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Determination of DPPH Free Radical Scavenging Activity of Polyphenolic Compounds in *Terminalia* by HPLC

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The spectrophotometric method for the evaluation of 1,1-diphenyl-2-picrylhydrazyl (DPPH•) free radical scavenging activity has certain shortcomings such as like interference from color pigments from natural products and lower sensitivity. A specific HPLC method for evaluating the DPPH free radical scavenging activity of natural products was developed. A gradient elution method was employed to obtain better efficiency of separation. The best chromatographic conditions are as follows. The mobile phase was a mixture of methanol and water (50:50, v/v) pumped at a flow rate of 1 mL/min, the DPPH peaks were monitored at 517 nm and the column temperature was 25 ºC. The proposed HPLC method was applied for the estimation of DPPH free radical scavenging activity of polyphenolic compounds such as gallic acid, vanillic acid, caffeic acid, *p*-coumaric acid and rutin and the extract of *Terminalia*. The 50 % radical scavenging activity (IC_{50}) determined by the HPLC method correlated well with that of spectrophotometry. The HPLC method can be used as a reliable method of estimate the antioxidant activity for natural products.

Keywords: Antioxidants, HPLC, DPPH free radical, Polyphenolic compounds, *Terminalia*.

INTRODUCTION

Herbs are used in many domains, including medicine, nutrition, flavouring, beverages, dyeing, repellents, fragrances, cosmetics[1-2]. Herbal medicine plays an important role in clinical therapy and is widely accepted as an alternative therapy, owing to its unmatched chemical diversity and minimum side effects. *Terminalia* is a well-known crude drug and a key herb ingredient in many important multi-herb remedies in traditional Chinese medicine (TCM) and it has been used for hundreds of years in China and other countries[3]. Polyphenolic compounds are commonly found in both edible and inedible plants and they have been reported to have multiple biological effects, including antioxidant activity[4,5]. Pharmacological studies revealed that polyphenolic compounds including gallic acid, vanillic acid, caffeic acid, *p*-coumaric acid were the main active components in *Terminalia* and all of them have strong antioxidative activities[6,7]. The reports from some publications showed that polyphenolic compounds had various pharmacological properties and could be used as antibacterial, antiinflammatory, antioxidant, anticancer[8]. Polyphenolic compounds in herbs can effectively prevent cerebrovascular and cardiovascular diseases and protect organism from oxidative damages due to their strong antioxidants activity[9-12].

DPPH• as a stable synthetical free radical species is often used for the evaluation of the general radical scavenging capabilities of various antioxidants[13-16]. Traditional spectrophotometry estimation method of DPPH scavenging effect is based on its strong spectral absorption band at 517 nm in methanol[17]. The spectrophotometry is used widely in the estimation of DPPH radical scavenging capabilities, but it is not applicable to evaluate the antioxidant activity of colored botanical extracts with a spectral absorption band near 517 nm due to interference by pigments[18-20]. Therefore, it is necessary to develop more reliable methods for evaluating the DPPH scavenging activitiy of herbal medicine.

Chandrasekar *et al.*[21,22] and Keleva *et al.*[23] developed a HPLC method to evaluate DPPH free radical scavenging activity of synthetic drugs. Compared with spectrophotometry, HPLC method can effectively separated sample from interfering substances with higher accuracy and reliability. In this paper, DPPH free radical scavenging activities of polyphenolic compounds was investigated using HPLC method. The gradient elution was applied to improve chromatographic peaks of

DPPH and eliminate the chromatography interference of other ingredients in the botanical extracts. The DPPH free radical scavenging capacity was quantified based on the linearity regression of antioxidant concentration and DPPH free radical scavenging rate. And the proposed method was used to determinate the antioxidant of *Terminalia* polyphenols. The purpose of this paper was to develop a HPLC method specific for the determination of the DPPH free radical scavenging activities of polyphenolic compounds in herb medicine.

EXPERIMENTAL

DPPH (the purity higher then 99 %) was obtained from Sigma. Gallic acid, vanillic acid, caffeic acid, *p*-coumaric acid and rutin (used as reference substance, the purity higher then 99 %) were purchased from National Institute of China for the Control of Pharmaceutical and Biological Products (NICPBP, Bejing, China). Methanol (HPLC-grade) was from Tedia (Fairfield, OH). All aqueous solutions were prepared with purified water (Wahaha, Hangzhou, China).

Sample preparation: The DPPH and all standard antioxidants including extracts of *Terminalia* were soluble in methanol. Fresh DPPH stock solution (10 mL) at a concentration of 2 mM/L was prepared on each day of analysis. The stock solutions of gallic acid, caffeic acid, rutin and extracts of *Terminalia* were prepared in methanol at a concentration of 1 mg/mL. The stock solutions of vanillic acid and *p*-coumaric acid were prepared in methanol at a concentration of 50 mg/mL and stored at -20 ºC. An aliquot of 10 µL of different concentrations of standard antioxidants or extracts of *Terminalia* in methanol were added to 100 µL of DPPH in solution (final concentration 1 mM/L). The mixture was vortexed for few seconds and left to stand in the dark for 30 min at room temperature.

HPLC analysis: The sample is filtered through 0.2 µm nylon membrane filter (Pall Gelman Laboratory, USA) and 20 µL of the sample is injected for HPLC analysis. The blank was prepared by adding 100 µL of methanol to 100 µL of DPPH stock Solution (final concentration 1 mM/L) and included before each run. Agilent1200 series high performance liquid chromatograph system (Aglient Technologies, USA), equipped with DAD diode array detector (G1315A), quaternary pump (G1311A), automatic sampler (SIL-10AP), UV variable-wavelength detector (1314A-UV) and column oven (CTO-10ASVP).The analysis column was a XDB-C_{18} column (150 × 4.6 mm, i.d, 5 µm) maintained at 25 ºC. The mobile phase consisted of methanol (A) and H_2O (B). A linear gradient of mobile phase A was applied during the analysis. The gradient profile was as follows: 0-15 min (50 % A), 15-20 min (90 % A). The flow rate of mobile phase was 1 mL min^{-1}. The injection volume was 20 µL. The DPPH peaks were monitored at 517 nm. The difference in the reduction of DPPH peak area (PA) between the blank and the sample was used for determining the percent radical scavenging activity of the sample.

$$\text{Radical scavenging (\%)} = [PA_{BLANK}\text{-}PA_{SAMPLE}]/\ PA_{BLANK} \times 100\ \%$$

RESULTS AND DISCUSSION

Optimization of mobile phase: In the present work, the gradient elution was used in HPLC method. As DPPH was soluble in methanol, for HPLC mobile phase optimization different ratios of methanol and water including 50:50, 70:30 and 80:20 (v/v) were examined. When the methanol content was 20 %, DPPH retention time was too short and the peak shape is not so better, DPPH retention time was found to decrease on increasing the organic phase content. DPPH eluted as a sharp peak at 14.955 min when the mobile phase ratio was 50:50. The method was specific for DPPH with a run time of 20 min. Good resolution and symmetrical peak shape was obtained as shown in Fig. 1a, which indicating that the

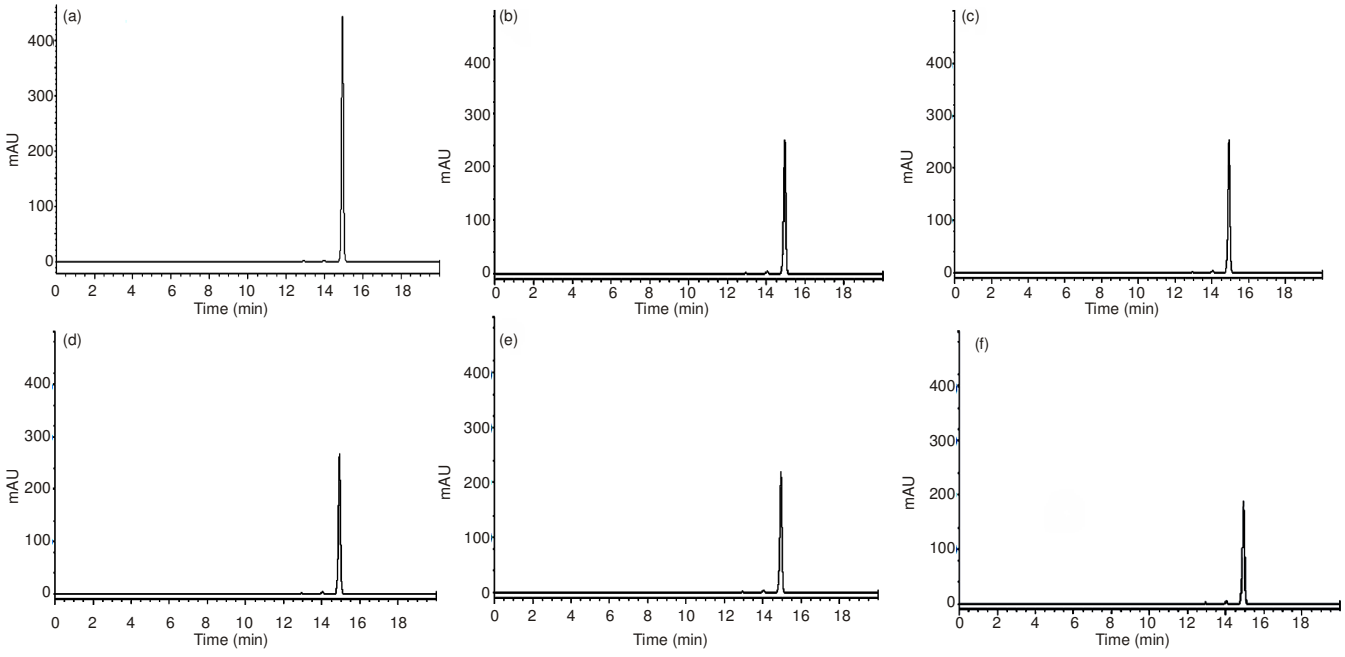

Fig. 1. (a) Blank and after incubation with; (b) gallic acid 10 µL; (c) caffeic acid 30 µL; (d) vanillic acid 30 µL; (e) *p*-coumaric acid 150 µL and (f) pure extracts of terminalia 40 µL. HPLC conditions: column, a Agilent1200 XDB-C_{18} (4.6 × 150 mm i.d, 5 µm) column; mobile phase, a mixture of methanol and water (50:50, v/v); flow rate, 1 mL/min; detection wavelength, 517 nm; column temperature, 25 ºC; injection volume, 20 µL

peak was well separated and the chromatography interferences of other ingredients in the *Terminalia* extracts was eliminated by the gradient elution method.

As shown in Fig. 1a, the HPLC chromatograms of DPPH blank solution with the concentration of 1 mM/L (as the blank). As gallic acid, caffeic acid, vanillic acid, *p*-coumaric acid and pure extracts of *Terminalia* was addition into DPPH blank solution, respectively, the peak area of DPPH was decreased obviously, the peak area and four polyphenolic compounds have dose-response relationship, which indicated that the proposed HPLC can be used to estimate the DPPH free radical scavenging activity of polyphenolic compounds.

Repeatability and reproducibility: The repeatability and reproducibility of the analytical method was confirmed from the peak area and retention times of the DPPH blank solution. The results as listed in Table-1, indicated good repeatability and inter-day precision with acceptable RSD (< 4 %).

TABLE-1
REPEATABILITY AND REPRODUCIBILITY OF THE RETENTION TIME AND PEAK AREA OF DPPH BLANK SOLUTION (n=6)

	Retention time (min)	RSD (%)	Peak area	RSD (%)
Day 1	14.955	0.25	3049	3.8
	14.937		2951	
	14.931		3250	
	14.926		3247	
	14.886		3101	
	14.856		3189	
Day 2	14.931	0.18	3047	4.0
	14.918		2940	
	14.95		3054	
	14.923		3205	
	14.876		3280	
	14.951		3189	
Day 3	14.942	0.15	3263	2.9
	14.922		3031	
	14.924		3200	
	14.891		3245	
	14.882		3078	
	14.899		3124	

Linearity graphs of standard antioxidants: The linearity regression of antioxidant concentration and DPPH free radical scavenging activity was tested (Table-2). Good linear correlation for all antioxidants was obtained. In addition, the 50 % radical scavenging activity (IC_{50}) of the antioxidants can be calculated based on the calibration equation as showed in Table-3. Because lower IC_{50} value means high radical scavenging activity, the radical scavenging activity was ranked as follows: gallic acid > caffeic acid > extracts of *Terminalia* > rutin > vanillic > *p*-coumaric acid.

TABLE-2
LINEARITY GRAPH OF STANDARD ANTIOXIDANTS BY HPLC METHOD

Standards	Calibration equation	Correlation coefficient (r)	Linear range (mg/mL)
Gallic acid	y=3507.4x+4.3541	0.9927	0.0025-0.025
Caffeic acid	y=1386.7x+6.1953	0.9889	0.005-0.06
Vanillic acid	y=24.286x+6.7143	0.9653	0.5-3.0
p-Coumaric acid	y=4.8386x+7.7857	0.9628	2.5-15
Rutin	y=709.79x+8.2867	0.9861	0.01-0.12
Extracts of *Terminalia*	y=1285.7x+4.0000	0.9893	0.01-0.07

TABLE-3
FREE RADICAL SCAVENGING ACTIVITY OF ANTIOXIDANTS AND EXTRACT OF *Terminalia*

Antioxidants (mg/mL)	IC_{50} concentration (mg/mL)	Maximum clearance rate (%)	Concentration of antioxidants (mg/mL)
Gallic acid	0.013	93.4	0.035
Caffeic acid	0.032	93.5	0.080
Vanillic acid	1.800	85.0	0.500
p-Coumaric acid	8.700	80.0	25.000
Rutin	0.058	94.0	0.140
Extracts of *Terminalia*	0.035	93.0	0.090

The DPPH radical scavenging activity of the antioxidants can also be evaluated *via* the comparison of the slope of their calibration equation. According to the calibration equation showed in Table-2, the DPPH radical scavenging activity was listed as follows: gallic acid > caffeic acid > extracts of *Terminalia* > rutin > vanillic > *p*-coumaric acid, which was agreement with the result from IC_{50}. According to IC_{50} and the slope of calibration equation, gallic acid and caffeic acid exhibit high radical scavenging activity as comparison with rutin, an acknowledged antioxidant. Gallic acid and caffeic acid are the main active components in *Terminalia*, so we could expected pure extracts of *Terminalia* have strong antioxidative activities.

Determination of IC_{50} values: The 50 % radical scavenging activity (IC_{50}) of the antioxidants and extracts of *Terminalia* is given in Table-3. Based on the IC_{50} values calculated from the calibration equation of various antioxidants, the IC_{50} value of the antioxidants and extracts of *Terminalia* were 0.013, 0.032, 1.8, 8.7, 0.058 and 0.035 mg/mL, respectively. As Fig. 2 showed, the maximum scavenging rate was ranked as follows: rutin (0.14 mg/mL) > gallic acid (0.035 mg/mL) > caffeic acid (0.08 mg/mL) > extracts of *Terminalia* (0.09 mg/mL) > vanillic (0.5 mg/mL) > *p*-coumaric acid (25 mg/mL).

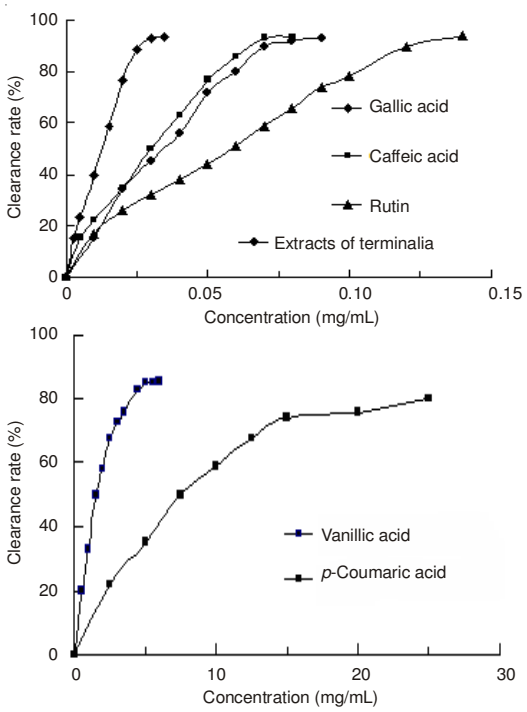

Fig. 2. Clearance rate of four polyphenolic compounds and rutin to DDPH free radicals

Conclusion

An HPLC method with good reliable and high reproducibility was developed specific for the determination of the DPPH free radical scavenging activities of polyphenolic compounds in herb medicine. Compared with the pre-existing HPLC-DPPH method, the proposed method improved peak shape and efficiency of separation and eliminated the chromatography interference of other ingredients in the *Terminalia* extracts by the gradient elution. And the results were more accurate and reliable. The DPPH radical scavenging activity of herb medicines can be evaluated *via* the comparison of the slope of their calibration equation and the 50 % radical scavenging activity (IC_{50}) of the antioxidants can be calculated based on the calibration equation. The IC_{50} values determined by the HPLC method agreed well with those by spectrophotometry. The proposed method can be used as alternative approaches of spectrophotometry in order to get more accurate results. HPLC method was successfully applied for the determination of antioxidant activity of polyphenolic compounds and it has potential application value in evaluating the antioxidant activities of traditional herb medicine.

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