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Full Length Research Paper

Improvement of the antioxidant activity of apigenin in linoleic acid system by o-prenylation

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With apigenin as raw material, 5-prenyloxy apigenin was synthesized and identified by high performance liquid chromatography - ultra violet - electrospray ionization tandem/ mass spectrometry (HPLC-UV-ESI/MS). And its antioxidant activity in linoleic acid system was superior to that of apigenin, which could support our hypothesis about antioxidant function regions of flavonoids.

Key words: Apigenin, o-prenylation, antioxidant, high performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-ESI/MS).

INTRODUCTION

A number of studies have shown that natural antioxidants from plant sources can effectively inhibit oxidation in food and reduce the risk of age-dependent diseases. Flavonoids, widespread in fruits, vegetables, teas and medicinal plants, have received the greatest attention, and have been studied extensively, since they are highly effective antioxidants, less toxic than synthetic antioxidants such as butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT) (Burda and Oleszek, 2001; Hollman et al., 1996).

In our previous study (Yang et al., 2009; Liu et al., 2010), by analyzing the position of the most active hydroxyl group, the composition of highest occupied molecular orbital (HOMO) and their relationships with the antioxidant activity of flavonoids, It was found that that B ring and the hydroxyl groups on B ring made a great contribution to the antioxidant activity of flavonoids. It was concluded that molecular structure of flavonoids could be divided into three functional regions: the crucial region of antioxidant activity (B ring), the accessorial region of antioxidant activity (C ring) and the adjustable region of solubility and affinity (A ring) (Figure 1). The division of the antioxidant functional regions suggested that the solubility of flavonoids in either water-or oil-phase could be improved by introducing hydrophilic and hydrophobic groups to A ring, which could make flavonoids fully play their antioxidant activity. The objective of this study was to check the aforementioned hypothesis by synthesizing o-prenylated apigenin and comparing the antioxidant activity of apigenin and its derivative in linoleic acid system.

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MATERIALS AND METHODS

Materials and chemicals

Leaves of *Adinandra nitida* (2007 production, moisture content 9.3%) for this study were purchased in Pingle, China, and identified by Professor Yousheng Zhang in Guangdong Academy of Agricultural Sciences. Isoprenyl bromide was purchased from Johnson Matthey Company. Linoleic acid (> 99%) was a product of AMRESCO Inc. (USA). HPLC grade methanol was supplied by DIKMA. Other chemical were of analytical grade.

Preparing apigenin from *Adinandra nitida* leaves

Apigenin was prepared according to our previous study (Liu et al., 2008), slightly modified as follows: the leaves of *A. nitida* (200 g) were extracted twice with 2000 ml of boiling water for 1 h and then filtered. After cooling, sulphuric acid was added to the extract in the ratio of 1:50 (by volume). The mixture was heated for 20 min and then filtered to collect the yellow precipitate. The precipitate was washed on the filter until neutral pH was obtained and then dried. About 21.5 g of raw product were obtained from the yellow powder. By recrystallizing for four times from ethanol, about 4.2 g of pure product were obtained.

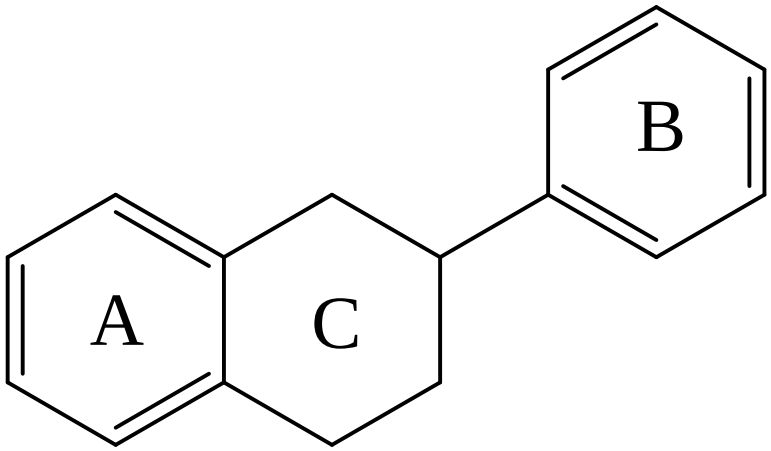

Figure 1. Basic structure of flavonoids.

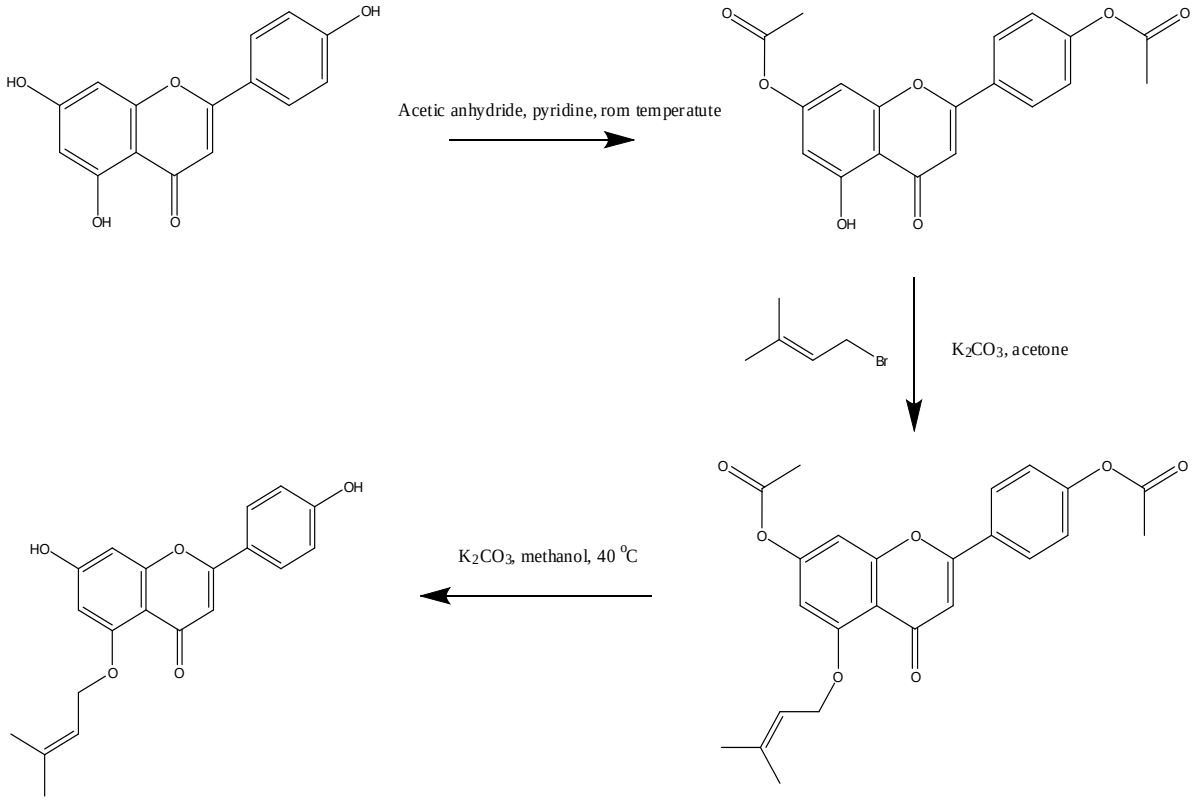

Figure 2. Synthetic route of 5-prenyloxy apigenin.

Synthesizing of 4′, 7-diacetoxyapigenin

4′, 7-diacetoxyapigenin was prepared according to our previous study (Xu et al., 2010), acetic anhydride (0.94 ml, 10.0 mmol) was added dropwise to a solution of apigenin (1.35 g, 5.00 mmol) in dry pyridine (80 ml) at room temperature. After stirring the mixture for 24 h at room temperature, it was poured into ice-cold water (1,600 ml). The white precipitate was separated by filtration, washed twice with a small amount of ice-cold water and recrystallized from methanol (300 ml). The resultant material was washed with distilled water and dried at 60°C to yield the product (0.842 g) as a slightly white solid.

Synthesizing of 5-prenyloxy apigenin

The synthetic route of 5-prenyloxy apigenin was shown in Figure 2. Isoprenyl bromide (0.8 mmol) was added dropwise to a solution of 4′, 7-diacetoxyapigenin (140 mg, 0.4 mmmol) and K_2CO_3 (1 g) in

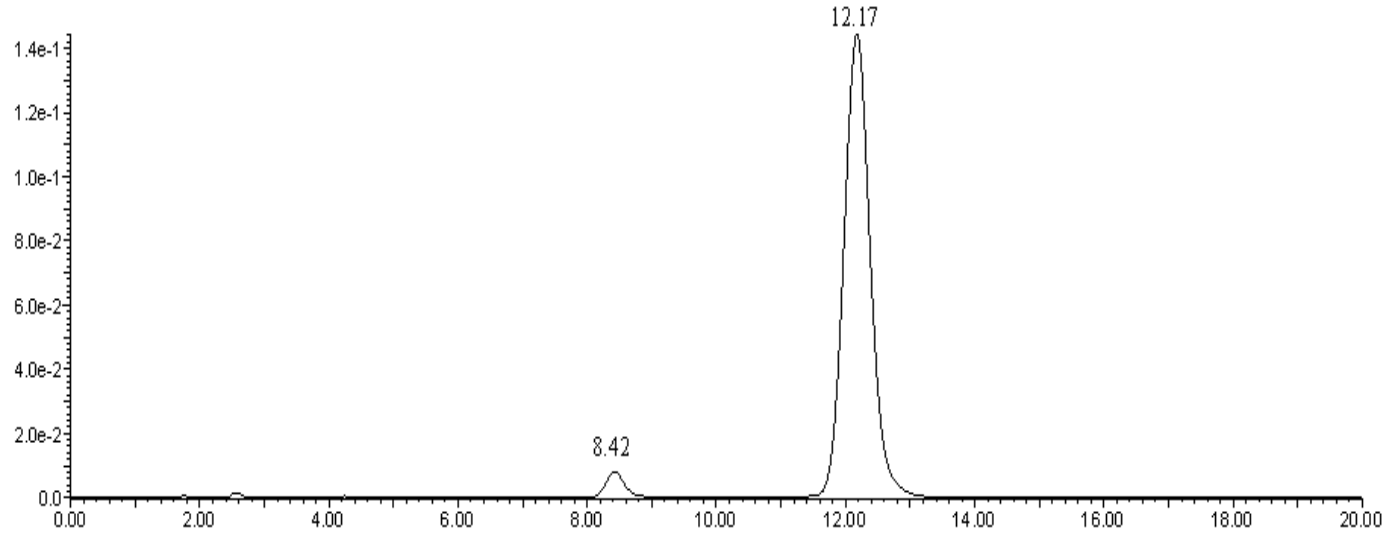

Figure 3. HPLC-UV profile of the product.

acetone (20 ml). The mixture was heated to reflux for 10 h. After removal of acetone under vacuum at room temperature, the residue was mixed with methanol (40 ml) and stirred for 2 h at 40°C. The resultant solution was poured into ice-cold water (400 ml, pH 5.0). The yellow precipitate was extracted by ethyl acetate. After removal ethyl acetate under vacuum at room temperature, about 108 mg of the product was gained.

Identification of the product by using HPLC-UV-ESI/MS

High performance liquid chromatography – electrospray tandem mass spectrometry (HPLC-ESI/MS) analysis was performed on a Waters ZQ 2000 mass spectrometer equipped with a Waters 1525 Binary HPLC Pump and a Waters 2487 Dual λ Absorbance Detector. Separation was on a reversed phase column, Diamonsil® C18 column (4.6 × 250 mm; 5 μm particle size, DIKMA, China). The mobile phase consisted of methanol and water (7:3). The flow rate was 1 ml/min. The wavelength for detection was set at 260 nm. Because of the acidic nature of flavonoids, the mass spectrometer was operated in negative electrospray mode to gain the best result with capillary voltage of 3 KV. Deprotonated molecular ions were observed at cone voltage of 20 and 80 V. Nitrogen was used as nebulizing gas with cone gas flow at 50 L/h, desolvation gas flow at 350 L/h. Source and desolvation temperatures were set at 100 and 250°C, respectively. The scan range was set from 100 to 800 m/z. The data were recorded and processed by Masslynx 4.1software.

Determination of antioxidant activity in linoleic acid system

The total antioxidant activities of the samples were carried out by use of a linoleic acid system (Zou et al., 2004). The linoleic acid emulsion was prepared by mixing 0.28 g of linoleic acid, 0.28 g of Tween 20 as emulsifier, and 50 ml of phosphate buffer (0.2 M, pH 7.0), and then the mixture was homogenized. A 0.5 ml ethanol solution of the samples (0.5 mg/ml) was mixed with linoleic acid emulsion (2.5 ml, 0.2 M, pH 7.0) and phosphate buffer (2 ml, 0.2 M, pH 7.0). The reaction mixture was incubated at 37°C in the dark to accelerate the peroxidation. The levels of peroxidation were determined according to the thiocyanate method (0.1 ml, 30%), sample solution (0.1 ml), and ferrous chloride (0.1 ml, 20 mM in 3.5% HCl). After the mixture was left for 3 min, the peroxide value was determined by reading the absorbance at 500 nm on a spectrophotometer.

Statistical analysis

The data obtained in this study were expressed as the mean of three replicate determinations and standard deviation (SD). Statistical comparisons were made with student's test. P values of < 0.05 were considered to be significant.

RESULTS AND DISCUSSION

HPLC-UV-ESI/MS analysis

Under the HPLC conditions mentioned in experimental section, good separation of the product was obtained in 20 min with UV detector recorded at 260 nm, as shown in Figure 3. And two components were detected by UV and MS. The ESI-MS spectra of component 1 (retention time 8.42 min) at cone voltage of 20 and 80 V were shown in Figure 4. This minor component was identified as apigenin by comparison with apigenin standard and the following ESI-MS analysis: 269 ($[M-H]^-$), 539 ($[2M-H]^-$).

The ESI-MS spectra of the main component (retention time 12.17 min) at cone voltage of 20 and 80 V were shown in Figure 5. From the ESI-MS spectrum of cone voltage 20 v, the $[M-H]^-$ and $[2M-H]^-$ of the main component were 337 and 675, respectively. It could be concluded that molecule weight of the compound was 338, which coincide with the molecular weight of 5-prenyloxy apigenin. When the cone voltage was increased to 80 v, the prenyl group of the compound was removed, the parent nucleus structure of the compound was identified

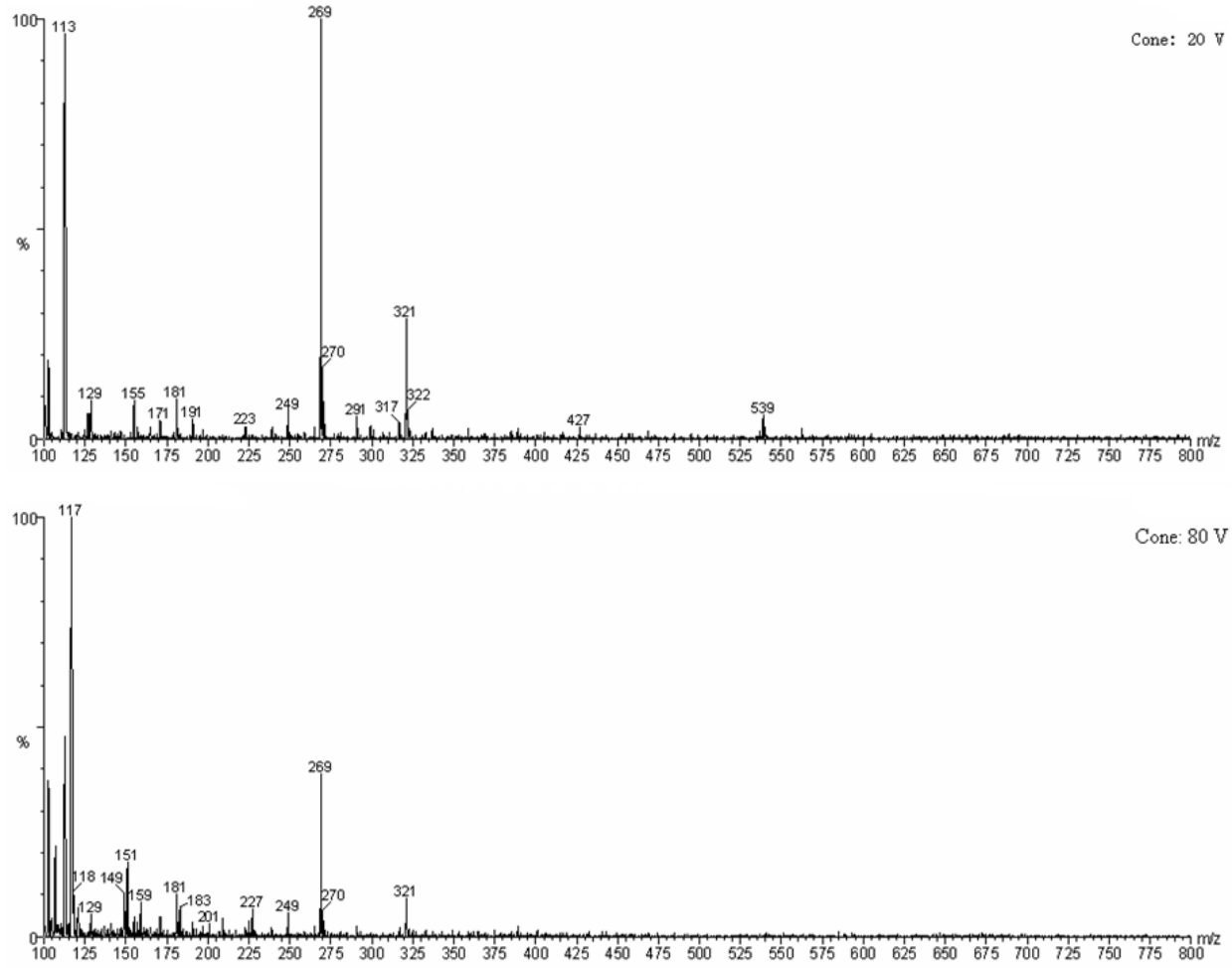

Figure 4. ESI-MS of compound 1 at cone voltage of 20 and 80 V.

as apigenin for the presence of the peak of 269. Based on the HPLC-ESI/MS analysis and the synthesis of 4′, 7-diacetoxyapigenin, 5-prenyloxy apigenin was successfully synthesized in this study. And the HPLC-UV analysis showed that the purity of 5-prenyloxy apigenin was more than 96%, which could be used for the following antioxidant assay.

Antioxidant activity in a linoleic acid system

The improvement of oil-solubility of flavonoids by using chemical modification had been reported. Li et al. (2005) found that the antioxidant activity of dihydromyricetin in lard system could be strengthened by etherifying with lauroyl chloride. But few studies about selective modification of flavonoids had been published. By using quantum chemistry method, the relationships between highest occupied molecular orbital (HOMO), the most active hydroxyl group of common flavonoids and their antioxidant activity were investigated in our previous study. And on the basis of these analyses, molecular structures of flavonoids could be divided into three function regions (Figure 1): crucial region for antioxidant activity (Ring B), accessorial region for antioxidant activity (Ring C), adjustive region for affinity and solubility (Ring A) (Figure 1). According to the division of the functional regions and the molecular structure of apigenin, it was presumed that the synthesis of 5-prenyloxy apigenin could improve the antioxidant activity and stability of apigenin in lipophilic systems. In this study, the antioxidant activity of the samples was determined by peroxidation of linoleic acid using the thiocyanate method at 37°C. During the linoleic acid peroxidation, peroxides are formed and these compounds oxidized Fe^{2+} to Fe^{3+}, the latter Fe^{3+} ion forms a complex with SCN^-, which has a maximum absorbance at 500 nm. High absorbance is an indication of high concentration of peroxide during the emulsion incubation. The antioxidant activity of the samples exhibited an amount dependent manner, as shown in Figure 6. The antioxidant activity of the samples followed the following order: 10 mmol/L 5-prenyloxy apigen in > 10

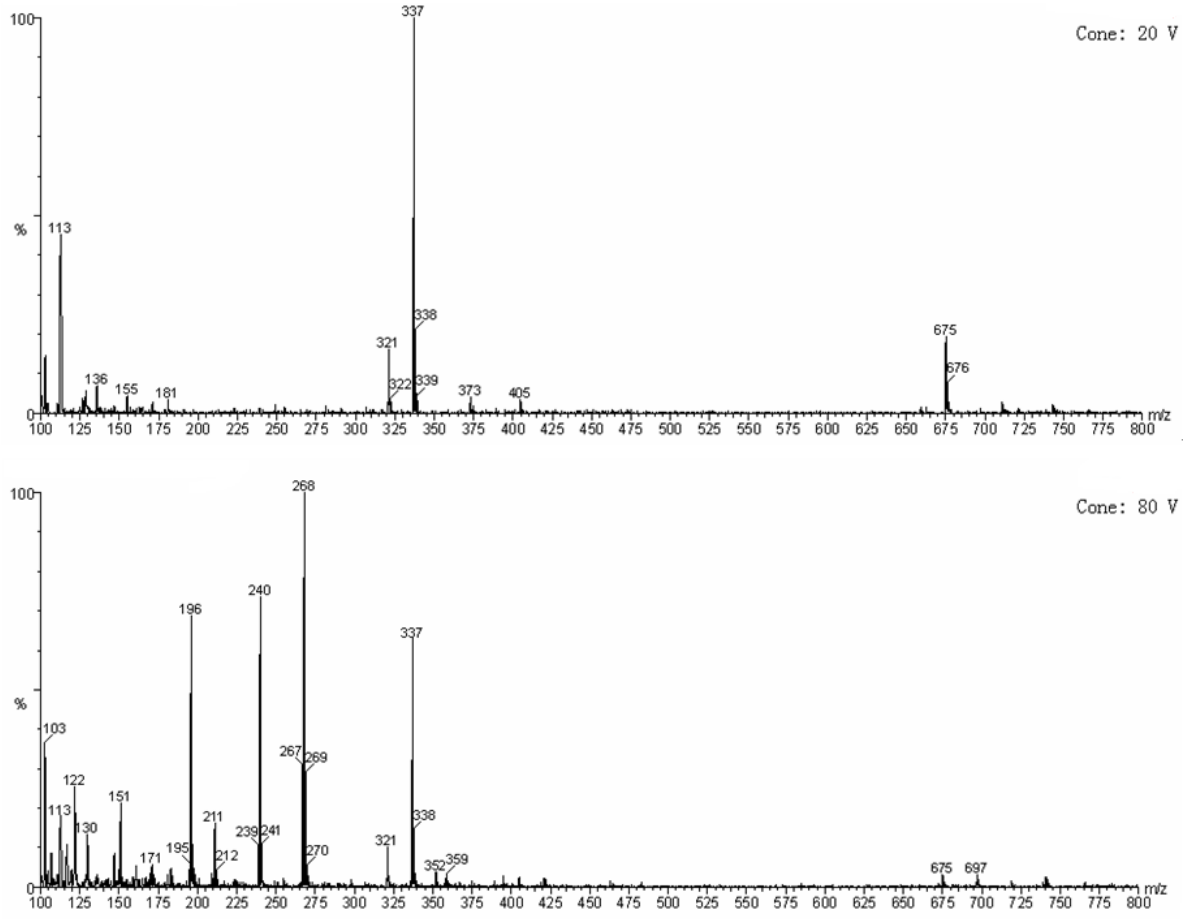

Figure 5. ESI-MS of compound 2 at cone voltage of 20 and 80 V.

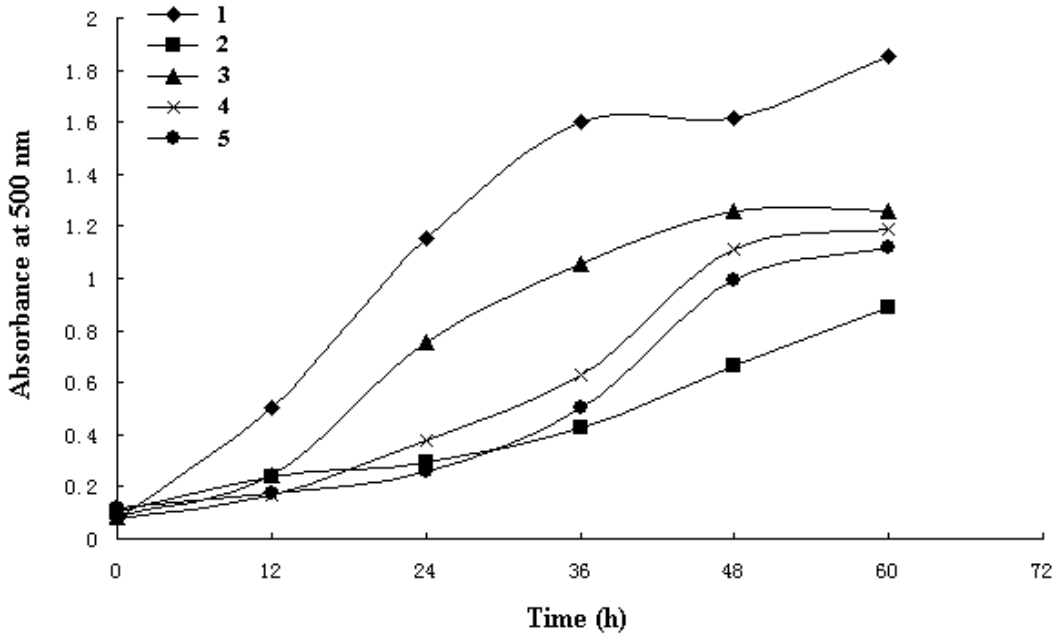

Figure 6. Antioxidant activity of the samples in linoleic acid system (1: Control; 2: 10 mmol/L 5-prenyloxy apigenin; 3: 1 mmol/L 5-prenyloxy apigenin; 4: 1 mmol/L apigenin; 5: 10 mmol/L apigenin).

mmol/L apigenin > 1 mmol/L apigenin > 1 mmol/L 5-prenyloxy apigenin > Control. At the concentration of 1 mmol/L, apigenin could be completely dissolved in the linoleic acid system and its number of free phenolic hydroxyl group was more than that of 5-prenyloxy apigenin, so the antioxidant performance of apigenin was superior to that of 5-prenyloxy apigenin. At the concentration of 10 mmol/L, the linoleic system could not contain 10 mmol/L apigenin while 10 mmol/L 5-prenyloxy apigenin could completely dissolve and perform the antioxidant activity. As a result, the antioxidant performance of 5-prenyloxy apigenin was higher than that of apigenin, which could support our hypothesis about antioxidant function regions of flavonoids.

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