In Vitro Antioxidant Effect of the Total Flavones of *Citrus Aurantium* L. var *Daidai* Tanaka Fruits

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ABSTRACT: OBJECTIVE To study the antioxidant effects of the total flavones of *Citrus aurantium* L. var *daidai* Tanaka fruits(TFEFD) *in vitro*. **METHODS** The scavenging effect of ·OH and DPPH· as well as the inhibitory effect of lipid peroxidation in mouse liver were measured by the salicylic acid, DPPH·, and thiobarbituric acid methods. **RESULTS** TFEFD could scavenge DPPH· and ·OH(IC_{50} 0.417 mg·mL^{-1} and 3.087 mg·mL^{-1}, respectively). It also significantly inhibited lipid peroxidation in mouse liver. There was a positive dose-effect relationship between the scavenging activity and the concentrations of TFEFD. **CONCLUSION** TFEFD has the capability to scavenge radicals and serve as one of the active natural antioxidants.
KEY WORDS: *Citrus aurantium* L.var *daidai* Tanaka fruits; total flavones; effective fraction; antioxidant effects

玳玳果总黄酮体外抗氧化作用的研究

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摘要：目的 探讨玳玳果总黄酮有效部位的抗氧化作用。**方法** 分别采用二苯代苦味酰基自由基法(DPPH·法)、水杨酸法

基金项目：福建省自然科学基金项目(2010J01189)；福建省科技厅重点项目(2010Y0030)；福建省科技计划项目(2010Y2004)
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(Fenton 反应法)和硫代巴比妥酸法(TBAS 法)等方法，进行玳玳果总黄酮有效部位抗氧化药效实验研究，评价玳玳果总黄酮对 DPPH·自由基、·OH 自由基的清除能力，测定玳玳果总黄酮对小鼠肝脂质过氧化的抑制作用。**结果** 玳玳果总黄酮有效部位对 DPPH·自由基及·OH 自由基均具有良好的清除作用，其自由基清除能力以半数清除率计分别为 0.417 mg·mL^{-1} 和 3.807 mg·mL^{-1}，同时对小鼠肝脂质过氧化具有显著的抑制作用，呈良好的量效相关性。**结论** 玳玳果总黄酮有效部位具有良好的抗氧化清除自由基作用。

关键词：玳玳果；总黄酮；有效部位；抗氧化

中图分类号：R285.5　　**文献标志码**：A　　**文章编号**：1007-7693(2012)02-0097-05

Free radicals play an important role in regulating the signal transduction of cells, growing of the cells, and inhibiting virus and bacteria. But the excess free radicals play a crucial role in the pathogenesis of several human degenerative or chronic diseases, such as cancer, rheumatoid arthritis, cardiovascular and pulmonary diseases, and various neurodegenerative diseases[1-2]. Studies on excavating the exogenous antioxidants may reduce the harmful effects of free radicals by inhibiting their generation, increasing their elimination and improving level of endogenous substance to interrupt the attack of free radicals[3].

Daidai(*Citrus aurantium* L. var *daidai* Tanaka) is a variation of *Citrus aurantium* Linn which belongs to Citrus of Rutaceae. It is distributed in Fujian, Sichuan and Zhejiang provinces of China. The immature fruit of daidai is called Citri Aurantii Amarae Fructus and the young fruit as Aurantii Immaturus Fructus which can be used as medicines. It has the efficacy of regulating qi-flowing for activating stagnancy[4-5]. Practical tests show that the main type of chemical components in daidai fruits are alkaloids, flavonoids, essential oils and organic acids, with a high content of total flavonoids [6-7].

The preparation technology of the effective fraction in total flavone from daidai fruits (TFEFD) was established[8]. Practical tests show that the main type of chemical components of the TFEFD is flavanone, which contains the hesperidin, naringin, neohesperidin etc. Their chemical structural formulas are listed as follows (Fig 1).

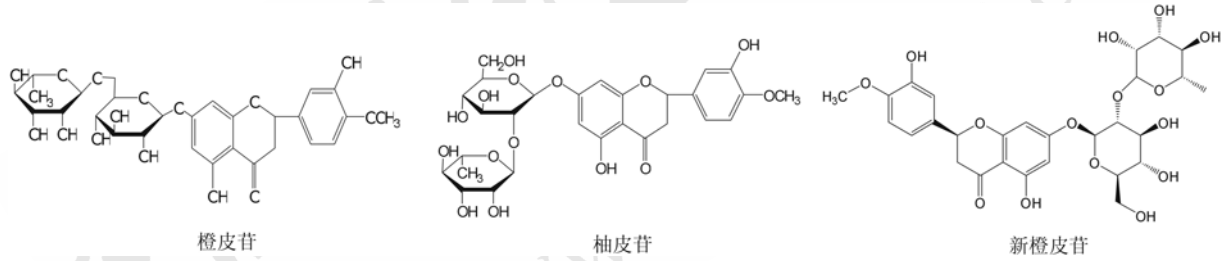

Fig 1　The chemical structures of main componerds in TFEFD

图 1　玳玳果总黄酮有效部位中主要成分的化学结构

In this study, the ·OH scavenging effect, the DPPH· scavenging effect and the inhibitory effect of TFEFD to lipid peroxidation of mouse liver were measured by the salicylic acid method, the DPPH· method, and the thiobarbituric acid method, respectively. The dose-effect relationship between the scavenging activity and the concentrations was investigated.

1　MATERIALS AND METHODS

1.1　Apparatus

UV-4802 double-beam UV-spectrophotometer (UNICO Shanghai Instruments Co., Ltd.); FA2004 electronic balance(Shanghai Precision & Scientific Instrument Co. Ltd.); TDL40B centrifuge (Shanghai Anting Scientific Instrument Co., Ltd.).

1.2　Materials and reagents

TFEFD was made in pharmaceutical analysis laboratory of Fujian University of Traditional Chinese Medicine, and the total flavones contents were 72.5% in the effective fraction; L-ascorbic acid reference substance (Vc) was provided by China Pharmaceutical Biological Products Analysis Institute(Stock 44150, lot. K17Q036, 95%); 2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Johnson Matthey Company; Thibarbituric acid was obtained from China National Medicine Group Shanghai Chemical Reagent Company(AR.>99.0%). All other chemicals were commercially available reagents made in China.

Kunming mice (SPF grade), ♂, certification

number: SCXK(HU)2007-0005, with body weight from 17 g to 23 g were obtained from Shanghai Slac Laboratory Animal Co. Ltd.

1.3 DPPH· scavenging test

Stock solutions of DPPH (2×10^{-4} mol·L^{-1})were prepared in absolute ethanol. Sample solutions containing 0.087–2.175 mg·mL^{-1} TFEFD were prepared in absolute ethanol.

Two milliliter sample solutions were mixed with 2 mL stock solutions of DPPH in the reaction tubes. The mixture was kept in the dark for 30 minutes. The absorbance was then measured at 517 nm using UV-4802 double-beam UV-spectrophotometer. Absolute ethanol was used as the blank. The radical scavenging activity of ascorbic acid was also determined. Analysis of the samples was run in triplicate.

Activity percentage was calculated using the equation:

$$\text{Activity}(\%)=[1-(Ai-Aj)/Ac]\times 100\%$$

Where Ac is the absorbance of solution of 2 mL DPPH and 2 mL absolute ethanol; Ai is the absorbance of 2 mL DPPH and 2 mL sample; Aj is the absorbance of 2 mL sample and 2 mL absolute ethanol.

1.4 ·OH scavenging test

Sample solutions with concentration of 1.032–8.256 mg·mL^{-1} TFEFD were prepared in distilled water. The scavenging activity of TFEFD on ·OH radical was determined using the method described by Smirnoff(1989). One milliliter sample solution was mixed with 1 mL of 8.8 mmol·L^{-1} H_2O_2, 1.0 mL of 9 mmol·L^{-1} $FeSO_4$, and 1 mL of 9 mmol·L^{-1} salicylicethanol solution. The mixture was kept at 37℃ for 30 minutes. The absorbance was then measured at 510 nm using UV-4802 double-beam UV-spectrophotometer. The radical scavenging activity of ascorbic acid was also determined. A blank solution was prepared as mentioned above except that H_2O_2 was replaced with distilled water.

Activity percentage was calculated using the equation:

$$\text{Activity}(\%)=[1-(Ai-Aj)\ /\ Ac]\times 100\ \%$$

Where Ac is the absorbance of control, Ai is the absorbance of sample and Aj is the absorbance of the solution without H_2O_2.

1.5 The inhibition of the TFEFD to lipid peroxidation of mouse liver

The mice were sacrificed and the liver was rapidly removed, weighed, and mixed with 9-fold normal saline at 0–4 ℃. The mixture was homogenized for 10 minutes, then centrifuged for 15 min at 3 500 r·min^{-1}. The supernatant was collected from the 10% liver homogenate.

Sample solutions with concentrations of 0.516–8.256 mg·mL^{-1} TFEFD were prepared in distilled water. Primarily, 0.3 mL liver homogenate was mixed with 0.2 mL of 0.02% H_2O_2, 0.2 mL of 0.03 mol·L^{-1} $FeSO_4$, and the 0.1 mL sample solutions. The mixture was kept at 37 ℃ for 40 minutes, then, added to 2.0 mL of a trichloroacetic acid solution(10%), and 1.0 mL 2-thiobarbituric acid (0.67%). The mixture was heated in a boiling water bath for 15 min and then cooled with flowing water immediately. The mixture was centrifuged for 15 min at 3 000 r·min^{-1}. The light density of the supernatant was measured at the wavelength of 532 nm. A blank solution was prepared as mentioned above except that TBA was replaced with distilled water. Analysis of the samples was run in quadruplicate. The inhibition rate was calculated using the equation:

$$I(\%) = [(A_{H_2O} - A)/A_{H_2O}]\times 100\%$$

Where A_{H_2O} is the absorbance of the solution where the sample was replaced with distilled water. A is the absorbance of the sample.

2 RESULTS

2.1 Scavenging effect on DPPH·

DPPH radical scavenging activity was observed to increase with sample concentrations (from 0.087 mg·mL^{-1} to 0.783 mg·mL^{-1}) (Fig 2). The clearance increased with the concentration of TFEFD. But the clearance became steady after the concentration of TFEFD reached 0.783 mg·mL^{-1}. Antioxidant activity was evaluated with IC_{50} values, the concentration at which radical scavenging activity was 50%. The IC_{50} of TFEFD was 0.417 mg·mL^{-1}, and the IC_{50} of Vc was 0.009 76 mg·mL^{-1}.

2.2 Scavenging effect on ·OH

·OH scavenging activity was observed to increase with sample concentration (from 1.032 mg·mL^{-1} to 8.256 mg·mL^{-1}). The clearance increased with the concentration of TFEFD. The TFEFD could scavenge ·OH with IC_{50} 3.087

mg·mL^{-1} (Fig 3). There was a positive dose-effect relationship between the scavenging activity and the concentrations. The IC_{50} of Vc was 0.118 mg·mL^{-1}.

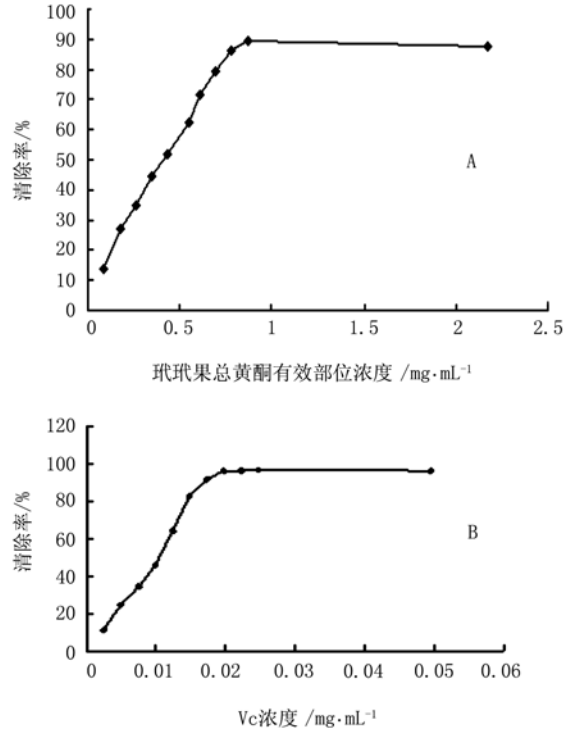

Fig 2 Scavenging effect of TFEFD and Vc on DPPH·
A–TFEFD; B–Vc

图 2 玳玳果总黄酮有效部位及 Vc 对 · OH 的清除作用
A–玳玳果总黄酮有效部位；B–Vc

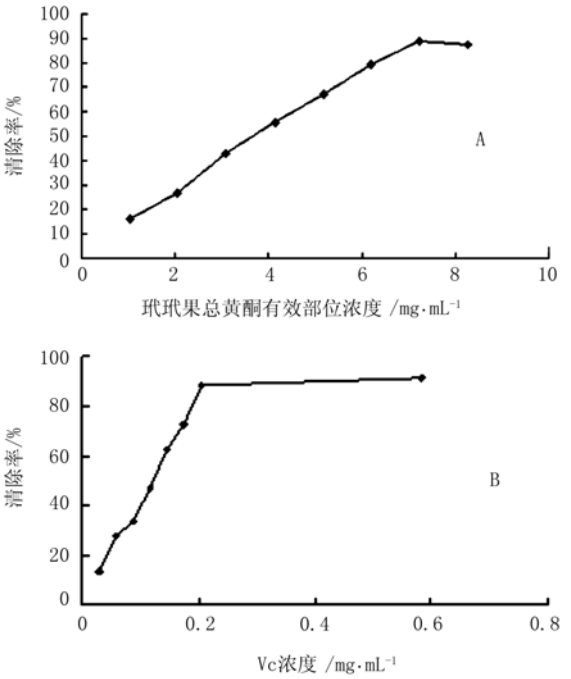

Fig 3 Scavenging effect of TFEFD and Vc on ·OH
A–TFEFD; B–Vc

图 3 玳玳果总黄酮有效部位及 Vc 对 · OH 的清除作用
A–玳玳果总黄酮有效部位；B–Vc

2.3 The inhibition of the TFEFD to lipid peroxidation of mouse liver

TFEFD inhibited effectively the lipid peroxidation induced by H_2O_2/Fe^{2+} in mouse liver *in vitro* (Tab 1). There was a positive dose-effect relationship between the inhibition rate and the concentrations of the TFEFD within the certain concentration range.

Tab 1 The inhibition effect of TFEFD on lipid peroxidation of mouse liver

表 1 玳玳果总黄酮有效部位对小鼠肝脂质过氧化的抑制作用

Grade	Concentration/mg·mL^{-1}	A_{532}	Inhibition rate/%
		0.701±0.002	
	0.561	0.625±0.016	10.7
	1.032	0.619±0.024	11.9
H_2O	2.064	0.482±0.016	31.1
TFEFD	3.096	0.391±0.009	44.2
	4.128	0.323±0.014	53.8
	5.160	0.172±0.008	75.1
	6.192	0.096±0.011	86.3
	7.244	0.094±0.015	86.5

3 DISCUSSION

DPPH· is a stable free radical agent in organic solvent. It has specific absorption at 517 nm because its lone pair electrons. The free radical scavengers can bleach its absorption by making the lone pair electrons matched. The change of DPPH· in absorbance is used to evaluate the ability of free radical scavengers. The more the absorbance reduced, the stronger the scavenging effects on DPPH· the test compounds exhibited. The TFEFD has the capability to scavenge DPPH· radicals. There was a positive dose-effect relationship between the scavenging activity and the concentrations in the certain concentration range.

The H_2O_2 can react with Fe^{2+}. Salicylic acid can capture the production of ·OH and generate colored substance, which has specific absorption at 510 nm. The free radical scavengers can bleach its absorption. The change of absorbance is used to evaluate the ability of free radical scavengers. The TFEFD has the capability to scavenge hydroxyl radical. There was a positive dose-effect relationship between the scavenging activity and the concentrations in the certain concentration range.

Liver MDA levels are dependent on free radical, in which H_2O_2/Fe^{2+} can be produced by Fenton reaction. The oxidation product is malondialdehyde. It can react with TBA and generate colored

substance, which has specific absorption at 530 nm. The free radical scavengers can bleach its absorption. Therefore, the change of absorbance is used to evaluate the ability of free radical scavengers. Our data shows that TFEFD is effective on the inhibition of lipid peroxidation in mice liver.

Vc, the commonly used and important water-soluble antioxidant, was chosen as the positive control. The control data proved the study method was feasible, stable and reliable.

Our data showed the total flavones of daidai fruits could scavenge DPPH· and ·OH with the inhibition effect on lipid peroxidation in mouse liver. There was a positive dose-effect relationship between the scavenging activity and the concentrations of TFEFD.

In conclusion, TFEFD has the capability to scavenge radicals and serve as one of the active natural antioxidants[9].

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收稿日期：2011-03-21