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Study on the Effect of Several Natural Products on Tyrosine Damage Induced by Peroxynitrite

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Abstract: Peroxynitrite ($ONOO^-$), a powerful oxidant, is produced by nitric oxide ($NO^·$) and superoxide anion ($O_2^{·-}$). Under the physiological condition, the $ONOO^-$ could oxidize the lipids, nitrifyproteins, damage DNA and others biomolecules, thereby harm human health. The study used natural products Capsanthin, Myricetin and Capsaicin as the research object and controlled with Vc, and developed the method of HPLC-DAD to separate the components of nitrification damage system, which could determine the inhibition rate of natural products on the formation of 3-nitrotyrosine (3-NT). The fluorescence spectrometry was employed to determine the ability of these substances to inhibit tyrosine dimer and inhibit the self-oxidation of phthalate. The results showed that Capsanthin, Myricetin and Capsaicin had strong inhibitory effect on the generation capacity of 3-NT and tyrosine dimer, and strong inhibitory effect on the self-oxidation of phthalate.

Keywords: HPLC-UV, Fluorescence Spectrometry, Peroxynitrite, Tyrosine Damage, Flavonoid Pigments, Pyrogallol

1. Introduction

Under the condition of oxidative stress, nitric oxide free radical ($NO^·$) and superoxide anion free radical ($O_2^{·-}$) will be combined to a cytotoxic substance, nitrite peroxide ($ONOO^-$) with strong oxidation, nitrification ability. $ONOO^-$ under neutral condition (pH 7.4) has a half-life of 1.9 s [1], the diffusivity and damage ability were higher than $O_2^{·-}$ and $NO^·$. The product of $ONOO^-$ induced tyrosine nitration to form 3-nitrotyrosine (3-NT). 3-NT has been used as a marker of protein nitration damage and its own accumulation can also cause oxidative damage in the body [2-5]. $ONOO^-$ oxidative damage of tyrosine not only produces tyrosine dimer, but also affects the physiological structure of proteins [6-8]. Therefore, research on free radical inhibitor is of great significance to protect human health.

Natural products are important ingredients for medicines, in recent years a variety of natural products have been found to inhibit $ONOO^-$ damage, a large number of studies have shown that flavonoids compounds have strong antioxidant activity [9-12], and also these compounds have obvious pharmacological effects on cardiovascular disease treatment and anti-tumor [13]. Studies have shown that the antioxidant effect of flavonoids is closely related to the phenol hydroxyl group in the adjacent position. ChangHui [14] reported 23 kinds of flavonoids on human leukemia HL-60 cell role, in which the effects on inhibiting the HL-60 cell proliferation were found double bond, ortho hydroxyl and hydroxyl groups in the flavonoids. Li Yongzheng [15] found that three hydroxyl isoflavones could activate AMPK pathway of the cervical cancer, increase the mitochondrial TSC1/TSC2 in complex formation and hinder the mediated protein of mTOR translation pathway, induce its apoptosis. Since multiple natural products have fall blood sugar [16], enhance immunity [17], antiviral [18], bacteriostatic [19] and other functions [20-22], and non-toxic harmless, natural products play important roles on human tumors, aging, the treatment of degenerative diseases such as diabetes, cardiovascular disease and prevention.

The experiment established HPLC to separate the component of the system, and studied the ability of several natural products, such as Capsanthin, Myricetin and Capsaicin, to inhibit the nitrification of tyrosine. We also explore the ability of these substances to inhibit the oxidative damage of

tyrosine by fluorescence spectrometry [23], and the antioxidant mechanism was investigated by inhibiting the oxidation of pyrogallol.

2. Materials and Method

2.1. Apparatus and Reagents

E2695 high performance liquid chromatography (Waters company, USA), SunFireRM C 18 (4.6×150 mm, including 5 μm) chromatographic column (Waters company, USA), U-3010 ultraviolet-visible spectrophotometer Hitachi (Japan), SH-3 Constant temperature magnetic stirrer (Beijing jinziguang science and technology development co. LTD), KQ-500 DB CNC ultrasonic cleaning device (Kunshan ultrasonic instrument co. LTD), PHS-25 digital pH meter (Shanghai precision scientific instruments company), DKB-450 A Water Bath (Shanghai senxin testing instrument co. LTD), BS–210 S electronic scales (Shanghai ShunYu constant flat scientific instrument co. LTD), PR 020 XXM 1 water purification machine (Beijing PALL pure water company), DL-300 hand-held centrifuge (Beijing dingguo biology company), SHB-III Circulating water multipurpose vacuum pump (Shanghai co. LTD). F-4500 fluorospectro photometer (Japan Hitachi).

Iso-amyl nitrite was purchased from the chemical reagent co. LTD. N-hexane, hydrogen peroxide (30%), potassium hydrogen phosphate, potassium dihydrogen phosphate, vitamin C, focal gallic acid and anhydrous ethanol were bought from the Tianjin fuchen chemical reagent factory. Manganese dioxide, sodium hydroxide, hydrochloric acid and sodium bisulfite were bought from Beijing chemical plant. L-tyrosine, L-3- nitro tyrosine, methanol, Capsanthin, Myricetin and Capsaicin were bought from Shanghai antispectrum science instrument co. LTD. Trimethyl-aminomethane and chlorinated hemoglobin were obtained from Beijing dingguo biotechnology co. LTD. Capsanthin, Myricetin and Capsaicin were allocated to 1×10^{-3} mol/L reserves used anhydrous ethanol., and Vc used deionized water to be configured as 1×10^{-3} mol/L.

2.2. Synthesis of Peroxynitrite

Use the Rao M. Uppu and William A. Pryor method to prepare the $ONOO^-$ solution, The $ONOO^-$ solution was diluted with 0.1 mol/L of sodium hydroxide solution before use. The concentration of nitrite was measured by UV-visible spectrophotometer.

2.3. HPLC method Has Been Used to Inhibit the Nitrification of Tyrosine in Several Natural Products

2.3.1. Production of the 3-NT Standard Curve

Weigh 11.3 mg 3-NT standard substance, pH 7.4 PBS buffer (0.05 mM) was used to constant 50 mL to acquire 1 mM 3-NT standard solution, and then diluted to 5 μM, 10 μM, 25 μM, 50 μM, 100 μM and 250 μM. Take above solution 1 mL filtered by 0.22 nm organic phase filter, then contents of 3-NT were measured by high performance liquid chromatograph (HPLC) and the 3-NT concentration was the x-coordinate, the peak area was the y-coordinate, and the standard curve was drawn. Chromatographic conditions were as follows: chromatographic column chooses the SunFire^{RM} C 18 anti-phase chromatographic column with a specification of 4.6×150 mm and a particle diameter of 5 μm. Mobile phaseV (methanol): V (PBS)=10:90 (Ph 7.4), the flow rate is 1.0 mL/min, sample quantity 10 μL, column temperature at 37°C, detection wavelength at 420 nm.

2.3.2. The Effect of Natural Products on 3-NT Generation Induced by Peroxynitrite

1.5 mL Tyr solution was firstly added in brown EP tube, secondly different volume of the natural products were added, the final concentration is 1.67 μM, 3.33 μM, 8.33 μM, 11.7 μM, 16.7 μM, 25 μM and 33.3 μM, then use the PBS solution to reach 2.9 mL mixture in 37°C water bath after incubation for 15 min, add 100 μL $ONOO^-$ solution in 37°C water bath 1h. The chromatographic condition is equal to 2.3.1.

2.4. The Inhibitory Effect of Several Natural Products on the Induction of Tyrosine Oxidation

4.40 mL Tris-HCl buffer (pH 7.4), 500 μL tyrosine (1.0×10^{-3} M), 50 μL chlorinated heme (1.0×10^{-6} M) and different volume of natural product (1.0×10^{-4} M) were added to 10 mL plastic centrifuge tubes, with no water ethanol as the blank control. Add 50 μL $ONOO^-$ to the reaction solution (the concentration of $ONOO^-$ concentration was determined by UV spectrophotometer and diluted to 1.0×10^{-2} M), with an instant vortex 10s, the subsequent reaction 20 min in 37°C water-bath. Take 3 ml of solution to a fluorescence cuvette. The fluorescence cuvette were determined by fluorescence spectrometry. Fluorescence spectrometer main parameters were as follows: scanning mode: Emission; data mode: Fluorescence; excitation wavelength: 317 nm; Emission wavelength range: 350-500 nm; scanning speed: 1200 nm/min.

2.5. The Inhibition Effect of Natural Products on the Spontaneous Oxidation of Pyrogallol

Take 2.80 mL Tris-HCl buffer (pH 8.2) and 100 μL deionized water into 5 mL colorimetric tube together, 0.10 mL (60 M) pyrogallol solution into another 5 mL colorimetric tube, the two colorimetric tube insulated at the same time in the 25°C water bath for 10 min. After that, the two tubes were quickly mixed and shaked. After 30s put in a fluorescence cuvette, the solution was determined by fluorescence spectrometry. Record fluorescence kinetics curves under the condition of the excitation wavelength at 446 nm and emission wavelength at 502 nm. The slope of the curve 0-5 min is V_0, change 0.10 mL water into natural product solution with different concentrations, the slope of the fluorescence curve is V_S. Main parameters of fluorescence spectrum were as follows: Scanning mode: Time; Data mode: Fluorescence; Excitation wavelength: 446 nm; Emission wavelength: 502 nm.

3. Results and Discussion

3.1. The Effect of Natural Products on the Inhibition of ONOO⁻ Induced Tyr

3.1.1. 3-NT Standard Curve

In this paper, the 3-NT chromatographic peak is obviously separated from other peaks and can be accurately quantified. Standard curve y=310046 x-337.73, R^2=0.9981.

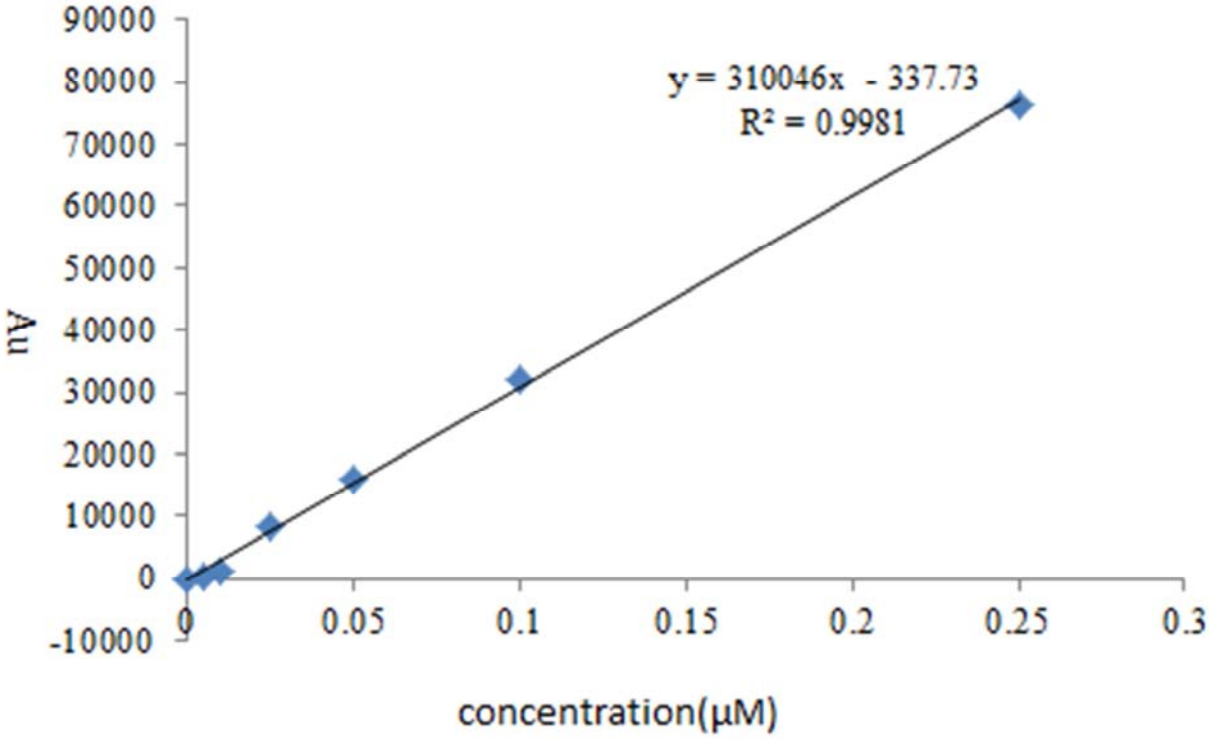
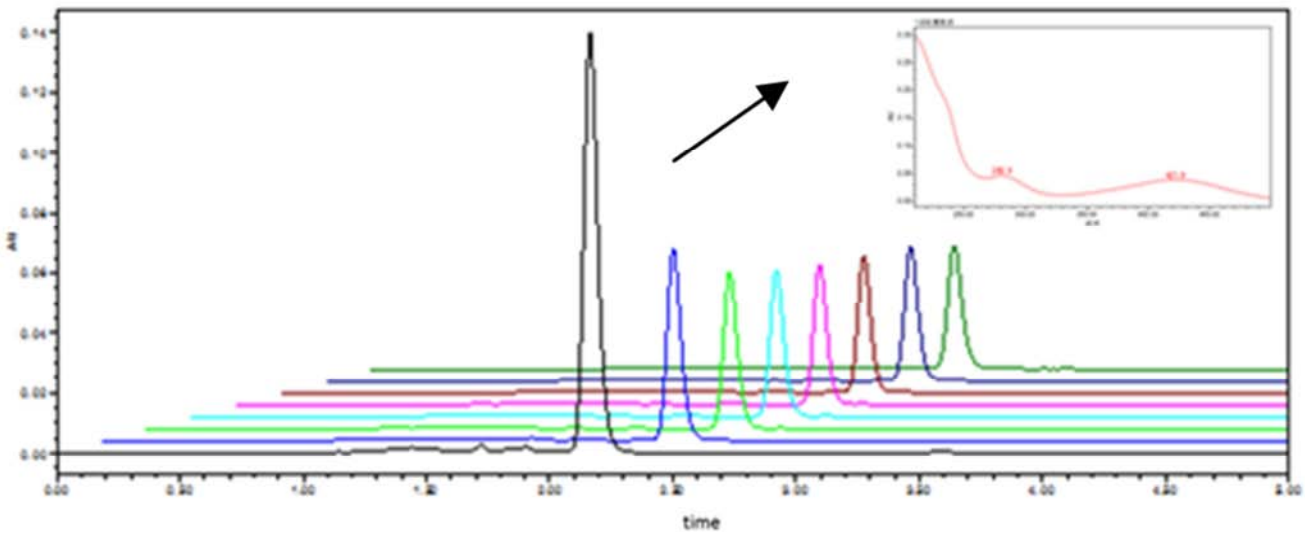
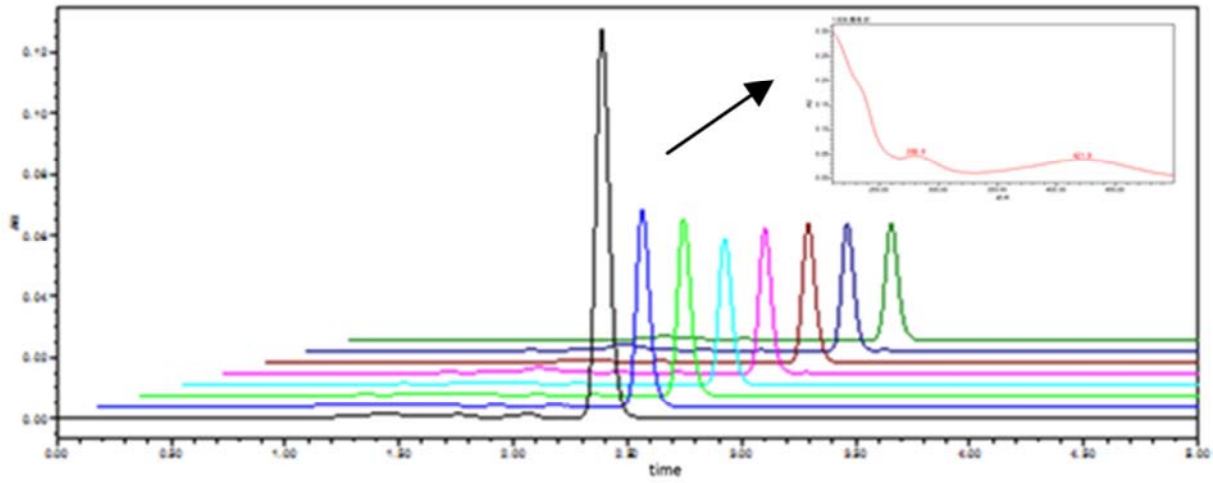


Figure 1. *3-NT standard curve.*

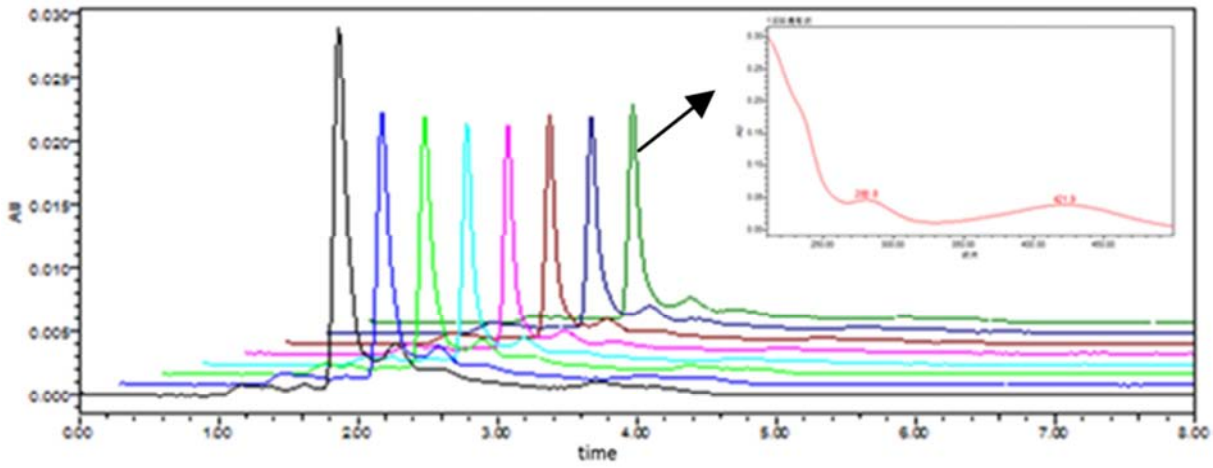
3.1.2. The Chromatograph of ONOO⁻ Induced Tyrosine Nitrification by Natural Products



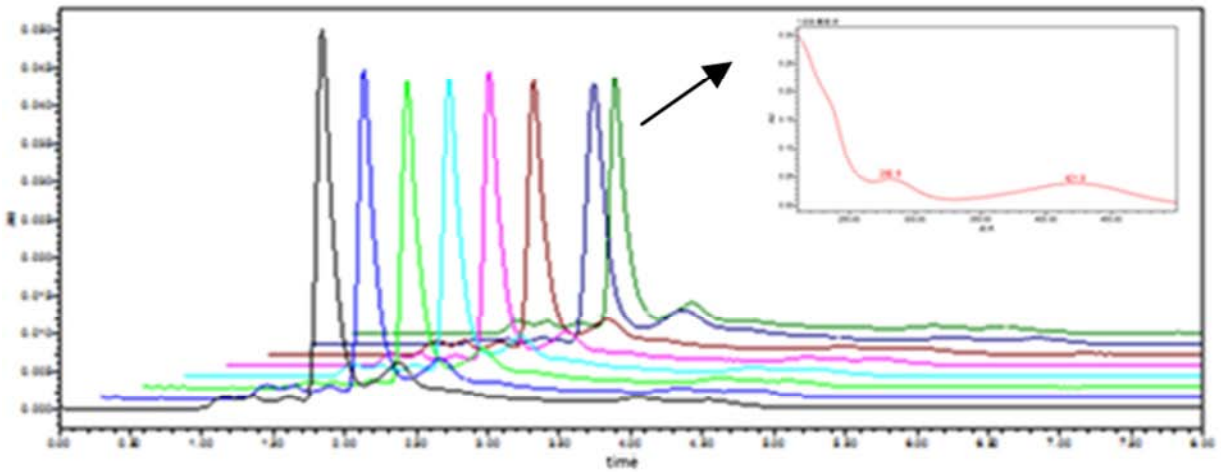
a



b



c



d

Figure 2. *Chromatography of Tyr induced by ONOO⁻ with the addition of different concentration of inhibitor (a-d: Capsanthin, Myricetin, Capsaicin and Vc. Final concentration of each natural products: 0, 1.67, 3.33, 8.33, 11.67, 16.67, 25, 33.3 μM).*

As shown in Figure 2, in the system of Capsanthin, Myricetin and Capsaicin, the production of 3-NT was decreased with the increase of natural product concentration, and the decrease in Vc was more obvious. This is because the phenolic hydroxylis contained in Capsanthin, Myricetin and Capsaicin can react with free radicals. In the process of inhibition of $ONOO^-$ damage, the phenol hydroxyl group is first associated with the $ONOO^-$ intermediates or with the decomposition of the NO_2 radical, then inhibits the formation of 3-NT.

3.1.3. Natural Products Inhibit $ONOO^-$ Nitrifying Tyrosine

The calculation of the concentration is based on the 3-NT curve and peak height in the spectrum. The results were shown in Table 1.

$$I\ (\%)=(1-C_1/C_0)\times 100\% \quad (1)$$

C_0 is $ONOO^-$ induced tyrosine nitrification to produce 3-NT concentrations without natural products.

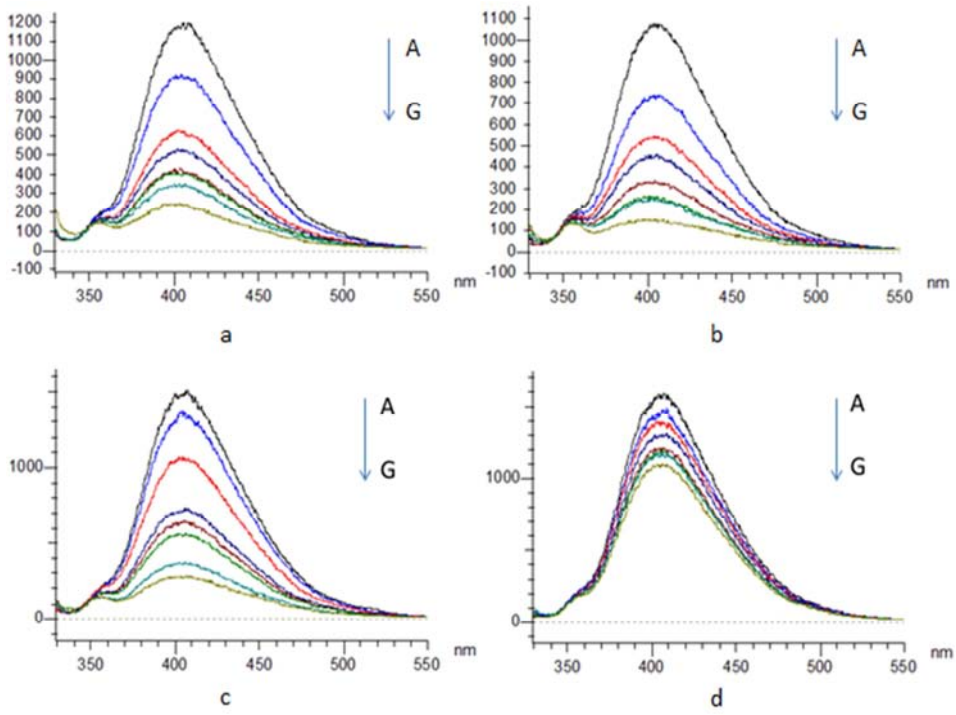
C_1 is $ONOO^-$ induced tyrosine nitrification to produce 3-NT concentrations with natural products.

Table 1. *Inhibitory of different pigments statistics.*

Sample	Concentration/μM	Inhibitory /%
Capsanthin	1.67	54.44
	3.33	62.45
	8.33	65.28
	11.67	66.53
	16.67	67.55
	25.0	68.57
Myricetin	33.3	70.70
	1.67	53.78
	3.33	58.57
	8.33	65.61
	11.67	67.18
	16.67	67.34
	25.0	70.13
	33.3	72.37
Capsaicin	1.67	26.01
	3.33	29.70
	8.33	33.46
	11.67	34.21
	16.67	37.26
	25.0	39.57
	33.3	41.35
Vc	1.67	13.69
	3.33	18.73
	8.33	21.98
	11.67	22.49
	16.67	28.16
	25.0	31.01
	33.3	32.53

The results showed that different concentrations of Capsanthin, Myricetin and Capsaicin had different inhibitory effects on $ONOO^-$ induced nitration of tyrosine. At low concentration 1.67 μM, several natural products still show good anti-nitrification activity, Capsanthin and Myricetin are particularly resistant to nitrification, which may be related to the functional groups in the structure.

3.2. The Natural Products Inhibit $ONOO^-$ Induced Tyrosine Dimer Generation



a. Capsanthin b. Myricetin c. Capsaicin d. Vc (A-G: 0, 0.2, 1, 1.4, 2, 3, 4 μM)

Figure 2. *The fluorescence spectra of tyrosine dimers with add natural products.*

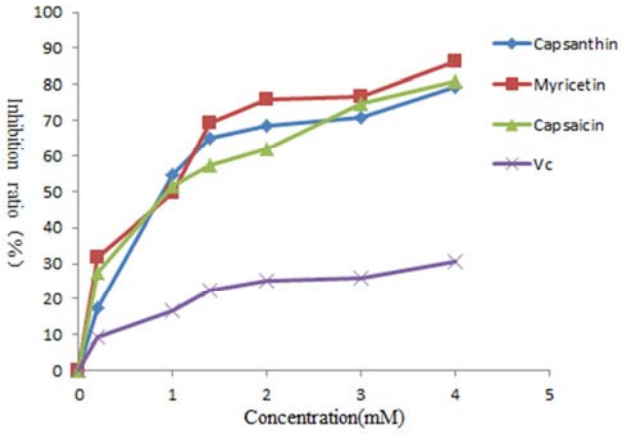


Figure 3. *The inhibition rate of tyrosine dimer was produced by the product.*

The results showed that several natural products had different inhibitory effects on $ONOO^-$ induced tyrosine oxidation, and the inhibitory ability was positively correlated with the material concentration. The fluorescence spectra of the production of tyrosine dimer with different concentrations of natural products were shown in Figure 2.

3.3. The Natural Products Inhibit $ONOO^-$ Induced Tyrosine Oxidation

The fluorescence value of lambda λex/em = 317/408 nm in the spectrogram is calculated, and the inhibition rate of natural products is calculated according to formula 2. The results were shown in Table 2.

$$I\ (\%)=(A_0-A_1/A_0)\times 100\% \quad (2)$$

A_0 is the fluorescence value without adding natural products, $ONOO^-$ induced tyrosine oxidation to produce tyrosine dimer.

A_1 is the fluorescence value with adding natural products, $ONOO^-$ induced tyrosine oxidation to generate tyrosine dimer.

Table2. *Inhibitory of different natural product statistics.*

Sample	Concentration/μM	Inhibitory /%
Capsanthin	0	0.00
	0.2	17.40
	1	54.60
	1.4	65.0
	2	68.67
	3	70.86
	4	79.22
Myricetin	0	0.00
	0.2	31.86
	1	49.51
	1.4	69.47
	2	75.96
	3	76.58
	4	86.48
Capsaicin	0	0.00
	0.2	27.45
	1	51.63
	1.4	57.36
	2	61.99
	3	74.88
	4	80.83
Vc	0	0.00
	0.2	9.38
	1	16.64
	1.4	22.43
	2	25.19
	3	25.77
	4	30.71

The calculated results showed that Capsanthin, Myricetin and Capsaicin had different inhibitory effects on $ONOO^-$ oxidative tyrosine. As the concentration of natural products increases, the production of tyrosine dimer decreases. When the concentration was 4 μM, the inhibition rate of Capsanthin, Myricetin and Capsaicin were 79.22%, 86.48% and 80.83%. The same concentration of Vc inhibition was only 30.71%. The inhibition rate of the molar concentration of several substances were significantly higher than that of Vc. The results showed that the antioxidant activity of several natural products were significantly stronger than that of Vc during the inhibition of $ONOO^-$ oxidative tyrosine.

3.4. The Natural Products Inhibit the Self-Oxidation of Pyrogallol

After testing, extract the rate of reaction of the fluorescent kinetics curve, the inhibiting ability of natural products to the self-oxidation of pyrogallol was calculated by formula 3. The self-oxidative rate diagram was made by the self-oxidizing rate of the x-coordinate, the natural product concentration was the y-coordinate, as shown in Figure 4. The inhibition abilities on pyrogallol self-oxidation by natural products were investigated. The concentration of natural products as x-coordinates, the inhibition rate was y-coordinates, as shown in Figure 5.

$$I\ (\%)=(V_0-V_S/V_0)\times 100\% \quad (3)$$

V_0 is the self-oxidation rate of pyrogallol without natural products.

V_S is the self-oxidation rate of pyrogallol with natural products.

Table 3. *Inhibitory of different natural product statistics.*

Sample	Concentration/mM	Inhibitory /%
Capsanthin	1.67×10^{-2}	7.71
	3.33×10^{-2}	19.96
	5.0×10^{-2}	30.22
	6.66×10^{-2}	36.97
	8.33×10^{-2}	48.19
	1.0×10^{-1}	63.21
Myricetin	1.67×10^{-2}	46.46
	3.33×10^{-2}	57.56
	5.0×10^{-2}	66.71
	6.66×10^{-2}	70.40
	8.33×10^{-2}	78.42
	1.0×10^{-1}	87.35
Capsaicin	1.67×10^{-2}	38.62
	3.33×10^{-2}	48.81
	5.0×10^{-2}	50.83

Sample	Concentration/mM	Inhibitory /%
Vc	6.66×10^{-2}	51.64
	8.33×10^{-2}	53.76
	1.0×10^{-1}	55.92
	1.67×10^{-2}	31.47
	3.33×10^{-2}	48.29
	5.0×10^{-2}	53.79
	6.66×10^{-2}	54.30
	8.33×10^{-2}	61.10
	1.0×10^{-1}	64.09

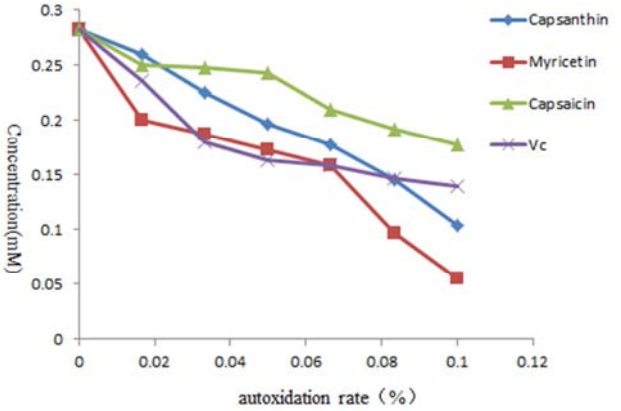


Figure 4. *The self-oxidation rate of pyrogallol.*

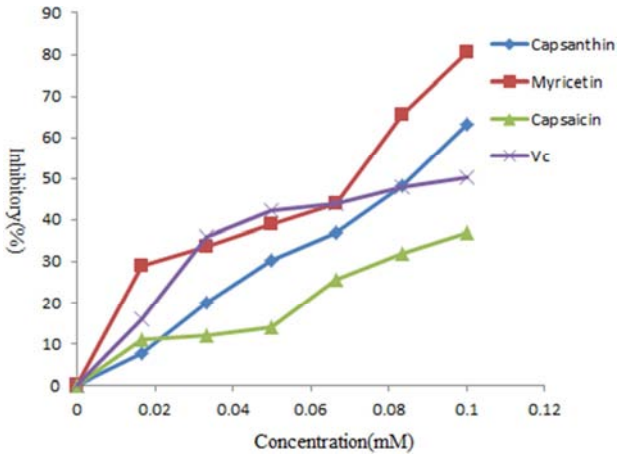


Figure 5. *The self-oxidative inhibitory of pyrogallol.*

The results showed that Capsanthin, Myricetin and Capsaicin had different ability on O_2^- inhibition. From Figure 5, it was observed that with the increase of the concentration of several natural products, the self-oxidation rate of pyrogallol decreases. When the concentration reached 0.1 mM, the inhibitory rate on O_2^- by Capsanthin, Myricetin and Capsaicin was 63.21%, 87.35% and 55.92%, the inhibitory of Vc was 64.09%. Capsaicin has a general effect on $O_2^{\cdot-}$, the antioxidant activity may be affected by its spatial structure in the system. From low concentration to high concentration, the inhibitory of Capsanthin and Myricetin were significantly higher than that of Vc, which showed good activity of scavenging hyperoxygen-free radicals in the system.

4. Conclusions

In this study, with the technique of fluorescence and HPLC-UV, the inhibition ability of Capsanthin, Myricetin and Capsaicin on $ONOO^-$ and O_2^- were investigated, these three flavonoids all have strong inhibition abilities, mainly because these three flavonoids have Ortho-phenol hydroxyl. The hydroxyl radical reacts with the free radical to release H^+, H^+ combined with OH, $\cdot NO_2$ and their oxidation products form a stable semiquinone free radical, thereby blocking the free radical reaction, this semiquinone structure can blocks free radical reactions. Intramolecular hydrogen bonding, C=C, and the existence of carbonyl and p-π conjugate system in the molecule enhanced its ability to remove the $ONOO^-$ by stabilizing free radical intermediates, extending the conjugate system and promoting the dispersion of electron clouds. Capsanthin, Myricetin and Capsaicin can realize the antioxidation function by scavenging superoxide anions.. This paper is significance to further explore the mechanism of natural products in human body and health care effect. This article analysis method for the composition of complex natural products free radical system can be analyzed accurately, and has a guiding effect on the anti-oxidation and anti-nitrification ability analysis of new natural products.

Acknowledgements

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