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A Study on the Antioxidant Activity of Volatile Oil from the *Citri Reticulatae* Pericarpium and *Citri Reticulatae* Pericarpium Viride of *Citrus Reticulata* 'Dahongpao'

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ABSTRACT

To study the antioxidant activity of volatile oil and its separated parts from the peel collected in different time of Citrus reticulata 'Dahongpao' such as Citri Reticulatae Pericarpium (CRP) and Citri Reticulatae Pericarpium Viride (CRPV) and Fructus Citri Immaturus (FCI), and nine representative constituents of essential oil from the peel of C. reticulata Blanco. Methods: The volatile oil and its separated parts and 9 characteristic constituents were tested the antioxidant activity by using the method as FRAP (ferric reducing/antioxidant power) and ABTS (2, 2'-azino-bis (3-ethylbenzthiozoline-6)-sulphonic acid), respectively. Results: The antioxidant activity value of the volatile oil and its separated parts and 9 representative components were obtained by the method as FRAP and ABTS. Conclusion: The volatile oil and its separated parts and 9 representative constituents have the antioxidant activity in some degree. The antioxidant activities of the volatile oil from the peel collected in different time of C. reticulata 'Dahongpao' is listed as the following sequence: FCI=CRPV=CRP.

Keywords: The peel of *C. reticulata* Blanco, *C. reticulata* 'Dahongpao', *Citri reticulatae* Pericarpium, *Citri reticulatae* Pericarpium Viride, Antioxidant activity, ABTS, FRAP

INTRODUCTION

Citrus reticulata Blanco belonging to the *Citrus L.* of Rutaceae is an important economical fruit plants planted all over the world which have many varieties such as *C. reticulata* 'Dahongpao', *C. reticulata* 'Chachi', *C. erythrosa* Tanaka, *C. reticulata* 'Tangerina', *C. reticulata* 'Unshiu', and etc. [1]. The peel collected in different time of above varieties can be used as two kinds of Chinese materia medica (CMM) such as Citri Reticulatae Pericarpium (CRP) and *Citri Reticulatae* Pericarpium Viride (CRPV) [2]. The CRP is the peel collected from September to December when the fruit is mature; The CRPV includes the little fruits fallen or collected in May and June which is called Fructus Citri Immaturus (FCI), and the peel of immature fruit collected from July to August which is also called CRPV [3]. The CRP and CRPV both belong to the qi-regulating CMM, while CRP is good at qi-regulating and energizing the spleen function, CRPV is adept in disintegrating stagnated qi and smoothing hepatitis in clinical[4,5]. The main bioactive components of CRP and CRPV include three kinds such as alkaloid, essential oil, and flavonoid. A lot of researches have

been done on the antioxidant activities of components from CRP and CRPV, and have gotten great progress. While the researches mainly focus on the antioxidant activities of flavonoid from CRP and CRPV, the researches focusing on the antioxidant activities of essential oils from CRP and CRPV are fewer relatively.

The essential oil of CRP and CRPV is a complicated system which includes hundreds of components. The components in essential oil of CRP and CRPV mainly include two classes such as monoterpenoid and sesquiterpenoid. The monoterpenoid is the main class in which the d-Limonene is the main component, besides there are γ-Terpinene, β-Myrcene, α-Pinene, β-Pinene, p-Cymene, Terpinolene, α-Thujene, and etc. The oxygenated monoterpene mainly includes Linalool, α-Terpineol, Thymol, Terpinen-4-ol, Thymol methyl ether, and etc. The content of sesquiterpenoid is small compared with that of monoterpenoid. The representative components of sesquiterpene include δ-Elemene, Copaene, Caryophyllene, Germacrene B, Germacrene D, (E, E)-α-Farnesene, γ-Elemene, δ-Cadinene, and etc. The oxygenated sesquiterpene mainly includes α-Sinensal, Elemol, Spathulenol, γ-Eudesmol, β-Eudesmol, Juniper camphor, and etc. Besides these, there are some important compounds such as Benzoic acid, 2-methylamino-, methyl ester which is an important characteristic compound in the essential oil of CRP and CRPV from *C. reticulata* 'Chachi', the alkanes such as Tricosane, Pentacosane, and n-Hexadecanoic acid [6-20]. This study has done the research on the antioxidant activities of essential oil and its separated parts from the peel collected in different time of *C. reticulata* 'Dahongpao', and 9 characteristic components including d-Limonene, γ-Terpinene, Linalool, Benzoic acid, 2-methylamino, methyl ester, α-Terpineol, Thymol, β-Caryophyllene, Decanal, Nerolidol from the essential oil of peel of *C. reticulata* Blanco. The structure of 9 representative compounds is represented in Figure 1.

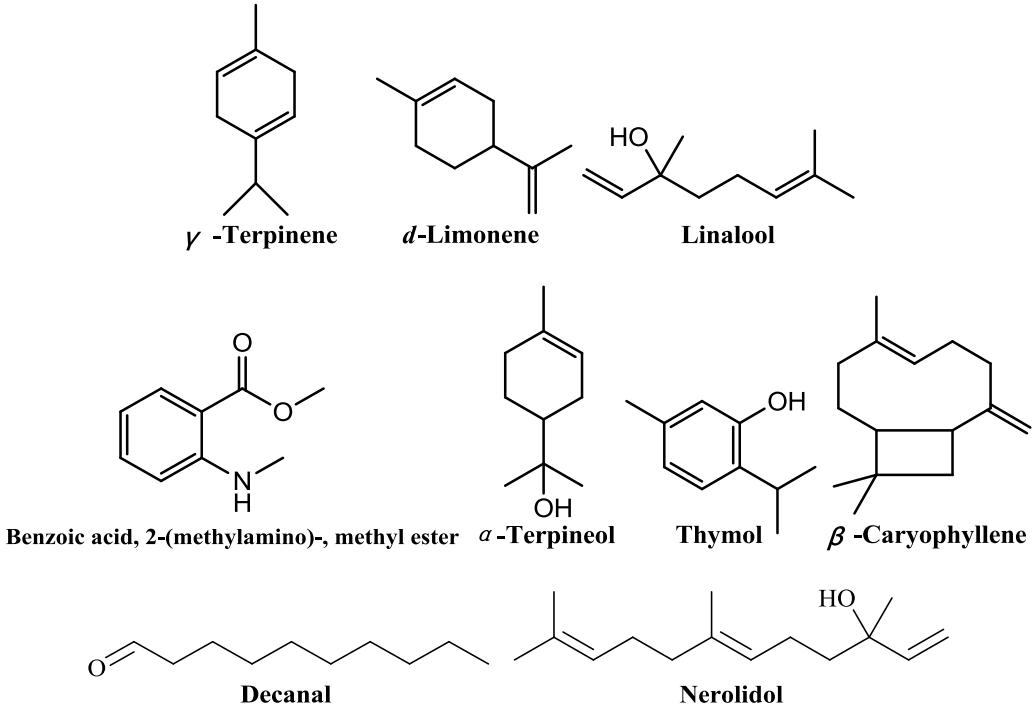

Figure 1: The structure of 9 representative compounds

The experiment method of antioxidant activity can be classified into two kinds as *in vitro* and *in vivo*. The character of *in vitro* method is the process is simple and convenience, and the cost is relatively lower, so the method is used widely. The manifestation of *in vitro* method is the scavenging activity to free radical, and the reducing capacity to metal ion of the antioxidant compound. The three representative *in vitro* methods are 1, 1-diphenyl-2-picrylhydrazyl (DPPH), 2, 2'-azino-bis (3-ethylbenzthiozoline-6) sulphonic acid (ABTS), and ferric reducing/antioxidant power (FRAP)[21]. This research used the method as ABTS and FRAP to study the antioxidant activity of the essential oil and its separated parts from the peel collected in different time of *C. reticulata* 'Dahongpao' planted in Bishan county of Chongqing municipality, and the nine characteristic components of essential oil of peel of *C. reticulata* Blanco.

EXPERIMENTAL SECTION

Materials

Materials and reagent: The peels were collected in different time of 2014 such as June 17 (FCI), September 15 (CRPV), December 15 (CRP) from the *C. reticulata* 'Dahongpao' planted in Bishan county of Chongqing municipality of China. The essential oil was extracted from FCI, CRPV, and CRP, respectively. The essential oil and its separated parts and the standards of 9 characteristic components can be seen in Table 1. The total antioxidant activity detection kits of ABTS and FRAP method were purchased from the Biyuntian biotechnology limited company.

Table 1: Information about the samples

The essential oil (EO) of peel	The EO from FCI	The EO from CRPV	The EO from CRP	The EO removed crystallization from FCI	The EO removed crystallization from CRPV	The EO removed crystallization from CRP	The crystallization being formed in -80°C from the EO of FCI	The crystallization being formed in -80℃ from the EO of CRPV	The crystallization firstly being formed in -4°C from the EO of CRP	The crystallization secondly being formed in -4°C from the EO of CRP
The nine characteristic components in EO of the peel of C. reticulata Blanco	d-Limonene	Linalool	Nerolidol	Thymol	β-Caryophyllene	Decanal	α-Terpineol	Benzoic acid, 2-methylamino, methyl ester	γ-Terpinene	

Instrumentation and equipment: The cell culture board with 96 hole, microplate reader (Gene company limited)

Method

The principal of method: The principal of FRAP method to detect the total antioxidant activity of a sample is that the antioxidant compounds in the sample can reduce the Ferric-tripyridyltriazine (Fe^{3+}-TPTZ) to Fe^{2+}-TPTZ with blue color, and then detect the absorbance light value of the Fe^{2+} in 593 nm to get the total antioxidant activity value of the sample [22].

The principal of ABTS method to detect the total antioxidant activity of a sample is that the ABTS can be oxygenated to $ABTS^{+}$ with blue color by some oxidant substances, while the amount of $ABTS^{+}$ will be decreased when the antioxidant compounds is there. The total antioxidant activity value of a sample can be calculated by detecting the absorbance light value of $ABTS^{+}$ in 734 or 405 nm. Trolox is an analogue of Vitamin E, and has nearly the same antioxidant activity compared with that of Vitamin E, so it can be used as a compared substance with other substances in the antioxidant activity detection experiment. For example, if the total antioxidant activity of Trolox is 1, the total antioxidant activity of other substances is expressed by the value compared with the value of Trolox in the same concentration [23].

The preparation of working solution: The preparation of FRAP working solution is as the following procedure. To make the 7500 μL dilution solution of TPTZ blend fully with 750 μL TPTZ, then add 750 μL buffer solution for detection into it which would be the 9000 μL FRAP working solution.

The preparation of ABTS working solution is as the following procedure. The ABTS radical reserve

solution was formed by ABTS solution 100 μL and the oxidant solution 100 μL (Caution: To be used after 12-16 hours in the real temperature and avoiding light). To make the ABTS radical reserve solution diluted by 80% Ethanol to become the ABTS working solution when it will be used (To make the outcome of absorbance light value in 734 nm of the diluted working solution subtract the absorbance light value in 734 nm of 80% Ethanol, should be in 0.7±0.05, usually the dilution ratio is 35-55).

The preparation of detected samples: According to the distribution of board which can be seen in Table 2, the detected samples were prepared as 0.15 and 0.3 mM solution by 80% ethanol, respectively.

The preparation for the detection of standard curve: The preparation of FRAP method is as the following procedure. The $FeSO_4 \cdot 7H_2O$ provided by the kit was weighted as 27.8 mg, then it was dissolved and kept to the volume as 1 mL, and then some 100 mM $FeSO_4 \cdot 7H_2O$ solution was diluted as 0.15, 0.3, 0.6, 0.9, 1.2, and 1.5 mM by 80% ethanol.
As for the ABTS method, the Trolox standard solution as 10 mM in the kit was diluted as 0.15, 0.3, 0.6, 0.9, 1.2, and 1.5 mM by 80% ethanol.

The detection of total antioxidant activity: As for FRAP method, 180 μL FRAP working solution was added to the detected hole of the board with 96 holes firstly, then 5 μL 80% ethanol was added to the blank hole for comparison secondly. According to the distribution of board in Table 2, 5 μL different concentration $FeSO_4$ standard solution was added into the detected hole for calculating the standard curve, and 5 μL each sample was added into the detected hole. Then, the absorbance light value was detected in 593 nm for three times in parallel to calculate the average value after 5 min incubation in 37°C. In the end, the standard curve was depicted and the regression equation was calculated, and then the total antioxidant activity of each sample was calculated by the regression equation, respectively.
As for the ABTS method, 200 μL ABTS working solution was added into the detection hole of the board with 96 holes firstly. 10 μL 80% ethanol was added into the blank holes for comparison. According to the board distribution which can be seen in Table 2, 10 μL Trolox standard solutions with different concentration were added into the detection holes for calculating standard curve, respectively, 10 μL each sample was added into the detection hole, respectively (It should be noticed that the solutions need to be blended softly). The absorbance light value in 734 nm was detected for three times in parallel to calculate the average value after incubation for 2-6 min in real temperature thirdly. In the end, the standard curve was depicted and the regression equation was calculated, and then the total antioxidant activity of different sample was calculated by the regression equation, respectively.

The Analysis of Data
The method of FRAP: The distribution of board can be seen in Table 2, in which the concentration of samples listed in row B and C is 0.15 mM, the concentration of samples listed in row D and E is 0.3 mM.

Table 2: The distribution of board

1	2	3	4	5	6	7	8	9	10	11	
blank e for parison	Fe2+ C1=0.15 mM	Fe2+ C2=0.3 mM	Fe2+ C3=0.6 mM	Fe2+ C4=0.9 mM	Fe2+ C5=1.2 mM	Fe2+ C6=1.5 mM	The hole for positive comparison				
olidol	2-methyl amino, benzoic acid methyl ester	Decanal	γ-Terpinene	α-Terpineol	β-Caryophyllene	d-Limonene	Linalool	Thymol	The EO from FCI	The EO from CRPV	T
e EO oved llizatio m FCI	The EO removed crystallization from CRPV	The EO removed crystallization from CRP	The crystallization being formed in -4°C from the EO of FCI	The crystallization being formed in -80°C from the EO of FCI	The crystallization being formed in -80°C from the EO of CRPV	The crystallization being formed firstly in -4°C from the EO of CRP	The crystallization being formed secondly in -4°C from the EO of CRP				
olidol	2-methyl amino, benzoic acid methyl ester	Decanal	γ-Terpinene	α-Terpineol	β-Caryophyllene	d-Limonene	Linalool	Thymol	The EO from FCI	The EO from CRPV	T
e EO oved llizatio m FCI	The EO removed crystallization from CRPV	The EO removed crystallization from CRP	The crystallization being formed in -4°C from the EO of FCI	The crystallization being formed in -80°C from the EO of FCI	The crystallization being formed in -80°C from the EO of CRPV	The crystallization being formed firstly in -4°C from the EO of CRP	The crystallization being formed secondly in -4°C from the EO of CRP				

The detected data is the average value of three times which can be seen in Table 3.

Table 3: The detected data

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.088	0.122	0.134	0.157	0.314	0.372	0.404	2.348				
B	0.156	0.152	0.196	0.157	0.142	0.185	0.133	0.157	0.168	0.177	0.196	0.168
C	0.214	0.166	0.184	0.167	0.182	0.159	0.143	0.184				
D	0.266	0.319	0.306	0.101	0.255	0.297	0.307	0.314	0.281	0.364	0.394	0.434
E	0.428	0.426	0.105	0.099	0.100	0.094	0.102	0.099				
F												
G												
H												

The standard curve is depicted by the standard substance as $FeSO_4$ which can be seen in Figure 2.

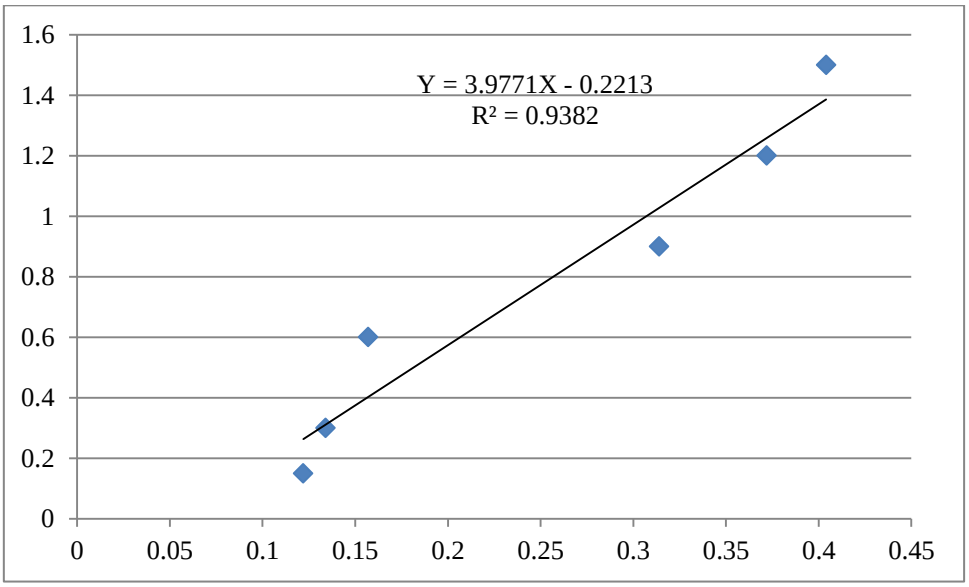

Figure 2: The standard curve depicted by the standard substance as FeSO4

The regression equation was calculated as 'Y=3.9771X - 0.2213' (X is the value of absorbance light, Y is the value of concentration), the correlation coefficient R2 is 0.9382. The antioxidant activity expression formula of each sample can be calculated by the regression equation. The total antioxidant activity was expressed by the concentration of the standard solution as FeSO4. For example, if the absorbance light value of a sample is equal to that of the standard solution as FeSO4 which concentration is 1 mM, the total antioxidant activity of this sample is 1 mM. As a result, the total antioxidant activity of a sample can be expressed as the concentration of the standard solution as FeSO4 which absorbance light value is equals to that of the sample divide the concentration of the sample. The antioxidant activity of each sample can be calculated by the regression equation through inputting the absorbance light value of each sample which can be seen in Table 4.

Table 4: The antioxidant activity value of each sample

Concentration	**Nerolidol**	**2-methyl amino, benzoic acid methyl ester**	**Decanal**	***γ*-Terpinene**	***α*-Terpineol**	***β*-Caryophyllene**	***d*-Limonene**	**Linalool**	**Thymol**	**The EO from FCI**	**The EO from CRPV**	**The EO from CRP**
0.15 mM	2.661	2.555	3.721	2.687	2.290	3.430	2.051	2.687	2.979	3.218	3.721	2.979
0.3 mM	2.789	3.491	3.319	0.601	2.643	3.200	3.332	3.425	2,988	4.088	4.486	5.016
Concentration	The EO removed crystallization from FCI	The EO removed crystallization from CRPV	The EO removed crystallization from CRP	The crystallization being formed in -4°C from the EO of FCI	The crystallization being formed in -80°C from the EO of FCI	The crystallization being formed in -80°C from the EO of CRPV	The crystallization being formed firstly in -4°C from the EO of CRP	The crystallization being formed secondly in -4°C from the EO of CRP				
0.15 mM	4.200	2.926	3.403	2.953	3.350	2.740	2.316	3.403				
0.3 mM	4.936	4.9	0.654	0.575	0.588	0.508	0.615	0.575				

The method of ABTS: The distribution of board can be seen in Table 5, in which the concentration of row B and C is o.15 mM, the concentration in row D and E is 0.3 mM.

Table 5: The distribution of board

	1	2	3	4	5	6	7	8	9	10	11	12
A	The blank hole for comparison	Trolox C1=0.15 mM	Trolox C2=0.3 mM	Trolox C3=0.6 mM	Trolox C4=0.9 mM	Trolox C5=1.2 mM	Trolox C6=1.5 mM	The hole for positive comparison				
B	Nerolidol	2-methyl amino, benzoic acid methyl ester	Decanal	γ-Terpinene	α-Terpineol	β-Caryophyllene	d-Limonene	Linalool	Thymol	The EO from FCI	The EO from CRPV	The EO from CRP
C	The EO removed crystallization from FCI	The EO removed crystallization from CRPV	The EO removed crystallization from CRP	The crystallization being formed in -4°C from the EO of FCI	The crystallization being formed in -80°C from the EO of FCI	The crystallization being formed in -80°C from the EO of CRPV	The crystallization being formed firstly in -4°C from the EO of CRP	The crystallization being formed secondly in -4°C from the EO of CRP				
D	Nerolidol	2-methyl amino, benzoic acid methyl ester	Decanal	γ-Terpinene	α-Terpineol	β-Caryophyllene	d-Limonene	Linalool	Thymol	The EO from FCI	The EO from CRPV	The EO from CRP
E	The EO removed crystallization from FCI	The EO removed crystallization from CRPV	The EO removed crystallization from CRP	The crystallization being formed in -4°C from the EO of FCI	The crystallization being formed in -80°C from the EO of FCI	The crystallization being formed in -80°C from the EO of CRPV	The crystallization being formed firstly in -4°C from the EO of CRP	The crystallization being formed secondly in -4°C from the EO of CRP				

The detected data is the average value of three times detection which can be seen in Table 6.

Table 6: The detected data

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.610	0.266	0.048	0.046	0.046	0.043	0.047					
B	0.582	0.607	0.597	0.600	0.597	0.587	0.585	0.601	0.522	0.584	0.609	0.605
C	0.597	0.610	0.605	0.586	0.551	0.593	0.596	0.574				
D	0.593	0.613	0.574	0.590	0.576	0.681	0.596	0.591	0.405	0.585	0.598	0.598
E	0.598	0.581	0.284	0.621	0.591	0.575	0.606	0.618				
F												
G												
H												

The standard curve was depicted by the Trolox as the standard substance which can be seen in Figure 3.

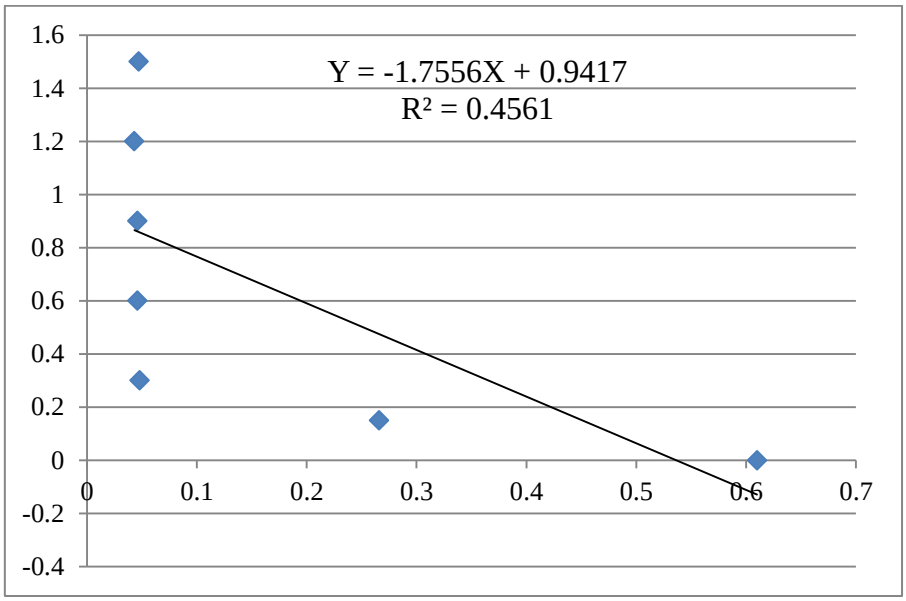

Figure 3: The standard curve depicted by the Trolox as the standard substance

The regression equation was calculated as 'Y=-1.7556X+0.9417' (X is the value of absorbance light, Y is the corresponding concentration), the correlation coefficient R^2 is 0.4506. The antioxidant activity of each sample can be expressed by Trolox-Equivalent Antioxidant Capacity (TEAC). For example, if the antioxidant activity of a sample is equal to that of the Trolox which concentration is 1 mM, the antioxidant activity of this sample is 1 mM. As a result, the total antioxidant activity of a sample can be expressed as the concentration of the standard solution as Trolox which has the same absorbance light value with the sample divide the concentration of the sample. The antioxidant activity of each sample can be calculated by the regression equation through inputting of the absorbance light value of each sample, respectively, which can be seen in Table 7.

Table 7: The antioxidant activity of each sample

Concentration	Nerolidol	2-methyl amino, benzoic acid methyl ester	Decanal	γ-Terpinene	α-Terpineol	β-Caryophyllene	d-Limonene	Linalool	Thymol	The EO from FCI	The EO from CRPV	The EO from CRP
0.15 mM	-0.533	-0.827	-0.707	-0.747	-0.707	-0.593	-0.567	-0.753	0.167	-0.8	-0.847	-0.56
0.3 mM	-0.33	-0.447	-0.22	-0.313	-0.23	-0.847	-0.35	-0.32	0.77	-0.36	-0.36	-0.283
Concentration	The EO removed crystallization from FCI	The EO removed crystallization from CRPV	The EO removed crystallization from CRP	The crystallization being formed in -4°C from the EO of FCI	The crystallization being formed in -80°C from the EO of FCI	The crystallization being formed in -80°C from the EO of CRPV	The crystallization being formed firstly in -4°C from the EO of CRP	The crystallization being formed secondly in -4°C from the EO of CRP				
0.15 mM	-0.707	-0.86	-0.8	-0.58	-0.173	-0.66	-0.7	-0.44				
0.3 mM	-0.36	-0.26	1.477	-0.497	-0.32	-0.227	-0.407	-0.477				

RESULTS AND DISCUSSION

For the method as FRAP, according to its principal, if the antioxidant activity of a sample is strong, the concentration of Fe^{2+} in this sample should be high, and the absorbance light value of this sample will be high, too. The antioxidant activity value of each sample can be gotten by the standard curve which can be seen in Figure 1. It is concluded that the nine representative compounds such as Nerolidol, 2-methyl amino, benzoic acid methyl ester, Decanal, *d*-Limonene, *γ*-Terpinene, Linalool, *α*-Terpineol, Thymol, *β*-Caryophyllene have the antioxidant activity in some degree, respectively.

The difference in the antioxidant activity of these compounds is little, the antioxidant activity of Decanal, *β*-Caryophyllene, Thymol, is relatively strong. The differences in the antioxidant activity of essential oil from the peel of *C. reticulata* 'Dahongpao' collected in different time such as FCI, CRPV, and CRP is as the following sequence, the antioxidant activity of FCI and CRPV is stronger than that of CRP, the antioxidant activity of CRPV is a little stronger than that of FCI. The antioxidant activity of essential oil removed the crystallization from FCI, CRPV, CRP, respectively, is as the following sequence, FCI=CRPV=CRP. The antioxidant activity of crystallization being formed in -4 and -80°C, respectively, from the essential oil of FCI, CRPV, CRP is listed as the following sequence, FCI=CRPV=CRP.

As for the method of ABTS, according to its principal, if the antioxidant activity of a sample is stronger, the concentration of $ABTS^+$ in this sample will be lower, and the absorbance light value of $ABTS^+$.in this sample will be lower, too. The standard curve as 'Y=-1.7556 X+0.9417' was gotten by the standard as Trolox, which correlation coefficient R^2 is not high, as a result, the antioxidant activity expressed value of each sample is not ideal, and most of the value is lower than zero. While compared with the absorbance value of blank, it can be seen that most absorbance value of samples is lower than that of blank which demonstrated that the concentration of $ABTS^+$ in each sample is lower than that of blank. According to the principal of this method, it can be concluded that each sample has the antioxidant activity in some degree. The reasons can be concluded as the following for the not ideal outcome of the antioxidant activity of each sample. Firstly, the concentration of $ABTS^+$ decreased violently and kept stability when the concentration of Trolox was more than 0.3 mM. The standard curve got in this condition is not ideal, and the value of correlation index R^2 is low, so the antioxidant activity value of each sample gotten by this curve is not ideal, too. Secondly, the ABTS could be oxygenated to $ABTS^+$ by the oxygen in the air to a certain degree in the course of detection, which would affect the experiment outcome. The antioxidant activity value of each sample gotten by the standard curve which can be seen in Figure 2 demonstrated that most of the sample has the antioxidant activity in some degree. As for the nine representative components, the antioxidant activity of Thymol is stronger than that of the other eight compounds. The antioxidant activity of essential oil of peel of *C. reticulata* 'Dahongpao' collected in different time can be listed as the following sequence: FCI=CRPV=CRP. The antioxidant activity of essential oil removed the crystallization being formed in low temperature from the peel of *C. reticulata* 'Dahongpao' collected in different time is listed as the following sequence: FCI= CRPV=CRP. The antioxidant activity of the crystallization being formed in -4 and -80°C, respectively, from the essential oil of peel of *C. reticulata* 'Dahongpao' collected in different time can be listed as the following sequence: FCI= CRPV=CRP.

Combined with the outcome of ABTS and FRAP, it can be concluded that the essential oil and its separated parts from the peel of *C. reticulata* 'Dahongpao' collected in different time, and 9 representative components of essential oil from the peel of *C. reticulata* Blanco has the antioxidant activity in some degree. The antioxidant activity of Thymol is relatively stronger in the nine representative components. The antioxidant activity of essential oil from the peel of *C. reticulata* 'Dahongpao' collected in different time can be listed as the following sequence: FCI= CRPV=CRP. Through the above analysis, it can be seen that there has great development value on the antioxidant activity of peel of *C. reticulata* Blanco. This research has provided some hints on how to deal with the 'rubbish' as peel of *C. reticulata* Blanco.

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