition and interference between active constituents in extracts, it was meaningful to focus on the diversity of antioxidant activity of different Mangnolia officinalis samples range from crude extracts to the chemical compounds. These informations would be helpful to reveal the distribution and composition of antioxidants of Mangnolia officinalis.

Determination of free radical scavenging capacity and evaluation of antioxidant activity based on CL assays

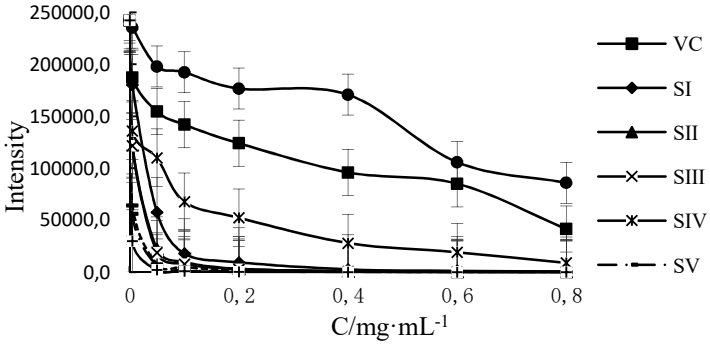
To study the scavenging effect of different Magnolia officinalis samples on O^{2-}•, H_2O_2 and •OH, three CL systems of Pyrogallol -luminol, H_2O_2- luminol and Fe^{2+}- H_2O_2- luminol were applied. The results presented in Figure 2 showed the chemiluminescence signals were reducing with the contents of tested samples added which revealed that Magnolia officinalis extracts and its main constituents exhibited a remarkable free radical scavenging activity. In particular, S_{VIII} (honokioe) and S_V (alkaloids) displayed clearly a higher activity than positive controls (Figure 3), while the activities of S_{VII} (magnolol) was weak. S_{III} showed a stronger scavenging activity than the other extracts. In terms of active constituents, S_V was stronger than the others, and S_{IV} was the weakest.



Pyrogallol -luminol CL system (a)

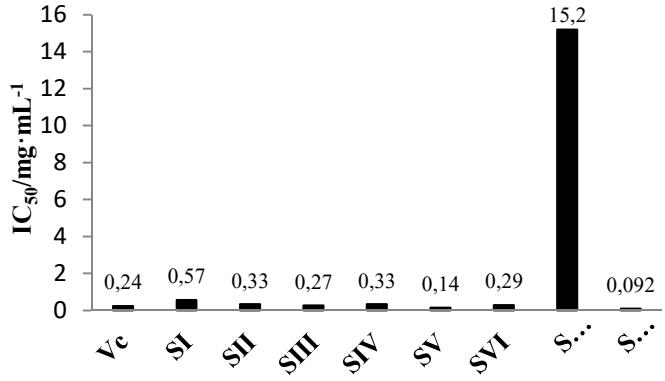


H_2O_2 -luminol CL system (b)

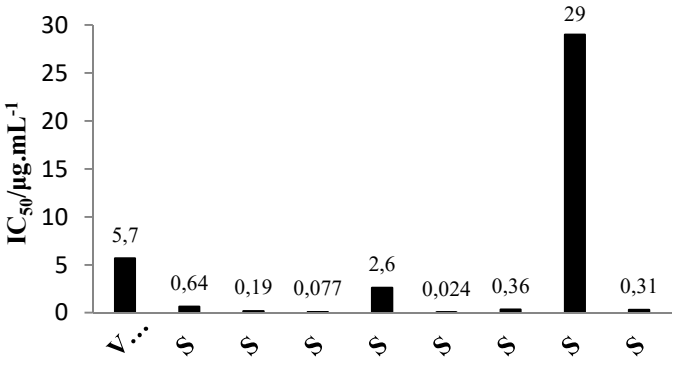


Fe^{2+}-H_2O_2 -luminol CL system (c)

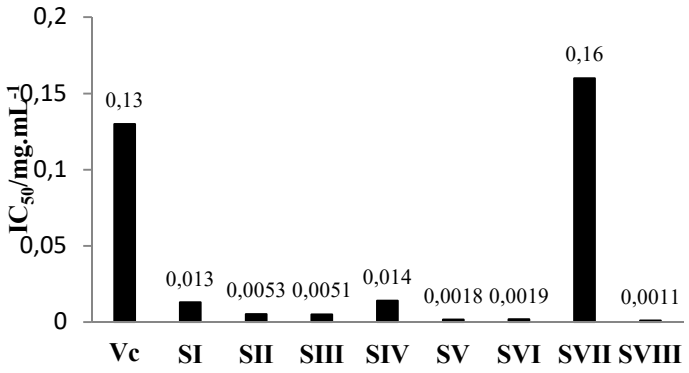
Figure 2. Effect of Magnolia officinalis on the chemiluminescence intensity of pyrogallol-luminol system (a), H_2O_2-luminol system (b) and Fe^{2+}-H_2O_2-luminol system (c). The values are presented as means ± S.D. (n= 5). Vc: ascorbic acid; S_I: water extract; S_{II}: 60% ethanol extract; S_{III}: 80% ethanol extract; S_{IV}: polysaccharides; S_V: alkaloids; S_{VI}: lignans; S_{VII}: magnolol; S_{VIII}: honokiol.



Pyrogallol -luminol CL system (a)



H_2O_2 -luminol CL system (b)



Fe^{2+}-H_2O_2 -luminol CL system (c)

Figure 3. Scavenging activity (expressed as the values of IC50) of Magnolia officinalis on O^{2-}• (a), H_2O_2 (b) and •OH (c). Vc: ascorbic acid; S_I: water extract; S_{II}: 60% ethanol extract; S_{III}: 80% ethanol extract; S_{IV}: polysaccharides; S_V: alkaloids; S_{VI}: lignans; S_{VII}: magnolol; S_{VIII}: honokiol.

To sum up, the IC50 values demonstrated the tested samples' antioxidant activity. Compared with the IC50 values, the tested samples showed the strongest scavenging activity on H_2O_2, followed by •OH and O_2^-•. In this study, alkaloid was the first time to be investigated about its free radical scavenging activity. Besides of the scavenging activity, however, the content in raw materials shouldn't be ignored to evaluate the impact of different active constituents on whole antioxidant activity of raw material.

Total phenol content determination

As shown in Table 1, the total phenols content increased with the liposolubility of solvent. The 80% ethanolic extract contained total phenols up to 228.3mgGAE/g, which was more than double that of water extract. The results coincided with the free radical scavenging activity among three extracts. i.e., the higher total phenols content, the stronger antioxidant activity of antioxidants. On the other hand, although alkaloids showed stronger activity than lignans (mainly are phenols, Chang, Li & Chiang, 2009), considering the gap between the content of alkaloids and phenols in raw materials, phenols were still more representative in evaluation of antioxidant activity of *Magnolia officinalis.* The results illustrated the key point was that there was a positive correlation between the antioxidant activity of extract with the total phenols content.

Table 1. Total phenols content of three kinds of extracts

Extract	Aqueous extract	60% ethanol extract	80% ethanol extract
Total phenols content[1] （mg GAE/g）	111.0	225.1	228.3

Experiments

Chemical and materials

Luminol was purchased from Sigma-Aldrich (St. Louis, MO, USA). Folin-Ciocalteu agent and gallic acid were purchased from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). Ascorbic acid, ethanol, chromotropic acid, Pyrogallol, DMSO were purchased from Chongqing Chemical Reagent Company (Chongqing, China). All chemicals were analytical

[1] Expressed as $mg \cdot g^{-1}$extract as gallic acid equivalent

grade. Water for experiments is redistilled water. The raw materials of Magnolia officinalis cortex were collected in August, 2012 from Wulong, Chongqing of China.

Extraction and separation of Magnolia officinalis cortex

Preparation of Extracts. The air-dried bark materials were pulverized by using mechanical grinder. Powdered bark samples (100g) were soaked for 12 hours by 800 mL water, 60% ethanol and 80% ethanol, respectively. Then, they were refluxed for 3 hours and then repeated two times, extracts were collected and filtered by Whatman No.1filter paper and the filtrate was subsequently concentrated by rotary evaporator at 50◦C (Jiapeng Rotavapor RE-2000A; China) and lyophilized (Freezing Dryer LGJ-10D, Beijing). The extracts were kept in a dark, dry and cool place for further use.

Preparation of polysaccharides. Preparation of polysaccharides was carried out in the way reported previously with some modifications [11]. The powdered bark samples (300g) were refluxed for 1 hour to remove lipids with petroleum ether, and then refluxed for another 1 hour with 95% ethanol to remove oligosaccharides. After the organic solvent was volatilized, the residues were extracted three times in a 8-fold volume of hot water and then filtered. The filtrates were concentrated at 55◦C. After centrifuged (3000 rpm, 10 min), the supernatant was precipitated by the addition of ethanol to a final concentration of 80% (v/v) at 4◦C overnight. Precipitates were collected by centrifugation (2000 rpm, 20 min), washed successively with ethanol and acetone. After lyophilized, 6.95g crude polysaccharides were obtained and polysaccharide content was measured by the phenol-sulfuric acid method with modifications [12]. Using glucose as a standard substance, the polysaccharides solution with concentration of 1.0% (w/v) were prepared by dissolving the polysaccharides in deionized water, 2.0 mL sulfuric acid and 0.4 mL phenol solvent was added into 0.2 mL polysaccharides solution. After a ten minutes boiling water bath and a ten minutes ice-water bath, the absorbance at 490nm was measured by ultraviolet-visible spectrophotometer (Puxi UV spectrophotometer TU-1810, Beijing).

Preparation of alkaloids. The extraction was performed using the modified method described by Kartal et al. [13]. Powdered bark samples (300g) were soaked overnight and refluxed three times with 60% ethanol (1:8, w/v) for 3 hours. After filtering and concentration, the concentrate was subsequently dissolved with distilled water, then treated with HCl (2%, v/v) to a final pH 2-3, and extracted with 100 mL petroleum ether to remove apolar impurities. The aqueous layer was brought to pH 10 with ammonium hydroxide and extracted three times with chloroform. The organic layer was evaporated under vacuum to obtain total alkaloids. The total alkaloids content was measured: Berberine was used as standard substance, 1.0mL sample solvent was mixed with 7.0mL phosphate buffer (pH6.0), then 3.5mL bromothymol blue was subsequently added in, and extracted with 8mL chloroform, after shaking for three minutes and boiling water bath for at least forty minutes, then the absorbance at 420nm was measured.

Preparation of lignans. Powdered bark samples (300g) were soaked overnight and refluxed three times with 80% ethanol (1:8, w/v) for 3 hours each time. Then the combined alcohol extracts were filtrated and centrifuged. The concentrate was extracted with ethyl acetate three times. The organic layer was evaporated under reduced pressure to obtain ethyl acetate extract, which was then dissolved in 50% ethanol and subjected to chromatographic separation on AB-8 macroporous adsorption resin with water, 80%, 90% ethanol at the rate of 1mL/ min, 2mL/min, 2mL/min. respectively. The 80% ethanol fraction was concentrated and then lyophilized to obtained lignans of Mangnolia officinalis [14]. The lignans content was measured by a chromatometry method with chromotropic acid and sulphuric acid as chromogenic agents which was detected at 540nm by UV spectrophotometer.

Purifacation of magnolol and honokiol. The purification of magnolol and honokiol was performed by silica column chromatography. The purity was confirmed by high performance liquid chromatography (Angilent HPLC 1260, Germany).

Free radical scavenging activity determine assays

The optimized CL assays of luminol in our lab were applied to determine the scavenging capacity of extracts prepared above on O2-•, H2O2 and •OH. The chemiluminescence signal of the three kinds of systems were measured, and then the free radical scavenging rate could be calculated. The value of IC50 signify the free radical scavenging capacity. The computational formula of scavenging rate was as fellows:

$$\text{Scavenging rate } (\%)=(I_0-I_t)/I_0 \times 100\%$$

Where I_0 was the chemiluminescence signal of the the essential control (DMSO), while It was the signal of the test sample. All samples were dissolved in DMSO.

Pyrogallol -luminol CL system. The scavenging activity of the superoxide anion (O^{2-}•) was assayed in the Pyrogallol -luminol CL system. 100μL Pyrogallol (5×10^{-4} mol/L) and 100μL sample solvent were added successively into a well which contained 100μL luminol (1×10^{-3}mol/L) at pH 11. Under the optimized conditions, the chemiluminescence signal (It(O^{2-}•)) was recorded by chemiluminiscence detector (Thermo Scientific Fluoroskan Ascent FL, US).

H_2O_2 -luminol CL system. The scavenging activity of the H_2O_2 radical was assayed in the H_2O_2 -luminol CL system. 100μL H_2O_2 (1×10-3 mol/L) and 100μL sample solvent were added successively into a well which contained 100μL luminol (1×10-3mol/L). Under the optimized conditions, the chemiluminescence signal （It（H_2O_2））was recorded by chemiluminiscence detector.

Fe^{2+}-H_2O_2 -luminol CL system. The scavenging activity of the hydroxyl radical (•OH) was assayed in the Fe^{2+}- H_2O_2-luminol CL system. 50μL H_2O_2 (1×10^{-3} mol/L), 50μL $FeSO_4$ (1×10^{-3} mol/L) and 50μL sample solvent were added successively into a well which contained 50μL luminol (1×10^{-3}mol/L). Under the optimized conditions, the chemiluminescence signal (It（•OH ））was recorded by chemiluminiscence detector.

Determination of total phenols contents

The amount of total phenolic was determined by the Folin - Ciocalteu method [15]. Briefly, 0.8 mL of extract and 4.0mL Folin–Ciocalteu phenol reagent were thoroughly mixed. After five minutes, 3.2 mL of 7.5% (w/v) sodium carbonate solution was added to the mixture and shaken thoroughly. The mixture was allowed to stand for thirty minutes in a dark condition and the absorbance at 765nm was measured by a UV spectrophotometer. A calibration curve was established with the gallic acid (range from 0.01 to 0.08 mg/mL, the regression equation of the calibration curve was $y=0.0083x+0.1958$, $R^2=0.99$), the results were expressed as mg gallic acid equivalents per gramme of the sample (mg GAE/g).

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Competing interest

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Ethical approval

This article does not contain any studies with human participants performed by any of the authors.

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