Structural elucidation and cellular antioxidant activity evaluation of major antioxidant phenolics in lychee pulp

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A B S T R A C T

Lychee pulp contains phenolic compounds that are strong antioxidants, but the identities of the major antioxidants present are unknown. In the present study, the major contributors to the antioxidant activity of fresh lychee pulp were identified and their cellular antioxidant activities were investigated. Aqueous acetone extracts of lychee pulp were fractionated on polyamide resin, and those fractions with the largest antioxidant and radical scavenging activities were selected using cellular antioxidant activity and oxygen radical absorbance capacity assays. Three compounds that were major contributors to the antioxidant activity in these fractions were obtained by reverse-phase preparative HPLC and identified as quercetin 3-O-rutinoside-7-O-α-L-rhamnosidase (quercetin 3-rut-7-rha), quercetin 3-O-rutinoside (rutin) and (−)-epicatechin using NMR spectroscopy, HMBC, and ESI-MS spectrometry. The concentration of quercetin 3-rut-7-rha was 17.25 mg per 100 g of lychee pulp fresh weight. This is the first report of the identification and cellular antioxidant activity of quercetin 3-rut-7-rha from lychee pulp.

1. Introduction

Epidemiological studies have shown that fruits protect against certain major chronic diseases such as cancer and cardiovascular disease (Manach, Scalbert, Morand, Rémy, & Jiménez, 2004; Scalbert & Williamson, 2000). The health effects of substantially increasing fruit consumption have been associated with the beneficial antioxidant activity of phenolic compounds in the fruit (Morton, Caccetta, Puddey, & Croft, 2000). Lychee (Litchi chinensis Sonn.) is a tropical to subtropical fruit (Ruenroengklin, Yang, Lin, Chen, & Jiang, 2009; Wang, Lou, Ma, & Liu, 2011). The pericarp, seed and pulp of lychee contain large quantities of phenolic compounds, which are potential sources of natural antioxidants (Khan, Asghar, Iqbal, Bokhari, & Khan, 2009).

Previous studies have focused on the lychee pericarp and seed, and identified several phenolics (Le Roux, Doco, Sarni-Manchado, Lozano, & Cheynier, 1998; Prasad et al., 2009; Xu, Xie, Hao, Jiang, & Wei, 2011; Zhang, Pang, Yang, Ji, & Jiang, 2004) and evaluated the activities of the purified compounds (Prasad et al., 2009; Xu et al., 2011). Recent work suggested that lychee pulp, which is the most commonly consumed part of the fruit, contains a large proportion of the total phenolics in the lychee. In ferric reducing antioxidant power and 2,2-diphenyl-1-picrylhydrazyl assays lychee pulp exhibits excellent antioxidant activities (Luximon-Ramama, Bahourun, & Crozier, 2003; Mahattanatawee et al., 2006; Saxena et al., 2011). However, the ability of these assays to predict in vivo activity has been questioned. The oxygen radical absorbance capacity (ORAC) values for flavonoids are not related to their cellular antioxidant activity (CAA) values (Wolfe & Liu, 2008). Based on cell culture, the CAA assay takes into account the bioavailability, uptake and metabolism of the antioxidants, and is a more biological method than the chemical methods commonly used in determination of antioxidant activity (Wolfe & Liu, 2007). Therefore, the CAA assay should be applied to lychee pulp.

Earlier research has focused on the in vitro biological activity of lychee pulp, and its chemical constituents have not been studied. Using high-performance liquid chromatography (HPLC) with photodiode array-mass spectrometry detection, Mahattanatawee et al. (2006) detected flavones (quercetin and kaempferol) and glycosides in lychee pulp from Florida. Bhoopat et al. identified two phenolics, trans-cinnamic acid and pelargonidin-3-O-glucoside, in lychee pulp from Thailand lychee pulp using HPLC with ultraviolet detection combined with electrospray ionization mass spectrometry (ESI-MS) (Bhoopat et al., 2011). Zhang et al. detected six individual phenolics including gallic acid, chlorogenic acid,
(+)-catechin, caffeic acid, (−)-epicatechin and rutin in lychee pulp from Southeast China lychee by HPLC (Zhang et al., 2013). However, none of these studies isolated and purified the biological compounds from lychee pulp or confirmed their identities by other methods. To our knowledge, the bioactive components of lychee pulp have not been studied extensively.

The objective of the present investigation was to identify the major antioxidant compounds in lychee pulp by ESI-MS and nuclear magnetic resonance (NMR) analyses. The antioxidant activities of the compounds were compared using ORAC and CAA methods.

2. Materials and methods

2.1. Plant material

Lychee (cv. Feizixiao) fruits were purchased at commercial maturity with bright red pericarp from a local fruit market in Guangzhou, China. The fresh fruits were selected for uniformity of maturity. Before manual removal of the pericarp and seed, each fruit was washed with tap water. The fresh lychee pulp was weighed and then immersed in chilled acetone/water (80:20, v/v).

2.2. Chemicals

6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid, (−)-epicatechin gallate (ECG), (+)-epigallocatechin (EGC), (+)-catechin hydrate, epicatechin, quercetin dehydrate, rutin, Folin–Ciocalteu reagent, 2,7'-dichlorofluorescin diacetate, fluorescein disodium salt and 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) were obtained from Sigma–Aldrich, Inc. (St. Louis, MO, USA). Macroporous adsorption resin, HPD826, was obtained from Cangzhou Bon Adsorber Technology Co., Ltd. (Cangzhou, China). Polyamide, 80–100 mesh, was purchased from Taizhou Luqiao Sijia Biochemical Plastic Factory (Taizhou, China). Dubelco’s modified eagle’s medium (DMEM), dimethyl sulfoxide, HPLC-grade methanol, acetic acid and acetonitrile were purchased from Thermo Fisher Scientific (Waltham, MA). HepG2 human liver cancer cells were obtained from the American Type Culture Collection (Rockville, MD, USA).

2.3. Extraction of phenolic compounds from lychee pulp

Fruit phenolics were prepared according to a previous method (Sun, Chu, Wu, & Liu, 2002). In brief, samples of freshly prepared lychee pulp (500 g) were blended for 5 min in 1000 mL of chilled acetone/water (80:20, v/v) using a Philips blender. Then each sample was homogenised (XHF-D, Ningbo Xin-zhi-Bio Technology Co. Ltd., Ningbo, China) for 5 min while in an ice-water bath. The homogenates were centrifuged at 2800 × g for 10 min (Changsha Xiangzi Instrument Co. Ltd., Changsha, China), and each pellet was re-extracted into chilled acetone/water (80:20, v/v) using a Philips blender. The eluents were concentrated on a rotary evaporator (Eyela N-1100, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) under vacuum at 45 °C and freeze-dried (Eyela FDU-2110) under vacuum at –80 °C.

2.4. Separation and purification of phenolic compounds from lychee pulp

A chromatographic fractionation flow chart of the aqueous acetone extracts is shown in Fig. 1. First, the concentrated extracts (40 mL in water) were fractionated on a HPD826 resin (Cangzhou Bonchem Co., Ltd., Cangzhou, China) column (ø 3.5 cm, length 40 cm). The elution was performed with 1 L of deionized water and 95% aqueous ethanol (v/v). The organic phase fraction was collected and analysed by HPLC, and then concentrated and fractionated on a polyamide resin (PR) column (ø3.5 cm, height 45 cm). Sequential elution was performed with 500 mL each of the following solvents: deionized water, 10% aqueous EtOH (v/v), 20% aqueous EtOH (v/v), 30% aqueous EtOH (v/v), 40% aqueous EtOH (v/v), 50% aqueous EtOH (v/v), 80% aqueous EtOH (v/v) and 100% aqueous EtOH (v/v). Eight fractions of 500 mL were collected and analysed by HPLC. Fractions with similar compositions were combined. The fractions containing compounds of interest, which exhibited good antioxidant activities, were purified by preparative HPLC with monitoring at 280 nm and elution with a mixture of water and methanol (65:35, v/v, for F2 and 60:40, v/v, for F3). The eluents were concentrated on a rotary evaporator (Eyela N-1100, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) under vacuum at 45 °C and freeze-dried (Eyela FDU-2110) under vacuum at –80 °C.

2.5. Identification of purified compounds from lychee pulp by ESI-MS

Mass spectra of the purified compounds from lychee pulp were obtained using a mass spectrometer (ABI, Foster City, CA, USA) equipped with a turbo ion-spray ionization source operating in both negative and positive ion ESI mode. The capillary voltages were 4500 V (negative) and 5500 V (positive). Continuous mass spectra were collected by accumulation of 10 multiple channel acquisitions scanning from 100 to 1000 m/z.

2.6. Identification of purified compounds from lychee pulp by NMR

1H NMR (400 MHz), 13C NMR (100 MHz) and heteronuclear multiple-bond correlation (HMBC) spectra of the purified substrates were recorded on a Bruker DRX-400 FT-NMR (Bruker, Billerica, MA, USA) spectrometer. Compounds P1 and P2 were
dissolved in deuterated dimethyl sulfoxide (DMSO-d6) and P3 was dissolved in methanol (methanol-d4). Chemical shifts (δ) are reported in parts per million (ppm) and coupling constants (J) are expressed in hertz (Hz).

2.7. Total phenolic contents

Total phenolic contents were determined using the method described by Dewanto et al. (2002). An aliquot (125 µL) of each fraction or standard solution was added to 0.5 mL of deionized water, followed by 125 µL of FCR. After 6 min, 1.25 mL of an aqueous 7% Na2CO3 solution was added to the mixture rapidly. Then, the final total volume was made up to 3.0 mL with deionized water and the mixture was incubated at ambient temperature in the dark for 90 min. Finally, the absorbance was recorded at 760 nm using a Shimadzu UV-1800 spectrometer (Shimadzu Inc., Kyoto, Japan). Gallic acid was used as the standard. The total phenolic contents are expressed as milligrams of gallic acid equivalents per 100 g of lychee pulp fresh weight (FW). The results are presented as means ± SD for three replications obtained from three samples.

2.8. Total flavonoid contents

Total flavonoid contents were determined by the method of Dewanto et al. (2002). An aliquot (250 µL) of each fraction or standard solution was mixed with 1.25 mL of deionized water and 75 µL of an aqueous solution of NaNO2 (5% mass fraction). The mixture was kept at room temperature for 5 min, and then 150 µL of an aqueous 10% AlCl3·6H2O solution was added. After 5 min, 0.5 mL of an aqueous 1 mol/L NaOH solution was added to the mixture. The mixed solution was then diluted to 3.0 mL with deionized water. (+)-Catechin was used as the standard. The absorption was measured at 510 nm against a blank. The results are expressed as milligrams of (+)-catechin equivalents per 100 g of lychee pulp FW. The data are stated as means ± SD for three replications obtained from three samples.

2.9. Analytical HPLC-diode array detector (DAD) analyses

The phenolic compounds in the lychee pulp were identified using a reverse-phase-HPLC-DAD method as described by Zhang et al. (2013). HPLC analysis was performed using an Agilent Zorbax SB-C18 column (250 × 4.6 mm I.D., 5 µm particle size, Agilent Technologies, Santa Clara, CA, USA) with a mobile phase flow rate of 1.0 mL/min. An Agilent 1100 DAD (Agilent Technologies, Santa Clara, CA, USA) was used for monitoring the eluent at 260, 280 and 320 nm. The mobile phase included solvent A (water/acetic acid, 996:4, v/v) and solvent B (acetonitrile), with the percentage of solvent B increased by the following binary gradient: 95:5 (A:B, v/v) at 0 min, increased to 75:25 (A:B, v/v) after 40 min, increased to 65:35 (A:B, v/v) after 45 min, and increased to 50:50 (A:B, v/v) after 50 min. The column was then equilibrated for 5 min with 95:5 (A:B, v/v). Peaks were identified using their retention times. Values expressed are as milligrams per 100 g of lychee pulp FW. The data are reported as means ± SD for three replications obtained from three samples.

2.10. Determination of ORAC

The ORAC assay was carried out using an Infinite® M200 PRO plate reader (Tecan Austria GmbH, Grödig, Austria) following the method reported by Wolfe et al. (2008). Wells of a black 96-well microplate (Corning Scientific, Corning, NY) were seeded with aliquots of either a sample (20 µL) in 75 mmol/L potassium phosphate buffer (pH 7.4, working buffer), standard (Trolox, 20 µL, concentration range 6.25–50 µmol/L), or a blank (working buffer, 20 µL, 75 mmol/L). Wells were prepared in triplicate for each type of the above solutions, and replicates were distributed throughout the microplate rather than in adjacent wells. No outside wells were used. Fluoroscein (200 µL, 0.96 µmol/L in working buffer) was added to each well. The plate was kept at 37 °C for 20 min in the plate reader. Then, 20 µL of freshly prepared AAPH in working buffer (119 mmol/L) was added to all wells except for the blank well. Excitation and emission spectra were recorded at 485 and 520 nm, respectively, every 4.5 min for 35 cycles. The ORAC results are reported as micromoles of Trolox equivalents (TE) per 100 g of lychee pulp FW. The ORAC values are presented as means ± SD for three replications obtained from three samples.

2.11. Measurement of CAA

The CAA assay protocol was adapted from Wolfe and Liu (2007). Briefly, human liver cancer cells (HepG2) were seeded into a black 96-well microplate (Corning) at a density of 6 × 10^4 cells in 100 µL of growth medium (DMEM containing 10% foetal bovine serum) per well. The microplate was kept in a carbon dioxide incubator at 37 °C for 24 h. After the growth medium was removed, cells were treated for 1 h with 100 µL of standard (quercetin) or purified compound with 2′,7′-dichlorofluorescin diacetate (25 µmol/L) in DMEM. Each well treatment was performed in triplicate. Then, 600 µmol/L AAPH in 100 µL of growth medium was added to every treated well, and the microplate was analysed using an Infinite® M200 PRO plate reader at 37 °C for 1 h. Excitation and emission spectra were recorded at 485 and 538 nm, respectively, every 5 min for 12 cycles. Calculations of the final CAA results followed the method of Wolfe et al., 2008. The CAA results are reported as micromoles of quercetin equivalents (QE) per 100 g of lychee pulp FW. The CAA values are presented as means ± SD for triplicate sets of data obtained from three replications experiment.

2.12. Statistical analysis

All results are presented as the mean ± SD for triplicate determinations of each sample. Data were analysed with one-way ANOVA followed by a Student–Newman–Keuls test. Statistical significance was defined as p < 0.05. All the statistical analyses were performed using SPSS statistical package version 13.0 (SPSS Inc. Chicago, IL, USA).

3. Results and discussion

3.1. Bio-guided selection of the main fractions with antioxidant activity from lychee pulp

The total phenolic content, total flavonoid content, ORAC and CAA values of the lychee pulp PR fractions and their percentage contribution to the total macroporous adsorption resin (MPAR) fraction are presented in Table 1. Fractions F2 and F3 from the PR exhibited higher total phenolic contents than fractions F1 and F2, and accordingly had higher percentage contributions to the total MPAR fraction of these four PR fractions. The total percentage contribution (75.45%) of F2 and F3 to the total phenolic content was more than five times that of F1 and F2 (13.48%). The total flavonoid contents of the four tested fractions were in a similar order to the total phenolic contents. The total percentage contribution (77.48%) of the fractions with higher flavonoid contents (F2 and F3) was five times that of the fractions with lower flavonoid contents (F1 and F2, 14.45%). Among the four fractions tested, F2 exhibited the highest ORAC values with the highest total phenolic contents and total flavonoid contents, followed by F3, then F1, and
Finally F4. The ORAC results of both F2 and F3 were three times higher than those of F1 and F4 (p < 0.05). The total percentage contribution (85.76%) of the ORAC values of F2 and F3 to the total MPAR ORAC was more than four times that of F1 and F2 (19.9%). The CAA values were in a similar order to the ORAC values, with F2 having the highest CAA value. The total percentage contribution (90.28%) of F2 and F3 to the total CAA value was much higher than the contribution of F1 and F4. However, there were some important differences between the CAA and ORAC results. The CAA value of F2 was five times higher than that of F3, although these fractions had similar total flavonoid contents, total flavonoid contents and ORAC values. In addition, F1 did not show CAA.

In different PR fractions, the total flavonoids content was positively correlated with the total phenolics content. Furthermore, the ORAC values of 4 PR fractions were positively correlated with their total phenolics contents, which was proved in many previous reports (Wolfe et al., 2008). However, the CAA values of different PR fractions were not consistent with their ORAC values. Although Fraction F2 and F3 had similar total phenolic content and showed equal ORAC antioxidant activity, the former exhibited much higher CAA value than the latter. The difference in phytochemical profiles in these two fractions might be the main reason accounting for the above discrepancy. And therefore, it is necessary to clarify the main phenolic compounds in different fractions. Moreover, Fraction F2 and F3 had higher total phenolics and flavonoids contents and exhibited higher antioxidant activity than fraction F1 and F4. Therefore, the phytochemical compounds of lychee pulp extracts with antioxidant activity are considered to be concentrated in PR fraction F2 and F3. These fractions were selected for isolation and identification of phenolic compounds.

3.2. Structural identification of compounds isolated from lychee pulp

Compounds isolated from the lychee pulp were identified using NMR and MS analyses. Compounds P1, P2 and P3 were identified as quercetin 3-O-rutinoside-7-O-α-L-rhamnoside (quercetin 3-rut-7-rha), rutin and (−)-epicatechin, respectively, based on following characteristics.

Quercetin 3-rut-7-rha (P1) from lychee pulp was obtained as a yellow amorphous powder. The structure of P1 was identified based on its 1H NMR, 13C NMR, HMBC and ESI-MS analyses (Fig. 2). Negative ESI-MS m/z: 755.4 [M–H]−, m/z 1512.3 [2M–H]−. Positive ESI-MS m/z: 779.2 [M+Na]+, m/z 1535.0 [2M+Na]+ and 1559.0 [2M+K]−. 13C NMR: 156.4 (C-2), 151.5 (C-3), 176.9 (C-4), 161.6 (C-5), 99.0 (C-6), 157.1 (C-7), 94.6 (C-8), 153.6 (C-9), 100.3 (C-10), 121.6 (C-1′), 115.6 (C-2′), 149.5 (C-3′), 146.4 (C-4′), 168.0 (C-5′), 121.6 (C-6′), 100.9 (C-7′), 100.3 (C-8′), 72.2 (C-2′′), 77.6 (C-3′′), 70.7 (C-4′′), 70.6 (C-5′′), 72.1 (C-6′′), 72.1 (C-7′′), 68.7 (C-8′′), 68.4 (C-9′′), 16.4 (C-6′′), 16.1 (C-6′′). Its molecular formula was determined to be C29H30O12 from the negative ESIMS ion at m/z 755.4. The 13C NMR spectrum displayed 27 carbon resonances assignable to a moiety of quercetin and a rutinose. These data were very similar to those of rutin, a known compound obtained in the present study, except for the presence of an additional rhamnose in P1 compared with rutin. The attachment of the rhamnose to C-7 via C-1′′′ was deduced from the HMBC spectrum, in which correlations were observed from H-1′′′ to C-7. Therefore, the structure of P1 was assigned as quercetin 3-rut-7-rha by comparison to the literature (Yoshida, Kondo, Ito, & Kondo, 2005).

Rutin (P2) was obtained as a yellow amorphous powder. The [M−H]− peak of P2 was observed at m/z 609.1 and its [M+Na]+ peak at m/z 633.5 in the ESI-MS spectrum. Its molecular formula was C23H24O11. Characteristic fragment ions at m/z 169.1 and 123.8 helped identify the compound as rutin as they matched those reported in the literature (Hokkanen, Mattila, Jaakola, Pirttilä, & Tolonen, 2009). Identification of P2 as rutin was confirmed by comparing its 13C NMR spectrum to published data (Hokkanen et al., 2009). The 13C NMR spectrum displayed 15 carbon resonances [157.0 (C-2), 133.7 (C-3), 177.8 (C-4), 161.8 (C-5), 99.2 (C-6), 161.6 (C-7), 94.0 (C-8), 156.9 (C-9), 104.3 (C-10), 122.0 (C-11), 116.6 (C-12), 148.9 (C-13), 146.2 (C-14), 116.7 (C-15), 121.6 (C-16′) for quercetin and 12 carbon resonances for rutinose [101.2 (C-1′), 101.7 (C-1′′), 76.9 (C-5′), 76.3 (C-3′), 74.6 (C-2′), 72.3 (C-2′′), 71.0 (C-4′), 70.8 (C-3′′), 70.4 (C-4′′), 68.7 (C-5′′), 67.4 (C-6′′), 18.2 (C-6′′)], which were in accordance with rutin (Wan, Yu, Zhou, Tian, & Cao, 2011).

(−)-Epicatechin (P3) was obtained as a white amorphous powder. Negative ESI-MS m/z: 289.1 [M−H]−, 13C NMR: 28.9 (C-4), 66.9 (C-3), 79.4 (C-2), 95.5 (C-8), 96.2 (C-6), 99.7 (C-4a), 115.3 (C-2′), 115.6 (C-5′), 119.3 (C-6′), 132.1 (C-1′), 145.3 (C-3′), 145.5 (C-4′), 157.0 (C-5′), 157.5 (C-7) and 157.6 (C-8a). These data were very similar to those reported for (−)-epicatechin (Prasad et al., 2009; Sun, Shi, Jiang, Xue, & Wei, 2007). The identification of P3 as (−)-epicatechin was confirmed by the presence of characteristic fragment ions at m/z 245.1, 179.1, and 137.1. Moreover, in the 1H and 13C NMR spectra of P3, the characteristic signals of nine protons and 15 carbons for (−)-epicatechin were observed (Fan et al., 2004; Pizzolattol, Venson, Junior, Smania, & Braz-Filho, 2002; Zhao, Yang, Wang, Li, & Jiang, 2006).

This is the first report of the isolation and identification of the compound quercetin 3-rut-7-rha in lychee pulp. Among the three compounds identified, quercetin 3-rut-7-rha was present in the highest concentration (17.25 ± 0.21 mg per 100 g of lychee pulp FW), followed by rutin (3.58 ± 0.27 mg per 100 g of lychee pulp FW) and (−)-epicatechin (2.31 ± 0.19 mg per 100 g of lychee pulp FW). In our previous work, we identified nine phenolic compounds in litchi pulp, including 3,4-dihydroxybenzoic acid, (−)-catechin, vanillic acid, caffeic acid, syringic acid, (−)-epicatechin, 4-methylcatechol, ferulic acid and rutin, by HPLC referring to the remaining time with corresponding standard compounds (Su et al., 2014; Zhang et al., 2015). Among these phenolics, three compounds were isolated in the present study, which were quercetin 3-rut-7-rha, rutin and (−)-epicatechin. However, the compound identified as 4-methylcatechol in our previous work was proved to be quercetin and a
In the present study, all the concentrations of phenolic components were less than 500 µg/100 g FW except for quercetin 3-rut-7-rha, rutin, and (-)-epicatechin, which were concentrated in fraction F2 and F3 by bio-guided isolation in the present study. The percentage contribution of quercetin 3-rut-7-rha, rutin, and (-)-epicatechin to the total phenolic content were 70.35%, 14.60%, and 9.42%. These results show that quercetin 3-rut-7-rha would be an important active compound in lychee pulp.

3.3. Antioxidant activity of the compounds isolated from lychee pulp and selected representative phenolic compounds

The antioxidant activity of the three compounds isolated from lychee pulp and four commonly used flavonoids were compared using the ORAC and CAA assays. The ORAC values are present in Fig. 3. Rutin and quercetin 3-rut-7-rha had the highest activities among the seven tested compounds, followed by catechin, epicatechin, quercetin, and ECG. EGC showed the lowest ORAC antioxidant activity. This result was consistent with previous reports (Aaby, Hvattum, & Skrede, 2004; Wolfe & Liu, 2008). The median effective concentration (EC50) values and CAA values of 7 flavonoid compounds are listed in Table 2. Quercetin had the highest activity followed by ECG, quercetin 3-rut-7-rha, EGC, catechin, epicatechin, and rutin. Epicatechin and rutin purified from lychee pulp had low and no activity, respectively. These results were consistent with the literature (Wolfe & Liu, 2008). Quercetin 3-rut-7-rha showed similar or a little lower CAA value than luteolin (37.1 ± 0.0 µmol of QE/100 µmol), morin (32.1 ± 2.1 µmol of QE/100 µmol), and myricetin (28.4 ± 0.9 µmol of QE/100 µmol), which were well studied flavonoids (Wolfe & Liu, 2008). Quercetin 3-rut-7-rha exhibited a much higher CAA value than other phenolics present in lychee pulp including caffeic acid, catechin, and ferulic acid (5.59, 2.5, and less than 2.5 µmol of QE/100 µmol, respectively) which were reported in previous reports (Wolfe & Liu, 2007, 2008). Antioxidant activity results showed that quercetin 3-rut-7-rha exhibited good ORAC and CAA antioxidant activities. However, rutin and (-)-epicatechin showed low CAA activity, though they had good ORAC values. The discrepancy between ORAC and CAA activity of these compounds could be at least partly attributed to their difference in chemical structure, which affects their ability.
to scavenge free radicals and the level of cellular absorption and metabolism (Wolfe & Liu, 2008).

Quercetin, with high CAA activity, is used as standard in CAA assay. However, quercetin 3-rut-7-rha, quercetin 3-glu and quercetin 3-rut (rutin) exhibited lower CAA values than quercetin because of their loss of the 3-hydroxy moiety on the C-ring (Wolfe & Liu, 2008). The position of glycosylation and the type of esterified sugar of flavonoids dramatically affected their CAA activity (Wolfe & Liu, 2008). As a result, rutin and quercetin 3-glu had no or low CAA activity but quercetin 3-rut-7-rha exhibited much higher CAA values. The differences in the CAA activity among the 3 quercetin glycosides could also partly be explained by the different twist angles of the B-ring compared to the A- and C-rings introduced by 7-glycosylation. The twist angle for rutin is almost 30°, whereas the angle for luteolin, which has base structure and similar CAA value to quercetin 3-rut-7-rha, is only around 20° (van Acker et al., 1996). The 7-glycosylation would decrease the torsion angle and enhance the CAA of quercetin 3-rut-7-rha.

As mentioned previously, CAA method takes account of the bioavailability, uptake and metabolism of the antioxidants and represents a more biologically relevant method for the determination of antioxidant activity than many other in vitro antioxidant activity analysis methods (Wolfe & Liu, 2007). Therefore, the extent of uptake into HepG2 cells of the detected flavonoids is also an important factor influencing their CAA activity. Flavonoids with several hydroxyl groups which presented in aglycons showed highest affinity for Caco-2 cell membranes (Tammela et al., 2004). Therefore, quercetin would be easily delivered into HepG2 cells. Despite the lack of research on the absorption of quercetin and its derivatives into HepG2 cells, we can take hints from the results on the absorption of flavonoids via intestine. Absorption of flavonoid glycosides requires some special conditions. Wolffram et al. reported that the absorption of quercetin-3-glucoside into brush border membrane of rat small intestine depends on a sodium-dependent glucose transporter (SGLT1) (Wolffram, Blöck, & Ader, 2002). Seink et al. found that intestinal absorption of quercetin-3-glucoside was regulated by lactase phlorizin hydrolase (LPH), an enzyme located in brush border membrane of intestinal cells (Sesink, Arts, Faassen-Peters, & Hollman, 2003). Therefore, the quercetin-3-glucoside would be partly uptaken into HepG2 cells in CAA assay, and then showed low CAA value. Rutin could not be absorbed unless it was hydrolysed by large intestinal microbe (Jaganath, Jaganath, Mullen, Edwards, & Crozier, 2006). Hence, we deduce that rutin can not passed through cell membrane into HepG2 cells showing no CAA activity. Quercetin 3-rut-7-rha, exhibited good water solubility in contrast to rutin because of the 7 glycosyl substituent in the C ring. Furthermore, quercetin 3-rut-7-rha would be absorbed more easily due to its 7-glycosidation (Chen, Lin, & Hu, 2005). Therefore, quercetin 3-rut-7-rha would be absorbed by cells and exhibited higher CAA activity due to its molecular structure, 7-glycosidation and good solubility compared to rutin and querce- tin-3-glucoside.

Besides its highest CAA and ORAC activity, the content of quercetin 3-rut-7-rha in lychee pulp was higher than the other phenolics including rutin, (-)-epicatechin and (+)-catechin etc. Therefore, quercetin 3-rut-7-rha would be the main antioxidant active substance in lychee pulp.

4. Conclusion

Major antioxidant phenolics in lychee pulp extracts were isolated by the guidance of CAA and ORAC assays. These compounds were purified by reverse-phase preparative HPLC and identified as quercetin 3-rut-7-rha, rutin and (-)-epicatechin by ESI-MS, 1H-NMR, 13C-NMR and HMBC analyses. Quercetin 3-rut-7-rha exhibited strong CAA activity, which was similar to luteolin and higher than morin or myricetin. This is the first report of the presence of quercetin 3-rut-7-rha in lychee pulp and its CAA activity.

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