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Original Article

Enhancing the antioxidant activity of immature calamondin by heat treatment

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Summary The purpose of this study was to investigate the enhancing effect of heat treatment (110 and 130 °C for 0.5, 1.0, 1.5 and 2.0 h, and 150 °C for 1.5 h) on antioxidant activity and phenolic compounds of immature calamondin (*Citrus mitis* Blanco). The results indicated that heat treatment could enhance the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging potency, oxygen radical absorbance capacity (ORAC) and total phenolic content. However, the major flavonoid, 3′,5′-di-*C*-β-glucopyranosylphloretin (DGPP), decreased drastically after being heated at ≥130 °C over 1.5 h. The increasing ratio of absorbance at UV_{420} nm coincided with the change of the antioxidant activity. Therefore, it was concluded that the browning products resulted in the increase of the antioxidant activity of immature calamondin heated at ≥130 °C over 1.5 h, while the increase of antioxidant activity at 110 and 130 °C ≤ 1.0 h heating was due to increased phenolic content.

Keywords Antioxidant activity, browning products, flavonoid composition, heat treatment, immature calamondin.

Introduction

Calamondin (*Citrus mitis* Blanco), a hybrid of *Citrus reticulata* Blanco and *Fortunella* spp (Myrna *et al*., 1992; Moshonas & Shaw, 1996), is used to make a hot drink due to its potential health beneficial properties. In our previous study, 3′,5′-di-*C*-β-glucopyranosylphloretin (DGPP), naringin and hesperidin are found in water extract of immature calamondin peel, while nobiletin and tangeretin are only observed in an ethyl acetate extract (Yu *et al*., 2013). The largest quantity of DGPP is observed in calamondin peel, juice sac and leaf (Ogawa *et al*., 2001), especially in immature calamondin peel (Lou *et al*., 2012; Yu *et al*., 2013). The flavonoid composition of *Fortunella* species differs from those of the *Citrus* species (Ogawa *et al*., 2001; Sadek *et al*., 2009). Eight polymethoxyflavones including nobiletin and tangeretin (Tatum & Berry, 1978) and other flavonoids, such as, hesperidin, neohesperidin, narirutin and diosmin (Ramful *et al*., 2011), have also been reported in calamondin. The levels of total phenols and flavonoids of immature calamondin are found to be higher than those of mature calamondin. Antioxidant activity and the DPPH-free radical scavenging effect of immature calamondin are also found to be higher than those of mature calamondin (Lou *et al*., 2014a,b). Therefore, this study aimed to further clarify the above observation on immature calamondin.

It is well known that dried citrus peel is used in traditional Chinese medicine (Chiu & Chang, 1998; Zang, 2005; Choi *et al*., 2011). Heat treatment might change the amount of extractable phenolic compounds and antioxidant activity of citrus peel (Jeong *et al*., 2004; Xu *et al*., 2007; Chen *et al*., 2011; Choi *et al*., 2011). The antioxidant activity of citrus peel extract increases as heating temperature increases (Jeong *et al*., 2004). The flavanone glycosides might be destroyed when heated to 120 °C for 90 min or 150 °C for 30 min (Xu *et al*., 2007). Therefore, heat treatment may be used to release bound phenolic compounds from citrus as well as increasing their antioxidant activity (Gil-Izquierdo *et al*., 2002; Xu *et al*., 2007; Choi *et al*., 2011). In our previous study, the antioxidant activity and total phenolic content of immature calamondin increase significantly after heat treatment at 150 °C for 1.5 h (Lou *et al*., 2014b). It has been concluded that high temperatures could enhance the extraction of phenolic compounds; however, some flavonoids such as DGPP could be degraded. Therefore, the increase of antioxidant activity could not simply be explained by

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the increase of the total phenolic content. The antioxidant activity of immature calamondin after heating might also be increased by the browning products (Lingnert & Eriksson, 1981; Lee, 1992; Dittrich *et al.*, 2003; Yilmaz & Toledo, 2005), as the colour of the extract from heated immature calamondin was obviously darker than that of the unheated immature calamondin. In this study, we investigated the changes in total phenolic content, antioxidant activity and browning products of immature calamondin after heat treatment at 110, 130 and 150 °C for 0–2 h, respectively. Then, activated carbon-treated hot water extract of immature calamondin was subjected to analysis to elucidate the major effective antioxidant compounds in the heated immature calamondin.

Materials and methods

Materials

Calamondin (*Citrus mitis* Blanco) was collected from a calamondin estate in the Jao-Si region, Ilan, Taiwan in November 2010. Calamondin with whole green appearance was collected and sorted as immature calamondin and had an average weight of 10.12 ± 1.30 g. The average soluble solids sugars and titratable acidity of the immature calamondin were 2.47% and 0.40%, respectively. After washing, immature calamondin was cut into c., 0.5 cm slices. They were then lyophilised (freeze-dried) for 48 h. Prior to extraction, the lyophilised immature calamondin slices were pulverised in a blender and passed through a 60-mesh sieve. The obtained powders of immature calamondin were stored in a suitable brown bottle with screw cap at −18 °C.

Chemicals

Methanol, ethanol, ethyl acetate and acetonitrile were LC grade from Merck Chemical Co. (Darmstadt, Germany). Acetic acid, Na_2CO_3, 2,2-diphenyl-1-picrylhydrazyl (DPPH), disodium fluorescein (FL) and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) were analytical grade. 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid, 97% (Trolox), standards of flavonoids, including naringin, hesperidin, diosmin, neohesperidin, quercetin, naringenin, hesperetin, limettin, nobiletin and tangeretin, and standards of phenolic acids, including caffeic acid, syringic acid, gentisic acid, ferulic acid, ellagic acid, *p*-coumaric acid, sinapic acid, protocatechuic acid and gallic acid, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Powder-activated carbon was extra pure reagent from Nippon Shiyaku Co. (Osaka, Japan). DGPP was extracted from immature calamondin by hot water according to Lou *et al.* (2012). The extract was separated by a semi-preparative HPLC, and the compound was collected. It was then subjected to liquid chromatography coupled with mass spectrometer (LC/MS^n) for identification. The estimated purity is 90.4%, based on area of HPLC chromatogram by UV 280 nm detection. After lyophilisation, the residue was redissolved in deionised water and stored at −18 °C for further use.

Heat treatment

The sliced immature calamondin was subjected to hot air drying in an oven at 110 °C and 130 °C for 0.5, 1.0, 1.5 and 2.0 h, separately, and 150 °C for 1.5 h. The obtained dried sliced immature calamondin was then lyophilised for 48 h. Prior to extraction, the lyophilised immature calamondins were pulverised in a blender and passed through a 60-mesh sieve. The obtained powders of hot air-dried immature calamondin were stored in a suitable brown bottle, filled with nitrogen, with screw cap at −18 °C.

Preparation of hot water extract

Three grams of dried and powdered immature calamondin was extracted with 90 mL deionised hot water (90 °C) for 1 h in a shaking water bath at the same temperature with the shaking rate of 100 rpm. After centrifugation at 5000 ***g*** for 10 min, the supernatant of the extract was collected. The residue was extracted by the same procedure two more times. Three resulting solutions were mixed, and an excess amount of methanol was added to remove pectic substances. The obtained solution was filtered with a Whatman No. 1 filter paper and transferred into a 250-mL flask. It was then dried by rotary vacuum evaporator at 40 °C. To dissolve the residue, a suitable volume of deionised water was added to the flask for the extract. The obtained solutions were poured into brown bottles with screw cap and stored at −18 °C until further use. Triplicate determinations ($n = 3$) were carried out during the study.

Treatment with activated carbon

To elucidate the effect of the browning products on the antioxidant activity, we used activated carbon to remove the browning colour substances from hot water extracts and investigate the antioxidant activity of the colourless hot water extracts. Powder-activated carbon was added to the obtained hot water extracts with a ratio of 1:2, w/v. After mixing by a Votex for 1 min, the activated carbon-treated extracts were subjected to centrifugation at 5000 g for 5 min. The supernatant was filtered by a 0.22-μm nylon membrane and collected for analysis.

HPLC analysis of flavonoid composition

The extracts of hot water were subjected to a HPLC analysis (Shimadzu LC-10AT) with a reverse phase column (Lichrospher C18e, 250 × 4.6 mm, 5 μm; Merck, Darmstadt, Germany) using a gradient with 2% (v/v) acetic acid in water as solvent A and 0.5% acetic acid in water/acetonitrile (1:1, v/v) as solvent B (Schieber *et al.*, 2001). The gradient was carried out as 0–10 min, 5–10% B; 10–55 min, 10–55% B; 55–60 min, 55–80% B; 60–65 min, 80–100% B; 65–70 min, 100% B; and 70–75 min, 100–5% B. The flow rate was 1 mL/min. Photodiode array (PDA) detection was performed between 220 nm and 350 nm, with a resolution of 2 nm. Eleven flavonoids used as standards were available in our laboratory as follows: naringin, hesperidin, diosmin, neohesperidin, quercetin, naringenin, hesperetin, limettin, nobiletin, tangeretin and DGPP. The phenolic compounds were identified by comparing their retention time and the UV spectra to those of standards. They were quantified from peak area at 280 nm by an external standard method (Lou *et al.*, 2014b), using calibration curves. Their concentrations were expressed as milligram per 100 g dry weight.

Determination of total phenolic content

A hundred microlitres of hot water extract from samples, or standard solution (gallic acid), was mixed with 100 μL of Folin-Ciocalteu's phenol reagent for 3 min (Taga *et al.*, 1984). The mixture was added to 1 mL of 20% Na_2CO_3 solution and incubated in the dark for 30 min at room temperature (25 ± 1 °C). After incubation, the absorbance was measured at 750 nm against the blank. The standard curve was determined with gallic acid, and the total phenolic content was expressed as mg gallic acid equivalent (GAE) per 100 g dry extract using the standard curve. A blank without sample was also measured. All samples were analysed in triplicate.

DPPH radical scavenging activity

The DPPH radical scavenging activity of immature kumquat extracts was estimated according to a slightly modified method of Yamaguchi *et al.* (1998). After 0.5 mL of immature kumquat extract was mixed with 0.5 mL of 0.5 mM DPPH in methanol for 30 min, the mixture was subjected to HPLC analysis with reverse phase column (Thermo ODS-2 Hypersil, 250 × 4.6 mm, 5 μm) under photodiode array (PDA) detection at 517 nm. The mobile phase was methanol/water (7:3, v/v), and the flow rate was 1 mL/min. The change in peak area of DPPH was determined after the reaction. A blank was also measured. Radical scavenging activity was expressed as per cent inhibition and was calculated using the following formula:

$$\%\ \text{DPPH radical scavenging activity} = (1 - \text{Peak area in sample}/\text{Peak area in blank}) \times 100$$

Oxygen radical absorbance capacity assay

The reaction was carried out in 75 mM phosphate buffer (pH 7.4) in cuvettes (Ou *et al.*, 2001). Fifty microlitres of plant extract solution and 50 μL of disodium fluorescein (70 nM final concentration) were mixed in the cuvette and pre-incubated for 15 min at 37 °C. Twenty-five microlitres of APPH solution (221 mM final concentration) was then added, and fluorescence was recorded for 70 min at excitation and emission wavelengths of 485 and 520 nm every 5 min. A blank sample containing phosphate buffer in the reaction mix was measured. Five calibration solutions of Trolox (10, 20, 30, 40 and 50 μM final concentration) were also tested to establish a standard curve. All samples were analysed in triplicate. The area under the curve (AUC) was calculated for each sample by integrating the relative fluorescence curve. The net AUC of the sample was calculated by subtracting the AUC of the blank. The regression equation between net AUC and Trolox concentration was determined, and Oxygen radical absorbance capacity (ORAC) values were expressed as μmol Trolox equivalents (TE)/g dry extract using the standard curve established previously.

Determination of absorbance at $UV_{420\ nm}$

According to Kim and Lee's method (2009), with minor modification, the hot water extracts of immature calamondin obtained by different drying conditions were diluted with distilled water to 20-folds. The absorbance of 200 μL resulting solution was then determined by ELISA reader at 420 nm. The absorbance represented the advanced browning products. A blank was also measured.

Statistical analysis

The data were subjected to analysis of variance (ANOVA) and the significance of the difference between means was determined by Duncan's multiple range test ($P < 0.05$), using SAS (SAS Inst., Cary, NC, USA).

Results and discussion

Change in antioxidant activity related to heat treatment

The ORAC- and DPPH-free radical scavenging potency of hot water extracts from immature calamondin after

drying at 110 °C, 130 °C for 0.5, 1.0, 1.5 and 2.0 h, and, at 150 °C for 1.5 h is shown in Table 1. The ORAC of fresh immature calamondin was 1276 ± 164 μmole TE/g dry extract. No significant changes were observed after drying at 110 °C until 2 h. However, the ORAC increased significantly at 130 °C for 1 h. The ORAC increased drastically to 2041 ± 109 μmole TE/g dry extract at 130 °C for over 1.5 h and to 2425 ± 173 μmole TE/g dry extract at 150 °C for 1.5 h.

The DPPH scavenging potency increased slightly after drying at 110 °C for 1.5 h and 130 °C for 0.5 h. No change was found at 110 °C below 1.5 h. However, similar to the changes in ORAC, the DPPH scavenging potency was enhanced tremendously after heating at 130 °C for over 1.5 h, from 14.3 ± 2.3% per mg mL^{-1} up to 23.9 ± 0.4% per mg mL^{-1}. The highest scavenging potency was observed at 150 °C for 1.5 h with a value of 29.8 ± 0.6% per mg mL^{-1}.

These results indicated that heat treatment could enhance the antioxidant activity of immature calamondin. Heated at 110 °C, only the DPPH scavenging potency increased, slightly, during 1.5 h. Interestingly, the antioxidant activity of immature calamondin increased dramatically after heating at 130 °C for 1.5 h. In our previous study, we also observed that the antioxidant activity of hot water extract from immature calamondin was enhanced by heating at 150 °C for 1.5 h (Lou *et al.*, 2014b). These were in agreement with an earlier report that the antioxidant activity of citrus peel extract increased as heating temperature increased (Jeong *et al.*, 2004).

Table 1 The DPPH scavenging effect and oxygen radical absorbance capacity (ORAC) of hot water extracts from immature calamondin affected by heat treatments

Heat treatments (°C)	(h)	Scavenging potency*(%) per (mg mL^{-1})	ORAC potency (μmole Trolox per gram dry extract)
Raw material		14.3 ± 2.3^{e}	1276 ± 164^{d}
110	0.5	15.8 ± 1.2^{de}	1348 ± 106^{d}
	1.0	15.7 ± 0.6^{de}	1317 ± 265^{d}
	1.5	17.1 ± 1.2^{cd}	1409 ± 94^{cd}
	2.0	17.6 ± 0.8^{cd}	1479 ± 239^{cd}
130	0.5	17.2 ± 0.7^{cd}	1448 ± 105^{cd}
	1.0	17.9 ± 0.5^{c}	1689 ± 41^{c}
	1.5	23.9 ± 0.4^{b}	2041 ± 109^{b}
	2.0	23.0 ± 0.5^{b}	2020 ± 211^{b}
150	1.5	29.8 ± 0.6^{a}	2425 ± 173^{a}

*Scavenging potency = Scavenging effect (%)/Concentration in the reaction (mg mL^{-1}).

$^{a–e}$Mean values (n = 3) in the same column with different superscripts are significantly different (P < 0.05).

Total phenolic content and flavonoid composition during heat treatment

The total phenolic content of hot water extract from fresh immature calamondin was lower than that of heat-treated fresh immature calamondin (Fig. 1). The total phenolic content of immature calamondin heated at 110 °C increased when heated for 2 h, while no change was observed by heating for 0.5 to 1.5 h. On the other hand, the total phenolic content of immature calamondin increased significantly after heating at both 130 °C and 150 °C for over 1.5 h. The changes were quite similar to that of DPPH scavenging potency and ORAC during different heat treatments. It is therefore suggested that high total phenolic content might contribute to high antioxidant activity by high temperature heating.

To elucidate the main effective phenolic compounds, the hot water extracts from heated immature calamondin were subjected to HPLC for flavonoid composition analysis. The changes in flavonoid composition of hot water extracts from immature calamondin after heat treatment are shown in Table 2. Six flavonoids, 3′,5′-di-*C*-β-glucopyranosylphloretin (DGPP), hesperidin, nobiletin, naringin, diosmin and tangeretin were observed in the immature calamondin. The content of DGPP (2802 ± 246 mg 100 g^{-1} dry extract) was remarkably higher than other flavonoids. The other three flavonoids that were high are hesperidin, naringin and

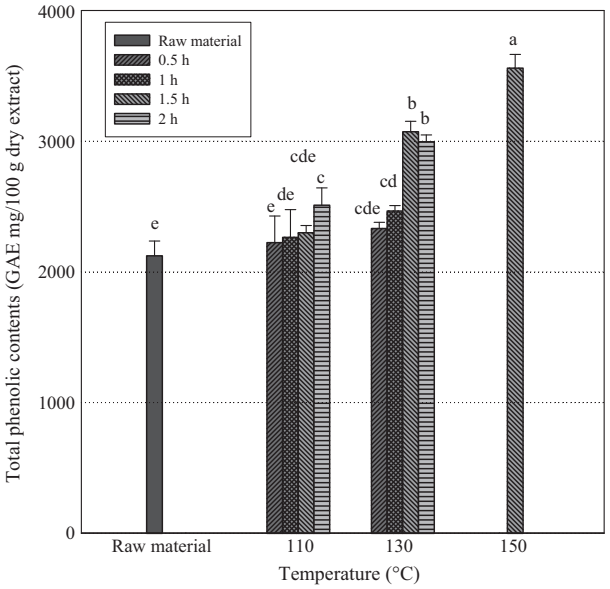

Figure 1 Total phenolic content of hot water extracts from immature calamondin with different heat treatments. Mean values (n = 3) with different letters are significantly different (P < 0.05).

Table 2 Profiles of phenolic compounds of hot water extracts from immature calamondin affected by heat treatments

Heat treatments		Phenolic compounds (mg per 100 g dry extract)						
(°C)	(h)	Naringin	Hesperidin	Diosmin	Nobiletin	Tangeretin	3′,5′-di-C-β-glucopyranosyl-phloretin	Total (mg per 100 g dry extract)
Raw material		111 ± 10^{ab}	239 ± 19^{c}	43 ± 3^{c}	145 ± 12^{cd}	29 ± 2^{cd}	2802 ± 246^{d}	3370 ± 247
110	0.5	111 ± 12^{ab}	211 ± 5^{d}	37 ± 5^{cd}	133 ± 5^{d}	21 ± 1^{e}	3331 ± 122^{b}	3845 ± 123
	1.0	103 ± 10^{bc}	201 ± 8^{d}	36 ± 11^{cd}	135 ± 2^{d}	30 ± 1^{cd}	3192 ± 27^{bc}	3696 ± 32
	1.5	115 ± 3^{ab}	166 ± 6^{e}	31 ± 3de	137 ± 3^{d}	29 ± 1^{cd}	3318 ± 59^{b}	3797 ± 59
	2.0	88 ± 4^{cd}	194 ± 9^{d}	62 ± 2^{b}	141 ± 7^{cd}	31 ± 2^{c}	3095 ± 146^{c}	3611 ± 146
130	0.5	123 ± 17^{a}	345 ± 12^{a}	38 ± 2^{cd}	168 ± 1^{ab}	40 ± 2^{ab}	3601 ± 129^{a}	4316 ± 131
	1.0	73 ± 2de	304 ± 22^{b}	31 ± 3de	174 ± 3^{a}	44 ± 2^{a}	3740 ± 28^{a}	4365 ± 36
	1.5	109 ± 9^{ab}	323 ± 5^{b}	26 ± 2^{ef}	159 ± 9^{b}	39 ± 5^{b}	1588 ± 37^{e}	2244 ± 40
	2.0	66 ± 4^{f}	236 ± 20^{c}	19 ± 3^{f}	110 ± 15^{e}	26 ± 3^{d}	1129 ± 144^{f}	1586 ± 147
150	1.5	69 ± 9^{f}	161 ± 4^{e}	106 ± 2^{a}	155 ± 9^{bc}	28 ± 1^{cd}	332 ± 15^{g}	851 ± 20

$^{a-g}$Mean values (n = 3) in the same column with different superscripts are significantly different ($P < 0.05$).

nobiletin, a more hydrophobic polymethoxyflavone. The content of DGPP increased after 110 °C heating from 0.5 to 2.0 h. However, hesperidin and naringin decreased during heating. The total content of identified phenolic compounds also increased slightly. During heat treatment at 130 °C, the DGPP increased during 0.5 and 1.0 h heating. Interestingly, the DGPP decreased tremendously to 1588–1129 mg 100 g^{-1} dry extract after heating for 1.5 and 2.0 h. The content of hesperidin, nobiletin and tangeretin increased for the first 1.5 h heating; however, they decreased after 2 h heating. The total identified flavonoid compounds increased for the first 1.0 h and decreased after over 1.5 h heating at 130 °C. During heat treatment at 150 °C, the DGPP and total flavonoid compounds decreased dramatically after 1.5 h heating.

These data indicated that heat treatment, at 110 °C for no more than 2.0 h and 130 °C for no more than 1.0 h, could enhance flavonoid content in hot water extract of immature calamondin. This might be due to some destruction of the cell wall structure by heat treatment, which led to better release of the flavonoids. Another opposite effect was observed, during heat treatment at 130 °C for over 1.5 h or 150 °C for 1.5 h, the DGPP degraded drastically to only half or less of its original content. Thus, some flavonoids can be degraded at high temperature heating, such 130 or 150 °C.

Compared with the changes of antioxidant activity, the antioxidant activity increased at 130 °C heating for 1.5 and 2.0 h, and 150 °C for 1.5 h; however, the total flavonoid compounds decreased in the same conditions. The total phenolic content increased significantly. Therefore, it is suggested that some unidentified compounds might also contribute to the antioxidant activity.

Contents of browning products during heat treatment

During heat treatment, the colour of the hot water extract turned darker than the extract from fresh immature calamondin. Considering that the browning reaction products might provide some antioxidant activity, we determined the absorbance at $UV_{420\ nm}$ of the hot water extract to determine the advanced reaction products of browning reaction (Kim & Lee, 2009). Figure 2 shows the absorbance of the hot water extract from fresh and heated immature calamondin.

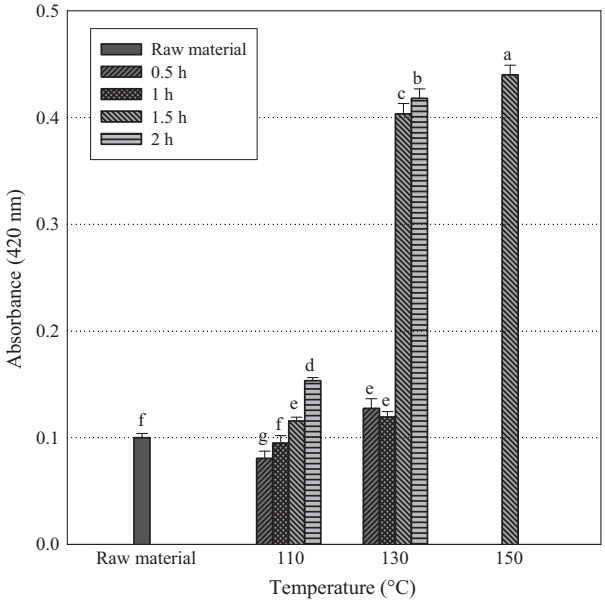

Figure 2 The absorbance at $UV_{420\ nm}$ of hot water extracts from immature calamondin with different heat treatments. Mean values (n = 3) with different letters are significantly different ($P < 0.05$).

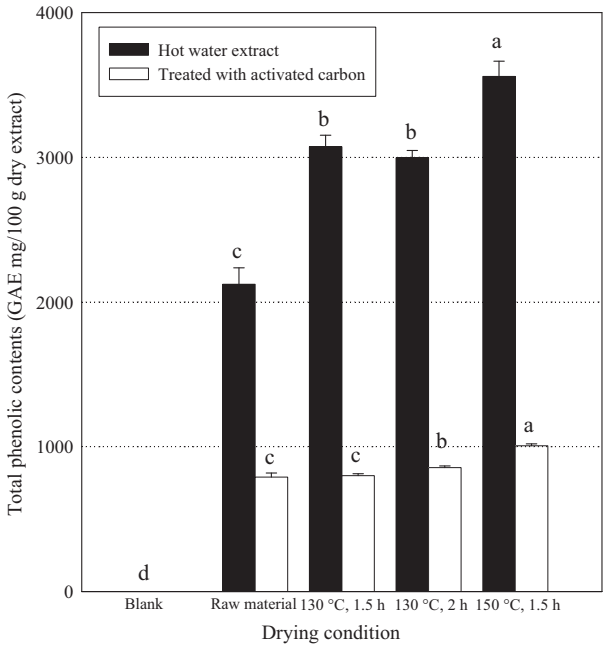

Figure 3 Effects of activated carbon-treated hot water extracts from immature calamondin after heat treatments on the total phenolic content. Mean values ($n = 3$) with different letters in the separated groups are significantly different ($P < 0.05$).

The results indicated that absorbance increased after heating at 110 °C for over 1.5 h. However, remarkable increase of absorbance was seen during heating at 130 °C for 1.5 and 2.0 h and at 150 °C for 1.5 h. They were almost threefold greater as compared to the fresh immature calamondin.

The trend of change in the $UV_{420\ nm}$ was similar to the change of antioxidant activity during heat treatment. These data hinted that not only total phenolic content, but also some browning products, might provide certain antioxidant activity in the hot water extract from heated immature calamondin.

Relation between browning products, total phenolic content and antioxidant activity

The pearson's correlations between $UV_{420\ nm}$ and antioxidant activity as well as total phenolic content and antioxidant activity were evaluated. The correlation coefficient between total phenolic content and DPPH scavenging potency was significant with $r = 0.993$ ($P < 0.001$), it was 0.986 ($P < 0.001$) between total phenolic content and ORAC. Similarly, the absorbance at $UV_{420\ nm}$ also highly correlated with the DPPH scavenging potency and ORAC with correlation coefficients of 0.933 ($P < 0.001$) and 0.942 ($P < 0.001$), respectively. This suggested that browning

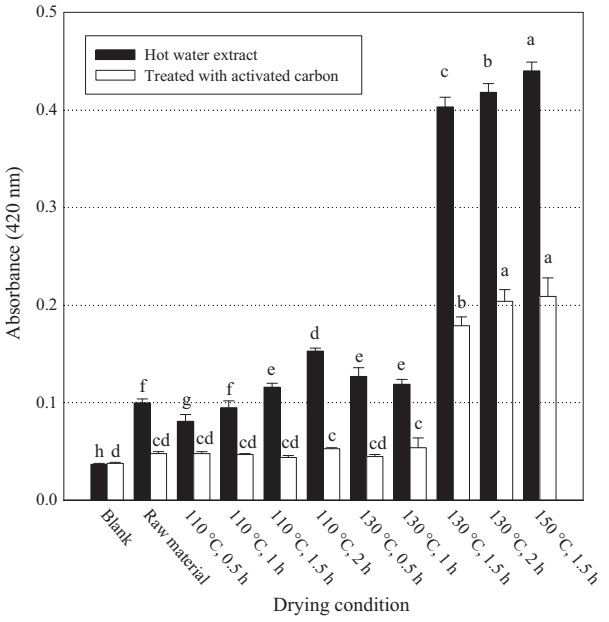

Figure 4 Effects of activated carbon-treated hot water extracts from immature calamondin after heat treatments on the browning products. Mean values ($n = 3$) with different letters in the separated groups are significantly different ($P < 0.05$).

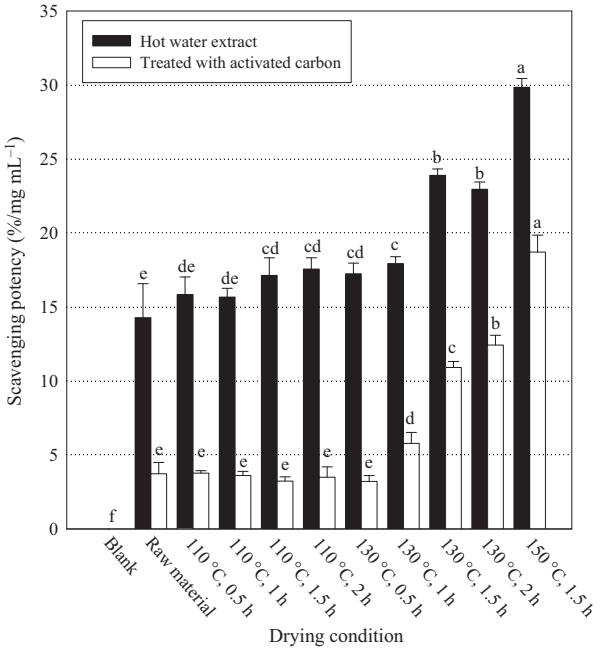

Figure 5 Effects of activated carbon-treated hot water extracts from immature calamondin after heat treatments on the DPPH radical scavenging potency. Mean values ($n = 3$) with different letters in the separated groups are significantly different ($P < 0.05$).

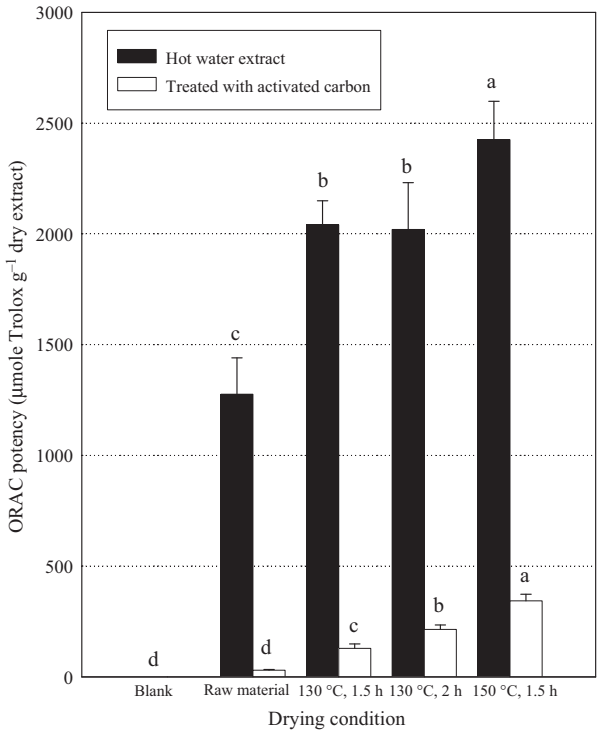

Figure 6 Effects of activated carbon-treated hot water extracts from immature calamondin after heat treatments on the oxygen radical absorbance capacity (ORAC). Mean values ($n = 3$) with different letters in the separated groups are significantly different ($P < 0.05$).

products could indeed contribute to antioxidant activity. Interestingly, the data of the relationship between $UV_{420\ nm}$ and antioxidant activity divided into two main groups. The higher absorbance group gave a higher antioxidant activity. This hinted that the drastic increase of antioxidant activity could be due to the drastic increase of browning products, as a gradually increase of the total phenolic content was observed during heat treatment.

To investigate the effect of the browning products on antioxidant activity, the hot water extracts were treated with activated carbon. The results are shown in Fig. 3. The total phenolic content decreased after treatment with activated carbon (Fig. 3). After treatment with activated carbon, the total phenolic content of the extracts heated by 130 °C for 1.5 and 2.0 h and 150 °C for 1.5 h decreased almost to the level equivalent to the content of the extract from the fresh immature calamondin. Figure 4 shows the browning products after treatment with activated carbon. The extract from immature calamondin heated under 110 °C for 0–2.0 h and 130 °C for 0–1.0 h exhibited an equal amounts of browning products as compared to the content of blank and fresh immature calamondin. Furthermore, the DPPH scavenging potency showed low or equal amounts between each other under the same heating conditions (Fig. 5). This hinted that no browning products might be involved in the effect on antioxidant activity; however, the low antioxidant activity might be due to the effect of the remaining total phenolic content. Interestingly, the DPPH scavenging potency and ORAC (Fig. 6) still increased after treatment with activated carbon heated at 130 °C for 1.5, and 2.0 h, as well as, 150 °C for 1.5 h. These changes coincided with the changes in browning products at the same heat conditions, while the changes in total phenolic content were relatively steady. The increasing ratio of the browning products was similar to the ratio of the DPPH scavenging potency and ORAC at a range of 2–3 folds. This indicated that browning products contributed to the antioxidant activity at high temperature heating. A positive correlation between browning and antioxidant activity of Maillard reaction products has been identified in model systems and in food (Manzocco *et al.*, 2001). Antioxidant activity could be improved due to formation of novel compounds having antioxidant activity during heat treatment or thermal processing (Choi *et al.*, 2006).

It is therefore suggested that the antioxidant activity of hot water extract from immature calamondin was affected by two main factors, that is total phenolic content and browning products. Heated at higher temperatures, for instance, >130 °C for more than 1.5 h, the high antioxidant activity of the extract might be mainly due to the browning products. Heated at lower temperatures, the total phenolic contents were the major responsible substances for the antioxidant activity.

Conclusions

Heat treatment at ≥130 °C over 1.5 h could significantly enhance the antioxidant activity of immature calamondin, which the major responsible substances for the increasing antioxidant activity were browning products. However, the low magnitude of increasing antioxidant activity at 110 °C and 130 °C ≤ 1.0 h is mainly due to the increase of total phenolic content.

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