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Research Article

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Study on the Antioxidant Activity and GC-MS Evaluation of Essential oil Obtained from the *Citri reticulatae* Pericarpium of *Citrus kinokuni* Tanaka

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ABSTRACT

Objectives: *In order to study the antioxidant activity of essential oil fractionated parts from the Citri reticulatae Pericarpium (CRP) of Citrus kinokuni Tanaka collected in different time and five representative compounds from the peel of C. reticulata Blanco.*

Methodology: *Firstly, essential oil (EO) were obtained from C. kinokuni Tanaka. The EO were treated with vaccum fractionation method while the different separated parts were analysed by shimadzu Gas chromatography-Mass spectrometer(GC-MS) along with the five characteristic compounds were tested for the antioxidant activity by using antioxidant activity techniques such as, ABTS (2,2' azino-bis (3-ethylbenzthiozoline-6)-sulphonic acid) and FRAP (ferric reducing/antioxidant power), respectively.*

Results: *According to FRAP method, the total antioxidant activity of EO obtained at 100°C is 3.19 at 0.15 mM concentration, which is comparatively more than other fractions of EO. While the antioxidant activity of EO obtained at 110°C and 160℃ is 2.377 and 2.189 at 0.3 mM concentration, respectively while at 0.15 mM concentration, the antioxidant activity is 1.627 and 1.005, respectively. The antioxidant activities of Limonene and Decanal is 2.095 and 2.019 at a concentration 0.3 mM, which are more than its other representative compounds. In ABTS method, the standard curve is not perfect, resulting the effect on the antioxidant activity of each sample. Antioxidant activity of EO at 240°C is 0.072 at 0.3 mM concentration while the rest of others EO samples and even 05 representative components, the antioxidant activities are below zero.*

Conclusion: *EO separated parts obtained from the CRP of C. kinokuni Tanaka and five representative compounds exist antioxidant activity upto certain extent.*

Keywords: *C. kinokuni* Tanaka; *Citri reticulatae* Pericarpium; GC-MS analysis; FRAP; ABTS; Antioxidant activity

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INTRODUCTION

Citrus reticulata Blanco concerned with the Rutaceae, Genus as *Citrus* L, is the most economical and vital fruit plants. It is planted worldwide, which have numbers of varieties such as *C. reticulate* 'Dahongpao', *C. reticulate* 'Chachi', *C. erythrosa* Tanaka, *C. reticulate* 'Tangerina', *C. reticulate* 'Unshiu' and many others. The EO obtained from *C. reticulata* Blanco shows a significant role in these sectors like, medications, cosmetics and so forth and its output represents about 30% of the entire EO yield in the world. The peel of different varieties collected in different time can be used as mainly two types of Chinese Materia Medica (CMM), likewise *Citri reticulatae* Pericarpium Viride (CRPV) and *Citri reticulatae* Pericarpium (CRP) [1-4].

The CRP gathered from the month of September to December, as fruit was matured. The CRPV involves the small fruits fallen or collected in May and June which is known as *Fructus Citri Immaturus* (FCI), while the peel from unmature fruit obtained from July to August which is likewise called CRPV [5]. The primary bioactive constituents of CRP and CRPV consist of three types, which are alkaloid, EO and flavonoids. Numbers of researches have been carried out on the antioxidant activities of main constituents from CRP and CRPV and have achieved incredible success. Furthermore, the researches were chiefly focused on the antioxidant activity of flavonoids from CRP and CRPV, while researches on the antioxidant activities of EO from CRP and CRPV is less moderately. The EO of CRP and CRPV is quiet complex system which incorporates many components. The components in the EO of CRP and CRPV predominantly contain two classes, likewise monoterpenoid and sesquiterpenoid.

The monoterpenoid is the chief class in which the d-Limonene is the fundamental component, besides it, there are *γ*-Terpinene, *β*-Myrcene, *α*-Pinene, *β*-Pinene, *p*-Cymene, Terpinolene, *α*-Thujene, and so on. The oxygenated monoterpene primarily incorporates Linalool, *α*-Terpineol, Thymol, Terpinen-4-ol, Thymol methyl ether, and others as well. The content of sesquiterpenoid is comparatively less than that of monoterpenoid. The representative constituents of sesquiterpene incorporate δ-Elemene, Copaene, Caryophyllene, Germacrene B, Germacrene D, (*E*, *E*)- *α*-Farnesene, γ-Elemene, δ-Cadinene *etc*. The oxygenated sesquiterpene chiefly contains α-Sinensal, Elemol, Spathulenol, γ-Eudesmol, β-Eudesmol, Juniper camphor, and so on. Besides these, there are some significant compounds, for example, Benzoic acid, 2-methylamino- methyl ester, which is a main compound in the volatile oil of CRP and CRPV from *C. reticulata* 'Chachi', the alkanes, for example, Tricosane, Pentacosane and n-Hexadecanoic acid [4,6-20]. This study demonstrated the research on the antioxidant activities of EO fractionated parts from the peel collected in different time of *C. kinokuni* Tanaka', and five characteristic components including Limonene, γ-Terpinene, *α*-Terpineol, Decanal, n-Hexadecanoic acid from the essential oil of *C. reticulata* Blanco. The structure of 5 representative compounds is represented in Figure 1.

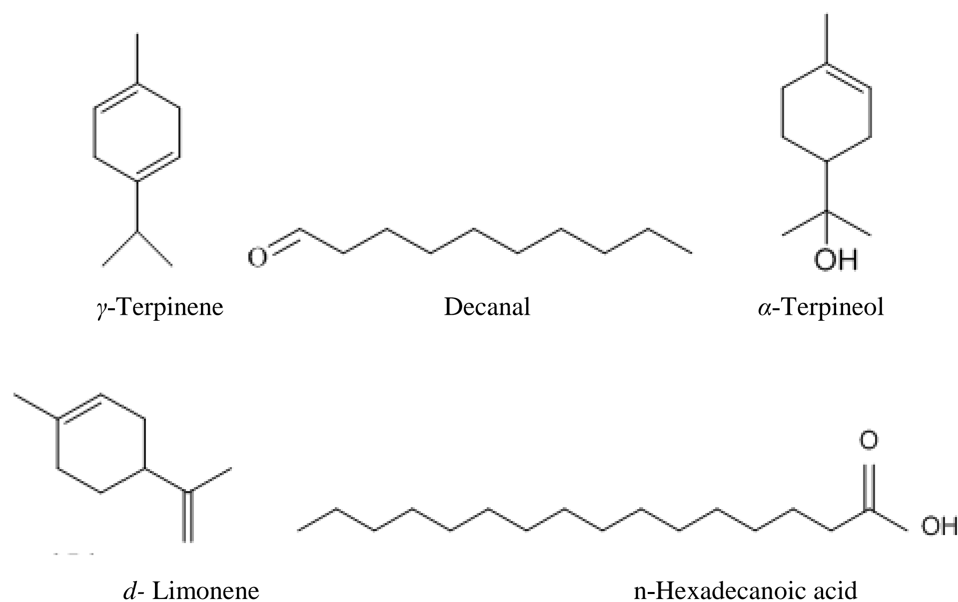

Figure 1. The structure of 5 representative compounds

The antioxidant activity experimental techniques can be grouped into two types, such as *in vitro* and *in vivo*. The *in vitro* process is simple, significant and the expenses are moderately lower, hence such techniques were utilized broadly. The indication of *in vitro* strategy is the scavenging activity to free radical and the reducing capacity to metal ion of the antioxidant compound. These three *in vitro* strategies are 1,1-diphenyl-2-picrylhydrazyl (DPPH), ferric reducing/antioxidant power (FRAP) and 2,2'-azino-bis (3-ethylbenzthiozoline-6) sulphonic acid (ABTS) [21]. This research utilized ABTS and FRAP technique to study the antioxidant activity of the fractionated parts of EO from the CRP of *C. kinokuni* Tanaka' collected in different time of 2016 planted in Nancheng county of Fuzhou, Jiangxi province of china and five characteristic components of EO from the peel of *C. reticulata* Blanco.

ESSENTIAL OIL EXTRACTION

Weighted samples were cutted around 0.5 × 0.5 cm large. The cut samples were swollen with about 10 times volume (v/w) 300-400 ml of pure water in a round-bottomed flask for soaking 0.5 h at 40°C, then extracted EO by Clevenger-type apparatus for 3-4 h [21]. Furthermore, the EO was obtained as per the procedure explained in Chinese pharmacopeia [20]. The volatile oil stored in another screw capped vials in a refrigerator at 4°C until needed [22].

Entire EO stored in refrigerator at a temperature below -80°C, So crystallization was formed. After that EO were introduced to vaccum fractionation method in order to achieve the fractions of EO from the CRP of *C. kinokuni* Tanaka at different temperature with the interval between it, as by increasing 30°C until get the fractions but unsuccessful in getting the fractions. Hence the temperature interval was set, as by increasing 10°C until get the fractions of EO.

Samples of EO from CRP of *C. kinokuni* Tanaka

Samples of EO were obtained by different temperature, such as EO obtained at 100°C, EO obtained at 110°C, EO obtained at 160°C, EO obtained at 170°C, EO obtained at 240°C and the crystallization being formed at -80°C.

GAS CHROMATOGRAPHY-MASS SPECTROMETER (GC-MS) ANALYSIS

GC-MS analysis of separated parts of EO was carried out with well-established methods with some modifications. Shimadzu GCMS-TQ8040 gas chromatograph (Japan) was used for GC-MS analysis. The gas chromatograph was coupled with Agilent DB-5 capillary column-(30 m × 0.25 mm i.d., 0.25 μm film thickness). The oven temperature was set from 60°C (3 min hold) to 250°C at 2.5°C min^{-1}, then hold it for 3 min. Helium was the carrier gas at a constant flow of 1 $ml.min^{-1}$. Ion-source and injector were remained at 200°C. The splitting ratio was 100:1. The solvent delay was 3 min. Electron impact mass spectra were taken at 70 eV. Scan at $scans.s^{-1}$ from *m/z* 25 to 450 amu [3]. 1 μl was the volume of injection [16].

The detected compounds in the fractions of EO from *C. kinokuni* Tanaka mentioned below in Table 1.

Table 1. Compounds detection by GC-MS in the essential oil of *Citrus kinokuni* Tanaka

Compounds	Retention index (RI[a])	RI[b]	CAS No.	Area percentage					
				100°C from EO	110°C from EO	160°C from EO	170°C from EO	240°C from EO	Crystallization being formed at -80°C from EO
Limonene	1018	1021	138-86-3	100%	95.14%	61.60%	5.65%	9.66%	47.77%
γ- Terpinene	1047	1049	99-85-4		4.86%	8.43%			3.65%
α -Terpineol	1143	1177	98-55-5			29.97%	77.75%	59.44%	2.37%
Terpinen-4-ol Phenol,	1137	1165	562-74-3				9.48%	11.15%	
2-methyl-5-(1-methylethyl)-	1262	1282	499-75-2				7.12%		
α-Farnesene	1458	1496	502-61-4					8.63%	
p-Cymen-7-ol	1284	1280	536-60-7					11.12%	
p-Cymene	1042	1020	99-87-6						1.00%
Decanal	1204	1215	112-31-2						6.30%
Nonanal	1104	1102	124-19-6						0.82%
n-Hexadecanoic acid	1968	1958	57-10-3						10.26%
Squalene	2914	2949	111-02-4						27.84%

Materials and Reagents

The peels were collected in different time of 2016 such as April to May, June to august and November from the *C.* '*kinokuni* Tanaka' planted in Nancheng county of Fuzhou, Jiangxi province of China. Detection kits of ABTS and FRAP were purchased from the Biyuntian biotechnology limited company.

SAMPLES INFORMATION

Samples of EO and five representative compounds were prepared as 0.15 mM and 0.3 mM concentration, such as EO at 100°C, EO at 110°C, EO at 160°C, EO at 170°C, EO at 240°C and the crystallization being formed at -80°C. Furthermore the five representative compounds includes, Limonene, *γ*-Terpinene, Decanal, n-Hexadecanoic acid and *α*-Terpineol.

Equipments

Microplate reader and the cell culture board 96 hole (Gene company limited).

The Principle Methodology

The FRAP technique is utilized to recognize and detect the total antioxidant activity of a sample. While 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 [23]. The principles of ABTS method used to detect and evaluate the total antioxidant activity of a sample is that, In which ABTS can be oxygenated to $ABTS^{+}$ with blue color by various oxidant substances, apart from this the amount of $ABTS^{+}$ will be decreased as by founding antioxidant compounds. The entire antioxidant activity values of each sample can be obtained by detecting the absorbance light value of $ABTS^{+}$ in 734 nm or 405 nm. Furthermore Trolox is the analogue of Vitamin E, which has almost the similar antioxidant activity as compared to Vitamin E, so it can be used for the sake of comparison with other substances in the detection of antioxidant activity process. Such as, if total antioxidant activity of Trolox is 1, so the total antioxidant activity of other substances is expressed by the compared value with the values of Trolox in the similar concentration [24].

FRAP Working Solution

The making of FRAP working solution involves few steps. In order to make dilution solution, 7500 µl of TPTZ completely mix with 750 µl TPTZ, and then add 750 µl buffer solution for detection into it which will be entirely 9000 µl FRAP working solution. The method of making ABTS working solution is mention below. In order to make ABTS radical reserve solution by taking ABTS solution 100 µl and oxidant solution 100 µl (Caution: It can be used only after 12-16 h in the absence of light in real temperature). Furthermore to prepare the ABTS radical reserve solution it should be diluted with 80% ethanol to get the ABTS working solution and then it should be used (To get the outcomes of absorbance light value in 734 nm of the diluted working solution subtract the absorbance light value in 734 nm of 80% Ethanol, range should be 0.7±0.05, usually the dilution ratio is 35-55).

The Detected Samples Preparation

The detected samples were prepared in the concentration of 0.15 mM and 0.3 mM solution by 80% ethanol. The entire samples distribution of board can be seen in Table 2.

The Standard Curve Preparation

The standard curve preparation for FRAP method is given below. The $FeSO_4{\bullet}7H_2O$ was taken from the kit and weighted as 27.8 mg, then it was dissolved to makes the 1 ml volume. Next this 100 mM $FeSO_4{\bullet}7H_2O$ solution was further diluted as 0.15, 0.3, 0.6, 0.9, 1.2, and 1.5 mM concentration by 80% ethanol. For ABTS method, 10 mM Trolox standard solution provided by the kit was diluted as 0.15, 0.3, 0.6, 0.9, 1.2, and 1.5 mM by 80% ethanol.

Total Antioxidant Activity Detection

For FRAP technique, first of all 180 µl FRAP working solution was added to the detected hole of the distribution board with 96 holes, then for comparison 5 µl 80% ethanol was added to the blank hole. According to distribution board in Table 2, 5 µl $FeSO_4$ standard solution in different concentration was added into the detected hole for the detection of standard curve while 5 µl from each and every sample were added in detected holes of the board. Absorbance light value was detected at 593 nm for three times in parallel to evaluate the average values after 5 min

incubation in 37°C. Finally standard curve was depicted and regression equation was used to calculate the total antioxidant activity of every sample was calculated, respectively. As according to ABTS technique, 200 µl ABTS working solution was added into the detection holes of the distribution board with 96 holes. For comparison 10 µl 80% ethanol was for blank holes. The distribution board which can be seen in Table 2, 10 µl Trolox standard solution with specified concentration were added into the detection holes in order to calculate the standard curve, respectively, From each and every sample 10 µl was added into the detected holes of distribution board, (To be noted that, solution need to be blended softly). The absorbance light values were detected in 734 nm for three times in parallel, in order to calculate the average values after incubation in real temperature for 2-6 min. The regression equation was calculated by getting the standard curve, then finally by using regression equation the total antioxidant activity of every sample were calculated, respectively.

Data analysis of FRAP method

The distribution of board can be seen in Tables 2 and 3, in which the concentration of samples listed in row B1 to B6 and C1 to C5 is 0.15 mM concentration while the samples listed in row B7 to B12 and C6 to C10 is 0.3 mM concentration.

Table 2. Distribution board

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	The blank hole for comparison	Fe^{2+} C1=0.15 mM	Fe^{2+} C2=0.3 mM	Fe^{2+} C3=0.6 mM	Fe^{2+} C4=0.9 mM	Fe^{2+} C5=1.2 mM	Fe^{2+} C6=1.5 mM	hole for positive comparison				
B	EO Obtained at 100◦C	EO Obtained at 110°C	EO Obtained at 160°C	EO Obtained at 170°C	EO Obtained at 240°C	The crystallization being formed at -80°C	EO Obtained at 100°C	EO Obtained at 110°C	EO Obtained at 160°C	EO Obtained at 170°C	EO Obtained at 240°C	The crystallization being formed at -80°C
C	α-Terpinol	Limonene	Decanal	γ-Terpinene	Palmitic acid	α-Terpinol	Limonene	Decanal	γ-Terpinene	Palmitic acid		
D												
E												
F												
G												

Table 3. Detected data

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	0.21	0.195	0.235	0.35	0.46	0.582	0.651	0.285				
B	0.307	0.224	0.191	0.184	0.19	0.196	0.41	0.39	0.37	0.19	0.26	0.34
C	0.238	0.198	0.192	0.182	0.184	0.261	0.36	0.352	0.179	0.186		
D												
E												
F												
G												

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

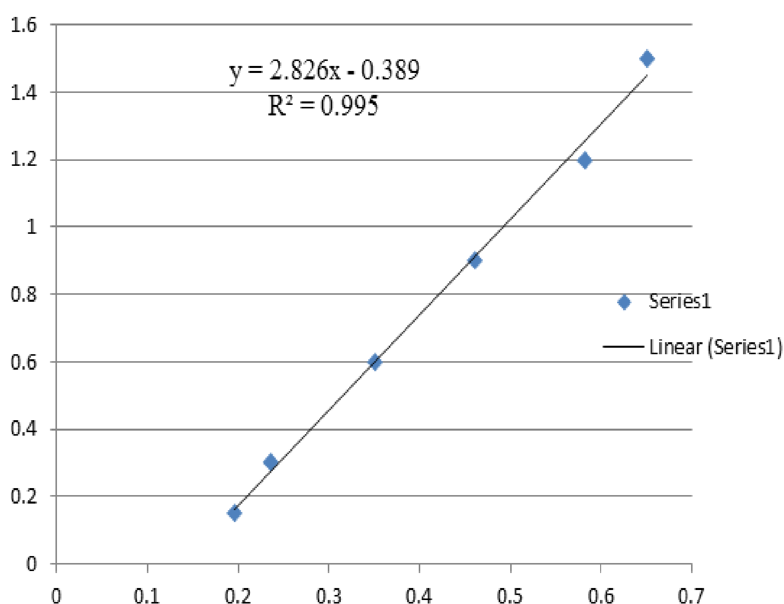

Figure 2. The standard curve depicted by the standard substance as $FeSO_4$

The regression equation was calculated as, the correlation coefficient R^2 is 0.995, (X is the value of absorbance light, while the value of concentration is Y). The antioxidant activity of each sample can be calculated by using regression equation. Totally the antioxidant activity was expressed by the concentration of the standard solution as $FeSO_4$ (Table 4). Such as, if the absorbance light value of a sample is same as that of the standard solution as $FeSO_4$ which concentration is 1 mM, totally the antioxidant activity of the 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 $FeSO_4$, which absorbance light value is equal to that of the sample divide the concentration of the sample. The antioxidant activity of each sample can be obtained by using regression equation by inputting the values of absorbance light of every sample which can be seen in Table 5.

Table 4. Total Antioxidant activity of each sample

Concentration	EOobtained at 100°C	EO obtained at 110°C	EO obtained at 160°C	EO obtained at 170°C	EO obtained at 240°C	The crystallization being formed at -80°C	α-Terpineol	Limonene	Decanal	γ-terpinene	Palmitic acid
0.15 mM	3.191	1.627	1.005	0.873	0.986	1.009	1.891	1.137	1.024	0.836	0.873
0.3 mM	2.566	2.377	2.189	0.493	1.153	1.906	1.162	2.095	2.019	0.39	0.455

Method of ABTS

According to the distribution board, which can be seen in Tables 5 and 6, 0.15 mM concentration of samples listed in row B1 to B6 and C1 to C5, while thesamples listed in row B7 to B12 and C6 to C10 is at 0.3 mM concentration.

Table 5. Detected data

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.687	0.488	0.402	0.224	0.045	0.041	0.041					
B	0.664	0.658	0.682	0.633	0.638	0.669	0.67	0.675	0.642	0.619	0.593	0.633
C	0.658	0.66	0.667	0.678	0.666	0.693	0.679	0.682	0.681	0.677		
D												
E												
F												
G												

Table 6. 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					
B	EO Obtained at	EO Obtained at	EO Obtained	EO Obtained at	EO Obtained	The crystallizationbeing	EO Obtained at	EO Obtained	EO Obtained at	EO Obtained	EO Obtained	The crystallizationbeing

	100◦C	110◦ C	at 160◦ C	170◦ C	at 240◦ C	formed at -80◦C	100◦ C	at 110◦ C	160◦ C	at 170◦ C	at 240◦ C	formed at -80◦C
C	α-Terpinol	Limonene	Decanal	γ-Terpinene	Palmitic acid	α-Terpineol	Limonene	Decanal	γ-Terpinene	Palmitic acid		
D												
E												
F												

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

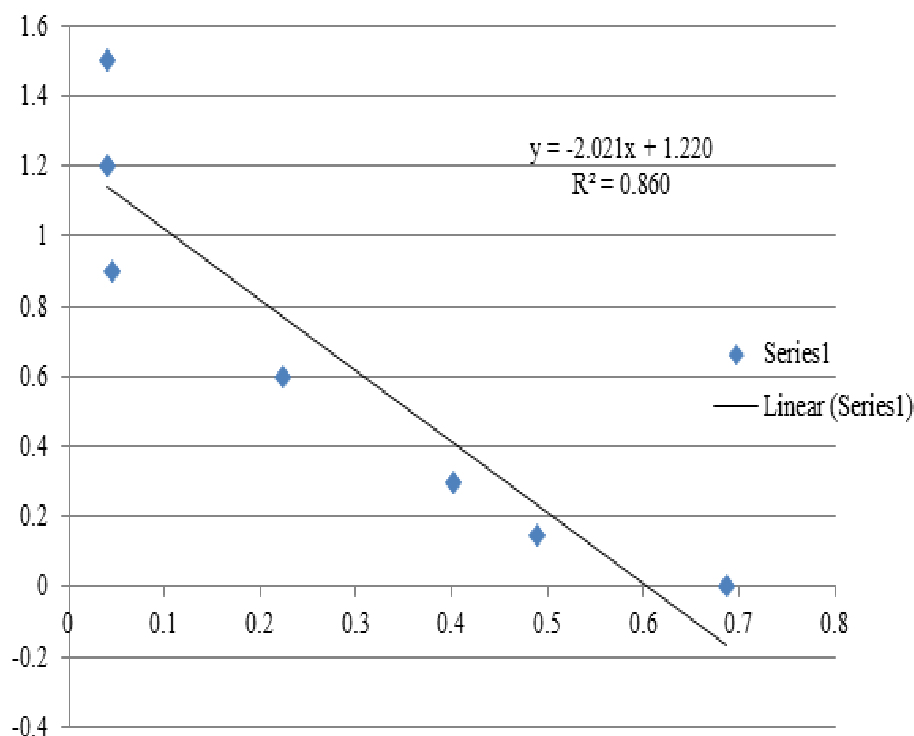

Figure 3. Standard curve by the Trolox as the standard substance

The regression equation was calculated as is Y=-2.021x+1.220. R^2=0.860 (X is the value of absorbance light while Y is the corresponding concentration) the correlation coefficient is R^2. Each sample antioxidant activity can be expressed by Trolox Equivalent Antioxidant Capacity (TEAC). Likewise, if a sample antioxidant activity of is similar to that of Trolox which concentration is 1mM, the sample antioxidant activity is 1 mM. Finally, the sample total antioxidant activity can be expressed as the concentration of the standard solution as Trolox, by having the same absorbance light value with the sample divide the concentration of sample. In order to calculate the antioxidant activity of each sample, which can be obatined by regression equation by inputting the absorbance light value of

every sample, respectively (Table 7).

Table 7. Total Antioxidant activity of each sample

Concentration	EO obtained at 100°C	EO obtained at 110°C	EO obtained at 160°C	EO obtained at 170°C	EO obtained at 240°C	The crystallization being formed at -80°C	α-Terpinol	Limonene	Decanal	γ-Terpinene	Palmitic acid
0.15 mM	-0.813	-0.732	-1.055	-0.395	-0.463	-0.88	-0.732	-0.759	-0.853	-1.002	-0.84
0.3 mM	-0.447	-0.481	-0.258	-0.103	0.072	-0.198	-0.602	-0.508	-0.528	-0.521	-0.494

RESULTS AND DISCUSSION

According to the principles of FRAP technique, if a sample antioxidant activity is strong, the concentration of Fe^{2+} will be high in the same sample and the absorbance light value will be high too. Each sample antioxidant activity value can be obtained by standard curve which can be seen in Figure 2. The five representative compounds such as, *α*-Terpineol, Limonene, *γ*-Terpinene, Decanal, Palmitic acid has the antioxidant activity upto some extent. The differences in the antioxidant activity of these compounds is that the antioxidant activity of *α*-Terpineol, Limonene and Decanal is relatively good enough which is more than 01 at 0.15 mM. Limonene and Decanal antioxidant activity increased at 0.3 mM while *γ*-Terpinene and palmitic acid decreased. Now the differences in the antioxidant activity of essential oil from the CRP peel of *C. kinokuni*Tanaka is that the antioxidant activity of EO obtained 100°C is comparatively more than others. The antioxidant activity of EO at 110°C, EO obtained 160°C and the sample in which Crystallization being formed at -80°C is more than 01 at 0.15 mM while it increased at a concentration of 0.3 mM. Furthermore EO at 240°C is less than 01 at 0.15 mM, meanwhile it increased at 0.3 mM concentration. In last the antioxidant activity of EO at 170°C decreased abruptly at concentration 0.3 mM. So it can be concluded that each sample of CRP essential oil exist antioxidant activity in certain extent.

As for as the principles of ABTS method is concerned, if the antioxidant activity of a sample is strong, lower will be the concentration of $ABTS^{+}$ in the sample and the absorbance light value of $ABTS^{+}$ in this sample will be lower as well. The standard curve is Y=-2.021x+1.220 was obtained by standard as Trolox, which correlation coefficient R^2 is 0.860. It is concluded that antioxidant activity expressed value of each sample is not that ideal and most of the value is lower than zero but EO at 240°C total antioxidant activity is 0.072 at a concentration of 0.3 mM. While comparing the absorbance light value of blank with others, that most of the absorbance light values 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 principles of this method, it can be concluded that every sample have antioxidant activity upto some extent. The reasons can be concluded as the following for not better results of the antioxidant activity of every sample. First of all, concentration of the $ABTS^{+}$ decreased violently and then kept stability as the Trolox

concentration was more than 0.3 mM. The standard curve obtained at this situation is not that ideal and the value of correlation coefficient R^2 as well. Hence, resulting the antioxidant activity values of every sample obtained through standard curve is not good enough. In addition to that, the ABTS could be oxygenated to $ABTS^+$ by the oxygen to some degree of extent in air at the time of detection, that would affect the experimental results. Every sample antioxidant activity values obtained by standard curve which can be seen in Figure 3, illustrated that mostly the samples have antioxidant activity upto certain degree of extent.

CONCLUSION

Collectively the results of ABTS and FRAP, it can be concluded that the different fractionated parts of CRP volatile oil and EO with crystallization at -80°C obtained from *C. kinokuni* Tanaka' and 05 representative compounds of essential oil from the peel of *C. reticulata* Blanco have antioxidant activity at certain extent. The antioxidant activity of alpha terpineol is relatively strong in the five representative components. From the above analysis, it can be seen that there has incredible progress value on the antioxidant activity of peel of *C. reticulata* Blanco. This exploration demonstrates the significance and usefulness of the peel (rubbish) from *C. reticulate* Blanco.

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