



Investigation of *in vitro* and *in vivo* antioxidant activities of flavonoids rich extract from the berries of *Rhodomyrtus tomentosa*(Ait.) Hassk



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ABSTRACT

This study investigated the *in vitro* and *in vivo* antioxidant activities of the flavonoids rich extract from *Rhodomyrtus tomentosa* Hassk (*R. tomentosa*) berries. The *in vitro* antioxidant assay demonstrated that the flavonoids rich extract (62.09% rutin equivalent) extracted by ethanol and purified by AB-8 macroporous resin was strong in reducing power, superoxide radical, hydroxyl radical and DPPH radical scavenging activity, as well as inhibiting lipid peroxidation. In the *in vivo* assays, the flavonoids rich extract significantly enhanced the activities of antioxidant enzymes in serums of mice after they were administered with the extract. The results suggested that the flavonoids rich extract from *R. tomentosa* fruits possesses potent antioxidant properties. In addition, the chemical compositions of flavonoids rich extract were identified by UPLC–TOF–MS/MS. Six flavonoids were tentatively identified as myricetin, quercetin, dihydromyricetin, kaempferol, quercetin 7,4'-diglucoside and vitexin. Therefore, *R. tomentosa* berries could be used as a new source of antioxidant ingredient.

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1. Introduction

Rhodomyrtus tomentosa(Ait.) Hassk (*R. tomentosa*), also named as *Rose Myrtle*, is an evergreen shrub in the family Myrtaceae and mainly distributed in southeast Asian countries, especially southern China, Japan and Thailand (Saising, Ongsakul, & Voravuthikunchai, 2011). The edible berries of *R. tomentosa* are of dark violet colour and bell shape (Salni et al., 2002), and have been historically used as a folk medicine to treat diarrhea, dysentery and traumatic hemorrhage (Geetha, Sridhar, & Murugan, 2010). In China, the berries are also widely used as functional ingredients to make traditional wines, jams and beverages. It has been revealed that *R. tomentosa* berries contain flavonoid glycosides, phenols, amino acids, organic acids, quinones, polysaccharide and other chemical constituents. In addition, *R. tomentosa* readily grows in sub-tropic and tropic areas, which makes it an ideal and economical source of plants to be used to develop new ingredients to promote health benefits (Langeland & Burks, 1998; Starr, Starr, & Loope, 2003).

Oxidative stress, defined as “an imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to damage” (Sies, 1997), is associated with higher risks of many ailments including diabetes mellitus, hypertension, obesity, dyslipi-

demia and inflammation (Hopps, Noto, Caimi, & Averna, 2010). Oxidative stress is induced by reactive oxygen species (ROS) such as superoxide anions ($\cdot\text{OOH}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\cdot\text{OH}$). ROS are “generated as by-products of aerobic respiration and metabolism” (Al-Gubory, Fowler, & Garrel, 2010), and modulated by antioxidant enzymes and non-enzymatic scavengers (Taleb-Senouci et al., 2009). Natural antioxidants obtained from plants and vegetables are generally needed to counteract the damage of ROS to cells.

Flavonoids are a large group of plant polyphenol secondary metabolites found widely in the leaves, seeds, bark and flowers of plants. Depending on their structural characteristics, flavonoids are divided into six classes, including flavanones, flavones, flavonols, isoflavonoids, anthocyanins and flavans (Julia & Johanna, 1998). It is already well recognised that flavonoids possess anti-lipoperoxidant, anti-tumoral, antiplatelet, anti-ischemic, anti-allergic, anti-inflammatory and anti-bacterial activities. In addition, flavonoids exhibit strong antioxidant capacities through scavenging oxygen free radicals, promote anti-oxidase or inhibit oxidative enzymes (Harborne & Williams, 2000). Therefore, the biochemistry and medicinal aspects of flavonoids have received increasing attention recently.

Studies on *R. tomentosa* mainly focused on the bioactive compounds from leaves and aerial parts because of their antibacterial, DNA damage prevention, and antioxidant activities (Lavanya, Voravuthikunchai, & Towatana, 2012; Limsuwan et al., 2009;

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Saising, Hiranrat, Mahabusarakam, Ongsakul, & Voravuthikunchai, 2008; Salmi et al., 2002; Tung et al., 2009) meanwhile only a few literatures concentrated on the anthocyanins extract from skins and whole berries (Cui et al., 2013; Liu, Guo, & Sun, 2012). However, study on the flavonoids and their antioxidant activities from *R. tomentosa* berries were relatively unknown. Therefore, in this study, the purified flavonoids rich extract from *R. tomentosa* fruits was investigated *in vitro* and *in vivo*. Research provides with baseline data on the flavonoids in the fruits of *R. tomentosa*.

2. Materials and methods

2.1. Plant materials and reagents

The fruits of *R. tomentosa* were purchased from Guangzhou Medicine Market (Guangzhou, Guangdong, China) and identified by the correspondent author Dr. Ruqiang Huang. A voucher specimen was storage at the School of Life Sciences, South China Normal University (Guangzhou, Guangdong, China).

Rutin was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). AB-8 macroporous resin was purchased from the Chemical Plant of Nankai University (Tianjin, China). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). HPLC grade acetonitrile was purchased from Burdick and Jackson (Muskegon, MI, USA). All other chemicals used for analysis were analytical grade, obtained from Guangzhou Chemical Reagent Corporation (Guangzhou, Guangdong, China).

2.2. Preparation of flavonoids rich extract

The air-dried berries were powdered (40 mesh) and extracted (4 h each, 2 times) with 95% ethanol under reflux (70 °C). The extracts were combined and evaporated to near dryness under vacuum at 50 °C. The extract was then extracted with petroleum ether for 2 times and the water-soluble fraction was purified by AB-8 macroporous resin eluted with 40% ethanol. The eluted fractions were concentrated and dried. Then the pure flavonoids rich extract were obtained and stored at 5 °C for the following experiment.

2.3. Determination of total flavonoids content

The total flavonoids content was determined using spectrophotometric method as described by Jia, Tang, and Wu (1999) with some modification, and rutin was used as a standard. 1.0 mL total flavonoids solution was diluted to 5.0 mL with 40% aqueous ethanol. 0.3 ml NaNO₂ (1:20, w/v) was added to the solution. After 5 min, 0.3 ml Al(NO₃)₃ (1:10, w/v) was added and mixed thoroughly. After 6 min, 2.0 ml NaOH (1 M) was added to the solution which was then brought up to 10.0 mL by 40% aqueous ethanol. The mixed solution was incubated at room temperature for 10 min. The absorbance was measured at 510 nm by spectrophotometer (UV2550, Shimadzu, Japan) using quartz cuvettes (10 × 10 mm). The solution containing all the reagents except replacing the sample with 40% aqueous ethanol was used as a blank. The standard curve equation was $A = 1.963 \times C$, $R^2 = 0.9997$ where A was the absorbance and C was the concentration of rutin equivalent (RE) mg/mL. The total flavonoids content in the berries was calculated and expressed as mg rutin equivalent per g dry weight. And the purity of the flavonoids rich extract was expressed as $(m_{\text{rutin equivalent}}/m_{\text{extract}}) \times 100\%$.

2.4. Antioxidant activity of flavonoids rich extract *in vitro*

2.4.1. DPPH radical scavenging assay

The DPPH radical scavenging activity of flavonoids rich extract was determined according to the method described by Hsu, Zhang, Peng, Travas-Sejdic, and Kilmartin (2008) with some modifications. A 2.0 mL of flavonoids rich extract (dissolved in ethanol) at various concentrations was mixed with 2.0 mL of 200 μM DPPH solution in ethanol. The mixture was kept at room temperature for 30 min before measuring its absorbance at 517 nm. Ascorbic acid was used as a reference standard. Radical scavenging activity was calculated as followed: $\text{RSA} (\%) = [(A_0 - A_1)/A_0] \times 100$ where A_0 was the absorbance of pure DPPH and A_1 was the absorbance of DPPH in the presence of various extracts. The result was expressed in the effective concentration of flavonoids rich extract needed to get to RSA 50% (EC₅₀).

2.4.2. Hydroxyl radicals ([•]OH) scavenging assay

The scavenging activity of hydroxyl radicals was determined by the method previously described by Mathew and Abraham (2006) with a slight modification. To a 1 mL of different concentrations of the flavonoids rich extract, 1 mL phosphate buffer (20 mM, pH 7.4) containing ferric chloride (1 mM), EDTA (1 mM), deoxyribose (2.8 mM), 0.1 mL ascorbic acid (1 mM) and 0.5 mL hydrogen peroxide (20 mM) were added. The mixture was incubated at 37 °C for 1 h before 1 mL of TBA (1%, w/v) and 1 mL of TCA (2.8%, w/v) were added. The mixture was subsequently incubated at 100 °C for 20 min, cooled down to room temperature and measured at 532 nm. The control was made under the same method except replacing the flavonoids rich extract with absolute methanol. Radical scavenging activity was calculated as followed: $\text{RSA} (\%) = [(A_0 - A_1)/A_0] \times 100$ where A_0 was the absorbance of the control and A_1 was the absorbance of the sample. Ascorbic acid at the same concentration as the samples was used as reference. The result was shown in the effective concentration of the flavonoids rich extract needed to get to RSA 50% (EC₅₀).

2.4.3. Superoxide radical (O₂^{•-}) scavenging assay

Superoxide radical scavenging ability of the extract was examined by a pyrogallol auto-oxidation system described by Stefan and Gudrun (1974). Briefly, reaction mixtures containing 0.5 mL of various concentrations of the flavonoids rich extract and Tris-HCl buffer (4.5 mL, 50 mM, pH 8.2) were incubated at 25 °C for 20 min, and then 70 μL of pyrogallol solution (6 mM, 10 mM HCl) was added. The absorbance at 325 nm was recorded immediately and every 30 s until the reaction time reached 5 min. Ascorbic acid was the reference. The scavenging rate was obtained as followed: $\text{RSA} (\%) = [1 - (A_1 - A_2)/A_0] \times 100$, where A_0 was the absorbance of without extract, A_1 was the absorbance in the presence of the extract and A_2 was the absorbance of without pyrogallol. The result was shown in the effective concentration of flavonoids rich extract needed to get to RSA 50% (EC₅₀).

2.4.4. Ferric-reducing antioxidant power (FRAP) assay

The ferric-reducing antioxidant power was determined according to Oyaizu (1986). A 2.5 mL aliquot of various concentrations of flavonoids rich extract was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. After 2.5 mL of 10% trichloroacetic acid was added, the mixture was centrifuged at 3000 rpm for 10 min. 2.5 mL of the upper layer of the solution was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride, and the absorbance was measured at 700 nm after 10 min. Ascorbic acid was used as positive reference. An increase in the absorbance of the reaction mixture indicated increased reducing

power, and the EC₅₀ value is the effective concentration at which the absorbance was 0.5 for reducing power.

2.4.5. Inhibition of lipid peroxidation

Lipid peroxidant value (POV) was measured according to Li, Zhang, and Zheng (2009). The flavonoids rich extract was dissolved in ethanol and mixed with fresh lard. The lipid system was thoroughly homogenised (70 °C) for 30 min, and stored in 65 °C water bath with stirring every 24 h. POV was determined using Na₂S₂O₃ titrimetric method and calculated as followed: POV (meq/kg) = $S \times N \times 1000/W$, where S was the volume of Na₂S₂O₃, N was the normality of Na₂S₂O₃, and W was the weight of extract.

2.5. Antioxidant activity of flavonoids rich extract in vivo

This animal experiment was carried out in accordance with EU Directive 2010/63/EU for animal experiments and the guidelines issued by the Ethical Committee of South China Normal University, and obtained permission from the Ethical Committee of South China Normal University.

2.5.1. Animal preparation and experimental design

Male Kunming mice (weighing 20 ± 2 g, 8 weeks old) were purchased from Laboratory Animal Center of Sun Yat-Sen University (Guangzhou, Guangdong, China). The mice were housed in cages with free access to food and water, and allowed to acclimate to animal room conditions (21 ± 2 °C, relative humidity 50–60%, and 12 h light and dark cycle) for 1 week prior to experiment. After adaptation, the mice were randomly divided into four groups (ten mice per group), a normal control group (NCG) and three flavonoids rich extract treatment groups (TGs). Mice in NCG were intro-gastric gavage administered with physiological saline (10 mL/kg body weight per day). Simultaneously, mice in TGs were respectively introgastric gavage administered with flavonoids rich extract in three different doses (5, 25, 125 mg/kg body weight per day) by gavage. All groups were fed once daily for 3 consecutive weeks.

After the final treatment with the extract or physiological saline, the mice were fasted for 2 h. Then blood was collected by plucking their eyeballs. Serum was separated from the blood by centrifugation (5000 rpm, 10 min, and 4 °C) and stored at –80 °C for further analysis.

2.5.2. Determination of in vivo antioxidant enzyme activities and MDA levels

The serum contents of malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were determined using commercial kits purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China).

2.6. UPLC–TOF–MS/MS analysis of flavonoids rich extract

The flavonoids rich extract of *R. tomentosa* fruits were resolved in methanol (HPLC grade) and analysed by Waters ACQUITY UPLC system (Waters, Milford, MA, USA) and Triple TOF™ 5600 mass spectrometer (AB Sciex, USA). Analytes were separated by ACQUITY UPLC BEH C18 column (10 cm × 2.1 mm, 1.7 μm) using flow rate at 0.3 mL/min at 30 °C. The mobile phase was consisted of eluent A (deionized water) and eluent B (acetonitrile) with a gradient elution: 0–2 min, 90% A; 2–19 min, B from 10% to 100% and maintained to 25 min; 25–30 min, 90% A. The injection volume was set at 3 μL. The MS conditions were set as followed: positive ion mode, desolvation gas flow at 600 L/h at 350 °C, cone gas flow at 60 L/h and source temperature at 100 °C, collision energy 10.0/35 V, nebuliser pressure 50 psi, ionisation voltage 5.5 kV, and scan range m/z 100–1000.

2.7. Statistical analysis

All analyses were performed in triplicate. Results were expressed as mean ± SD. Statistical analyses of the data were performed with one-way analysis of variance (ANOVA) or Student's *t*-test (SPSS 16.0). Significant differences ($p < 0.05$) between the means were determined using Tukey's multiple range test.

3. Results and discussion

3.1. Total flavonoids content

Total flavonoids content in the *R. tomentosa* berries was 5.21 ± 0.20 mg RE/g dry weight while the flavonoids rich extract purified by AB-8 macroporous resin contained 62.09 ± 2.63% RE, determined by an UV spectrophotometer. Lai et al. (2013) studied different maturity stage and environmental impacts on the amount of total flavonoids in *R. tomentosa* in Vietnam and reported about 1.52 ± 0.14 mg quercetin equivalent/g dry weight of total flavonoids and 1.60 ± 0.06 mg cyanidin-3-glucoside equivalent/g dry weight of total anthocyanins in the *R. tomentosa* berries at the fourth maturity stage. The protocol obtaining flavonoid rich extract from *R. tomentosa* berries in this study served its purpose effectively. In addition, flavonoids were reported in numerous fruits and vegetables, such as chili and pepper (83–1163 mg/kg dry weight), angular loofah (675.5 mg/kg dry weight), guava (1128.2 mg/kg dry weight), papaya shoots (1264 mg/kg dry weight), bilimbi (806 mg/kg dry weight), onion leaves (2720 mg/kg dry weight), cranberry (157–263 mg/kg fresh weight), bog whortleberry (184 mg/kg fresh weight), lingonberry (74–146 mg/kg fresh weight), and crowberry (100–102 mg/kg fresh weight), and black currant (18.2–38.3 mg/100 g fresh weight) (Häkkinen, Kärenlampi, Heinonen, Mykkänen, & Törrönen, 1999; Mian & Mohamed, 2001; Mikkonen et al., 2001). Compared with other fruits and vegetables, the extract from *R. tomentosa* berries in this study was rich in flavonoids by possessing more than 20 times of total flavonoids content than those in cranberry and even higher amount than those in other berries and vegetables.

3.2. Antioxidant activities of flavonoids rich extract in vitro

3.2.1. DPPH radical scavenging ability (DPPH – RSA)

Fig. 1A showed the RSA of flavonoids rich extract on DPPH (ascorbic acid as the positive control). At concentrations from 2.0 to 20.0 μg/mL, DPPH – RSA of flavonoids rich extract ranged 11.6–73.3% while the ascorbic acid was 15.9–87.1%. EC₅₀ values were 10.97 ± 0.18 μg/mL and 8.03 ± 0.11 μg/mL for total flavonoids extract and ascorbic acid, respectively ($p < 0.05$). The flavonoids rich extract had a strong scavenging activity on DPPH radical based on the fact that effective concentration to inhibit RSA was only 20% higher than pure ascorbic acid. Extract of *R. tomentosa* demonstrated DPPH – RSA in several studies. Afnani and Manaf (2011) reported IC₅₀ value 30 μg/mL and 80% of maximum inhibition at 100 μg/mL concentration. Maskam (2011) concluded that methanol crude extract had the highest RSA towards DPPH with IC₅₀ value at 107 μg/mL. Geetha et al. (2010) reported that the extracts from *R. tomentosa* required 36% to 182% higher concentrations than ascorbic acid to inhibit 50% of DPPH radical. Controversially, DPPH radical IC₅₀ value obtained from *R. tomentosa* reported by Cui et al. (2013) was 6.27 μg/mL while ascorbic acid in the same study was 17.4 μg/mL. Therefore, the purified total flavonoids extract obtained in this study was superior to most of the other reported values.

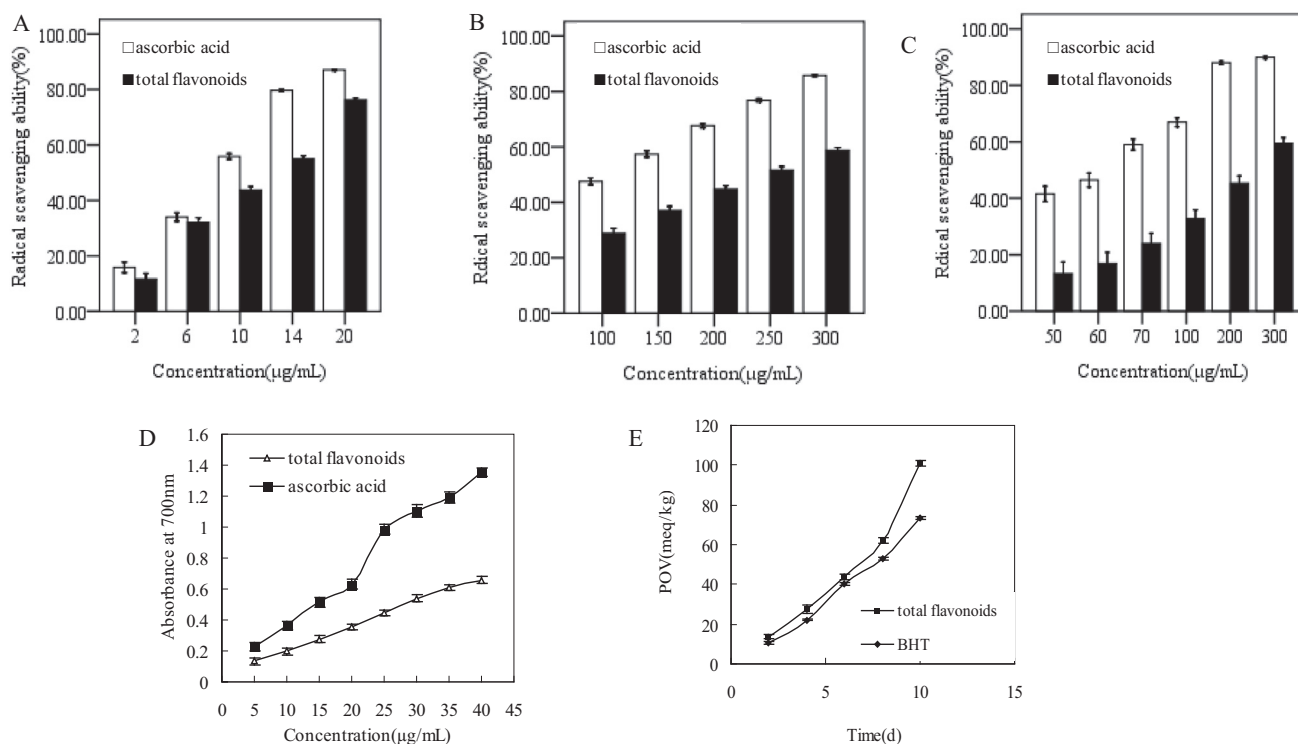


Fig. 1. *In vitro* antioxidant activities. Data was shown as mean \pm SD ($n = 3$). (A) DPPH radical scavenging assay; (B) $\cdot\text{OH}$ radical scavenging ability; (C) $\text{O}_2^{\cdot-}$ radical scavenging ability; (D) ferric-reducing antioxidant power assay; (E) inhibition effect on the lard oxidation.

3.2.2. $\cdot\text{OH}$ radical scavenging ability ($\cdot\text{OH}$ – RSA)

$\cdot\text{OH}$ radical, known as a highly toxic radical, can cause oxidative damage to biological macromolecules including lipids, proteins and nucleic acids (Ozyürek, Bektaşoğlu, Güçlü, & Apak, 2008). In the assay, $\cdot\text{OH}$ radical scavenging ability ($\cdot\text{OH}$ – RSA) was assessed by monitoring the degraded fragments of deoxyribose (Verma, Vijayakumar, Rao, & Mathela, 2010). As shown in Fig. 1B, the $\cdot\text{OH}$ – RSA of flavonoids rich extract and ascorbic acid both increased with the increase of concentrations, and the scavenging activity at 300 $\mu\text{g/mL}$ for flavonoids rich extract and ascorbic acid were 58.77% and 85.73%, respectively. In addition, ascorbic acid showed an excellent scavenging activity with EC_{50} value of $116.37 \pm 1.40 \mu\text{g/mL}$, and flavonoids rich extract was also observed to have scavenging activity with an EC_{50} value of $217.73 \pm 3.46 \mu\text{g/mL}$ ($p < 0.05$). Only Afnani and Manaf (2011) reported previously that 0.17 $\mu\text{g/mL}$ of *R. tomentos* extract could inhibit 50% (IC_{50}) of $\cdot\text{OH}$ radical.

3.2.3. $\text{O}_2^{\cdot-}$ radical scavenging ability ($\text{O}_2^{\cdot-}$ – RSA)

$\text{O}_2^{\cdot-}$ radical, originated either through metabolic process or oxygen activation by physical irradiation, is a very harmful compounds to cells (Kumaran & Karunakaran, 2007). In the assay, the spontaneous oxidation of pyrogallol can be inhibited by antioxidants and the scavenging ability can be investigated by measuring the change of absorbance at 325 nm. The results (Fig. 1C) indicated that in the range of 50–300 $\mu\text{g/mL}$, the flavonoids rich extract showed a dose-dependent inhibition on $\text{O}_2^{\cdot-}$ radical. The EC_{50} values of total flavonoids extract and ascorbic acid were $214.83 \pm 6.54 \mu\text{g/mL}$ and $60.55 \pm 1.35 \mu\text{g/mL}$, respectively ($p < 0.05$). It needed more than 3 times of concentration of flavonoids rich extract to have effects on $\text{O}_2^{\cdot-}$ – RSA than ascorbic acid in this study. There was no report on the oxygen free $\text{O}_2^{\cdot-}$ – RSA of *R. tomentos*.

3.2.4. Ferric-reducing antioxidant power (FRAP)

The FRAP assay is a simple and direct method to assess “antioxidant power”. The antioxidants can transform $\text{Fe}^{3+}(\text{CN})_6^-$ into

$\text{Fe}^{2+}(\text{CN})_6^-$ so that the reducing power can be monitored by measuring Prussian blue formation at 700 nm (Benzie & Strain, 1996). In Fig. 1D, a dose–response relationship was found in reducing ability of flavonoids rich extract (ascorbic acid as the positive control). At the concentration of 40 $\mu\text{g/mL}$, the reducing power of total flavonoids extract and ascorbic acid were 0.656 and 1.356, respectively. And the reducing ability of flavonoids rich extract ($\text{EC}_{50} = 28.67 \pm 1.37 \mu\text{g/mL}$) was lower than ascorbic acid ($\text{EC}_{50} = 13.75 \pm 0.88 \mu\text{g/mL}$, $p < 0.05$). Maskam (2011) reported the 36% maximum chelating ability at 100 $\mu\text{g/mL}$. 50% reducing power of anthocyanins rich extract from *R. tomentos* was 51.7 $\mu\text{g/mL}$ in another study (Cui et al., 2013). Our extract demonstrated stronger reducing power than those reported by others.

3.2.5. Inhibition effects on lipid peroxidation

In order to investigate the inhibition effects of flavonoids rich extract on lipid peroxidation, the lard auto-oxidation system was tested under controlled conditions. The results showed that both of total flavonoids extract and BHT had the highest POV values at tenth day, 101.08 meq/kg and 73.34 meq/kg, respectively (Fig. 1E). It indicated that the lipid peroxidative inhibition effect of flavonoids rich extract was stronger than BHT.

3.3. Antioxidant activities of total flavonoids extract *in vivo*

In the *in vivo* study, changes in the activities of SOD, GSH-Px and content of MDA in mice were investigated. The results shown in Table 1 indicated that the flavonoids rich extract treatment groups (TGs) exhibited significant increases in the activities of SOD and GSH-Px, along with significant decreases in the level of MDA in the serum compared with the normal control group (NCG) ($p < 0.01$). The enhanced activities of antioxidant enzymes, which were due to the increased mRNA expression, may provide an effective defense from the damage of free radicals. At dose of 125 mg/kg body weight, the total flavonoids extract showed the greatest antioxidant effect for ageing mice.

Table 1
Antioxidant activity of flavonoids rich extract in the serums of mice.

Group	SOD (U/mL)	GSH-Px (U/mL)	MDA (nmol/mL)
NCG	211.9 ± 21.7	41.35 ± 3.21	30.86 ± 4.15
TG I	235.2 ± 26.8*	47.23 ± 4.37*	27.63 ± 3.12*
TG II	260.6 ± 29.1*	50.52 ± 3.93*	21.91 ± 4.83**
TG III	282.1 ± 19.6**	52.1 ± 2.23**	20.23 ± 3.71**

NCG, normal control group; TG I, II and III, flavonoids rich extract treatment groups at a dose of 5, 25 and 125 mg/kg body weight, respectively.

Data were expressed as mean ± SD ($n = 10$).

* Significant at $p < 0.05$.

** Significant at $p < 0.01$ compared with NCG.

The free radical/oxidative stress theory of ageing, which was firstly conceptualised by Harman (1956), is currently one of the most popular explanations for how ageing occurs in the living organisms. As a consequence of normal aerobic respiration, O_2 is consumed by mitochondria, and H_2O is finally formed through the following process: $O_2 \rightarrow O_2^- \rightarrow H_2O_2 \rightarrow \cdot OH \rightarrow H_2O$. Free-radical scavenging enzymes, such as SOD, GSH-Px and CAT, are key components of the antioxidant defense system to protect cells and extracellular matrix from the oxidative injury. SOD can catalyse O_2^- into H_2O_2 which in turn is decomposed to water and oxygen by GSH-Px and CAT, therefore the formation of free radicals (O_2^- and its derivative $\cdot OH$) was prevented (Ames, Shigenaga, & Hagen, 1993). Lipid peroxidation is a free radical chain reaction which involves the oxidation of polyunsaturated fatty acids in cell membranes, resulting in oxidative cell damage (Najeeb et al., 2012). MDA, which is an end product of lipid peroxidation to damage the cell and cell membranes, also is one of the most frequently used biomarkers to evaluate the antioxidant activity *in vivo* (Rio, Stewart, & Pellegrini, 2005). Therefore, the results in our *in vivo* study suggested that flavonoids rich extract could exhibit antioxidant ability through affecting the production of free radicals in the progress of aerobic respiration and lipid peroxidation in our bodies.

The mechanism of non-enzymatic natural antioxidant mainly includes two aspects: (1) scavenging ROS directly, or inhibiting the generation of ROS; (2) promoting the expression of antioxidant

enzyme in the antioxidant system of body to scavenge ROS indirectly, or inhibiting the expression and activity of oxidase. Therefore, taking together of the results of *in vitro* and *in vivo* tests, flavonoids rich extract of *R. tomentosa* berries could exhibit antioxidant activity through direct scavenging effect on ROS as well as promote the expression of antioxidant enzyme to scavenge ROS. In all, flavonoids rich extract from *R. tomentosa* berries might be used as natural antioxidants and alternatives to prepare anti-ageing reagent.

3.4. Identification of total flavonoids extract

In this study, UPLC–TOF–MS/MS was used to determine the chemical compositions in the flavonoids rich extract from *R. tomentosa* berries. The extraction ion chromatograms and mass spectrum for tentative flavonoids were shown in Figs. 2 and 3, respectively. Table 2 contained their formula, retention times, MW, MS and MS^2 fragmentation ions. Six flavonoids exhibited their quasi-molecular ions $[M+H]^+$. Based on their typical fragmentation patterns as previously reported (Bonaccorsi, Caristi, Gargiulli, & Leuzzi, 2010; Ohkoshi et al., 2007; Stevens, Hart, Elema, & Bolck, 1996), compound 1 ($t_R = 5.896$ min), with a molecular ion at m/z 287 ($C_{15}H_{10}O_6$), was tentatively assigned to kaempferol with a characteristic fragment ion at m/z 171 ($[M-H-C_4H_4O_4]^+$); compound 2 ($t_R = 6.319$ min), with $[M-H]^+$ at m/z 627 ($C_{27}H_{30}O_{17}$), was tentatively identified as quercetin-7,4'-diglucoside and two fragment ions at m/z 465 and 303 were corresponded to quercetin-7-glucoside and quercetin, respectively; compound 3 ($t_R = 6.648$ min) had a molecular ion at m/z 321 ($C_{27}H_{30}O_{17}$) and two fragment ions m/z 155 $[M-C_8H_6O_4]^+$ and 137 $[M-C_8H_6O_4-H_2O]^+$ tentatively indicated that compound 3 was dihydromyricetin; compound 4 ($t_R = 6.758$ min), with M^+ at m/z 433 ($C_{21}H_{20}O_{10}$), was tentatively identified as vitexin and fragment ions at m/z 415 and 295 were corresponded to $[M-H_2O]^+$ and $[M-H_2O-C_4H_8O_4]^+$, respectively; compound 5 ($t_R = 6.900$ min), produced a $[M-H]^+$ at m/z 319 ($C_{15}H_{10}O_8$) which was fragmented into one major fragment at m/z 153 $[M-C_8H_6O_4]^+$, was tentatively identified as myricetin; compound 6 ($t_R = 7.474$ min), exhibited a

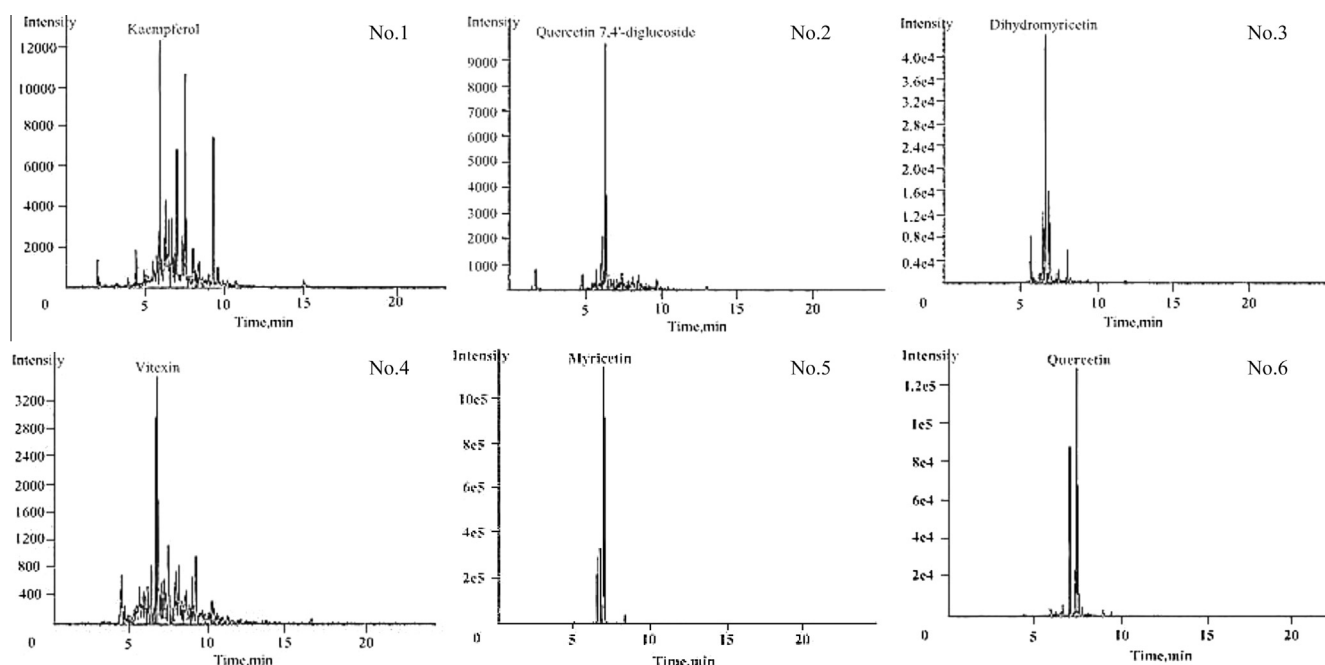


Fig. 2. The extraction ion chromatograms for six tentative flavonoids analysis by UPLC–MS/MS. No. 1: kaempferol; No. 2: quercetin-7,4'-diglucoside; No. 3: dihydromyricetin; No. 4: vitexin; No. 5: myricetin; No. 6: quercetin.

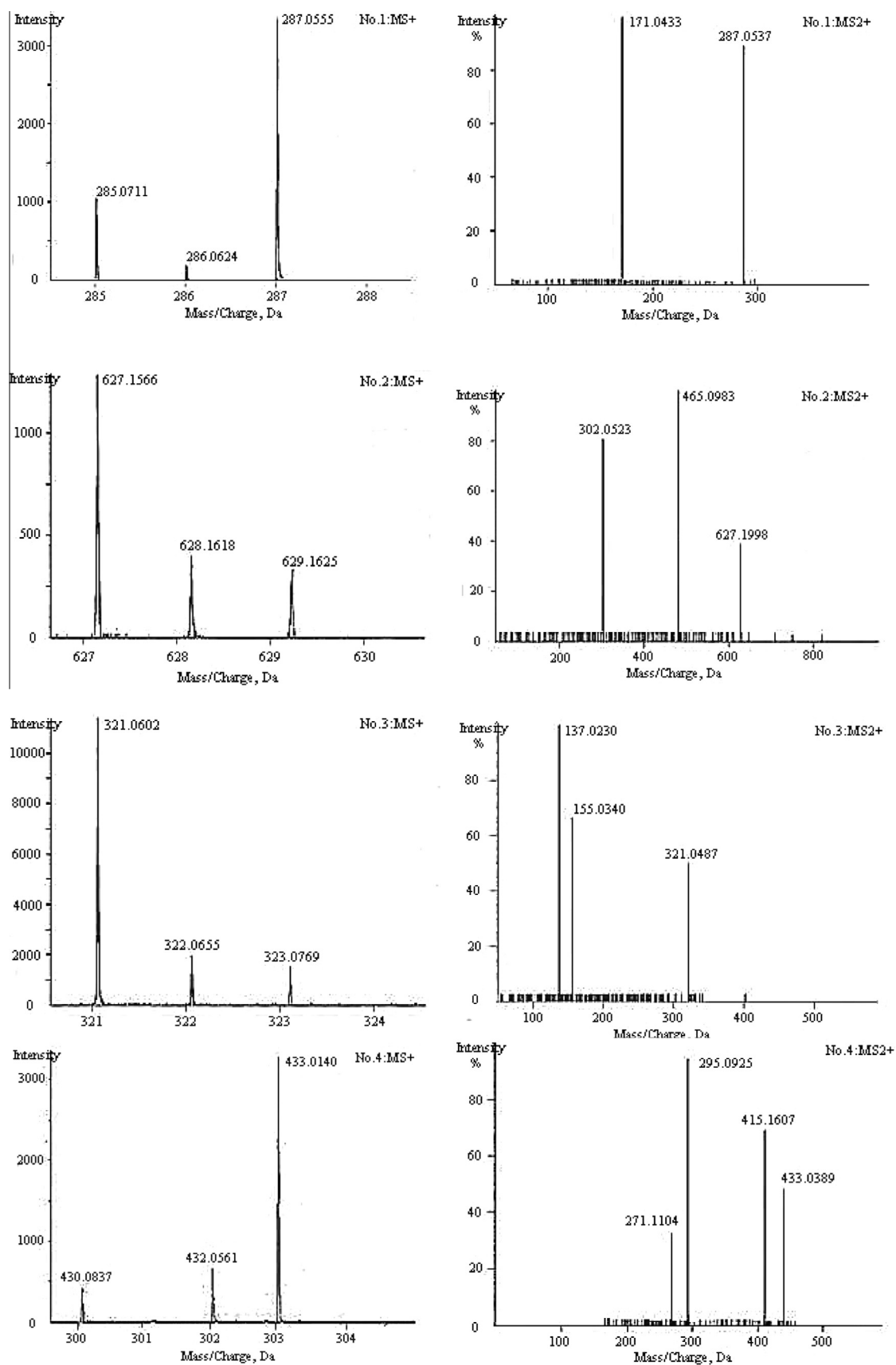


Fig. 3. Mass spectrum of identified flavonoids. No. 1: kaempferol; No. 2: quercetin-7,4'-diglucoside; No. 3: dihydromyricetin; No. 4: vitexin; No. 5: myricetin; No. 6: quercetin.

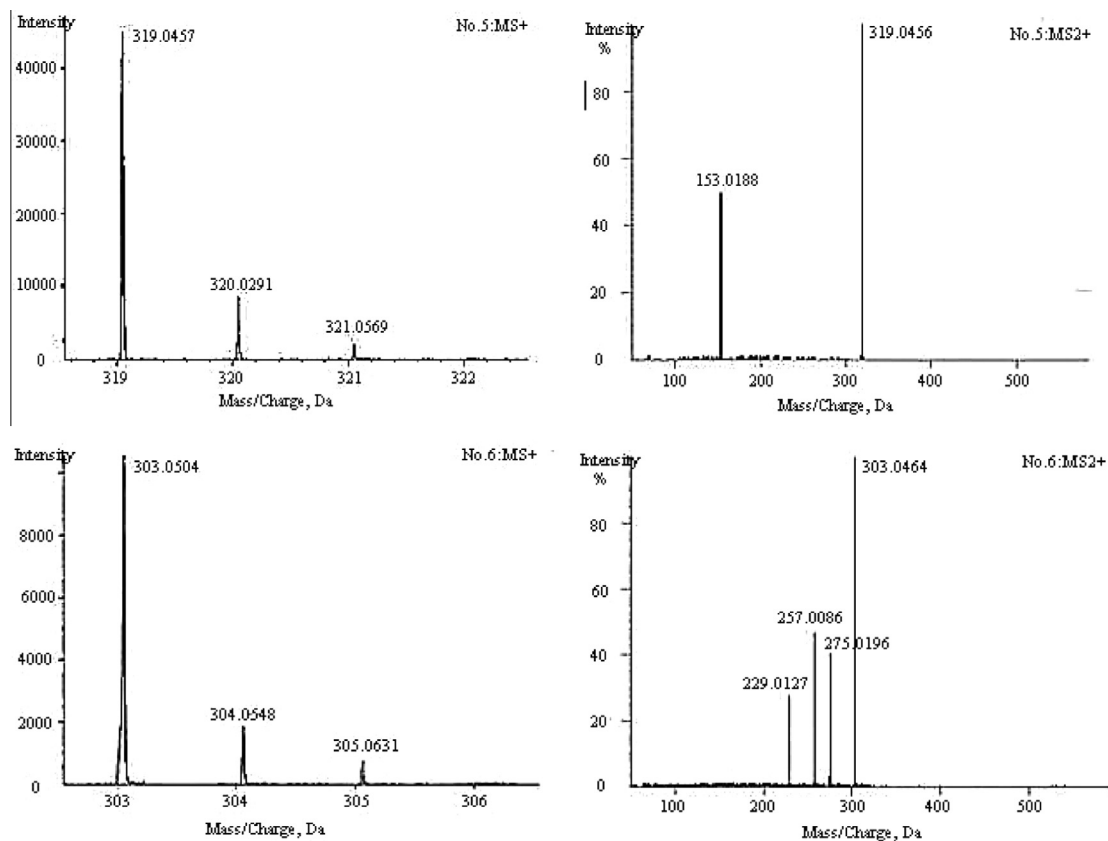


Fig. 3 (continued)

Table 2
Retention times, MS, and MS² fragmentation ions of the substances in flavanoids rich extract.

No.	Tentative identification	Formula	<i>t_R</i> (min)	MW	MS ⁺ (<i>m/z</i>)	MS ² fragment ions (<i>m/z</i>)
1	Kaempferol	C ₁₅ H ₁₀ O ₆	5.896	286	287	171
2	Quercetin-7,4'-diglucoside	C ₂₇ H ₃₀ O ₁₇	6.319	626	627	465, 303
3	Dihydromyricetin	C ₁₅ H ₁₂ O ₈	6.648	320	321	155, 137
4	Vitexin	C ₂₁ H ₂₀ O ₁₀	6.758	432	433	415, 295, 271
5	Myricetin	C ₁₅ H ₁₀ O ₈	6.900	318	319	153
6	Quercetin	C ₁₅ H ₁₀ O ₇	7.474	302	303	275, 257, 229

molecular ion at *m/z* 303 (C₁₅H₁₀O₇) and fragment ions at *m/z* 275, 257, 229 separately corresponding to [M–CO]⁺, [M–CO–H₂O]⁺, [M–2CO–H₂O]⁺, was identified as quercetin.

Lai et al. (2013) reported three types of flavonoids in *R. tomentosa*, myricetin-pentoside, miricitrin (3-*O*-rhamnoside-myricetin), and isorhamnetin-/rhamnetin-3-*O*-rhamnoside at the later stage of *R. tomentosa* maturity. The discrepancies between detected flavonoids in this study and those reported might be contributed by growing climate factors (sunlight, soil mineral levels, and pathogen attack, harvesting times, plant varieties, etc. (Dixon & Paiva, 1995; Lai et al., 2013). Compared with another berries, the presence of quercetin in *R. tomentosa* berries was in agreement with Häkkinen et al. (1999) who reported the ubiquity of quercetin in 25 edible berries. Besides being prevalent in berries, quercetin was detected in 41 of 62 tropical edible plants ranging from 14.5 to 1497.5 mg/kg dry weight in tropic plants grown in Malaysia (Miean & Mohamed, 2001). The existence of quercetin, myricetin, and kaempferol in a single variety of berries, however, was not reported in any of the 25 berries from Finland (Häkkinen et al., 1999). Vitexin (a.k.a. apigenin) was only detected in 11 of 62 tested tropic plants (Miean & Mohamed, 2001). In addition, dihydromy-

ricetin (a.k.a. ampelopsin) and quercetin-7,4'-diglucoside were also presented in the flavonoids rich extract.

To our best knowledge, there have been only a few studies concerning on the flavonoids profiles of *R. tomentosa* berries (Table 3). Liu et al. (2012) and Cui et al. (2013) studied the anthocyanin components of *R. tomentosa* berries, respectively. Lai et al. (2013) identified 19 phenolic compounds in *R. tomentosa* berries using HPLC–ESI–HR–MS and found that piceatannol was the major phenolic compound. In the study of Chen, Yu, and Yang (2011), two new quinones were isolated from *R. tomentosa* berries. In addition, other compounds such as stigmast-4-en-3-one, rhodomyrtonone, rhodomyrtonone I, rhodomyrtonone D, oleanolic acid and methyl gallate were also separated and purified from *R. tomentosa* berries (Hiranrat, Chitbankluoi, Mahabusarakam, Limsuwan, & Voravuthikunchai, 2011).

4. Conclusion

The results in this study demonstrated that flavonoids rich extract from *R. tomentosa* berries demonstrated remarkable antioxidant activities on the DPPH radical scavenging activity, reducing

Table 3
Representative bibliographies focusing on bioactive compounds from *R. tomentosa*.

Part (s)	Extraction method (s)	Compound (s)	Conducted assay (s)	Literature
Berries	Ethanol extraction; AB-8 macroporous resin chromatographic separation; UPLC–TOF-MS/MS identification	Myricetin; quercetin; dihydromyricetin; kaempferol; quercetin 7,4'-diglucoside; vitexin	– <i>In vitro</i> antioxidant activities: radical scavenging abilities (DPPH, hydroxyl, superoxide); ferric-reducing antioxidant power; lipid peroxidation inhibitory – <i>In vivo</i> animal studies: superoxide dismutase; glutathione peroxidase; malondialdehyde	This study
	Acetone extraction; Sep-Pak Plus cartridge fractionation; HPLC-DAD quantification; HPLC/HR-MS identification	Five ellagitannins; four stilbenes; three flavonols; five anthocyanins	N/A*	Lai et al. (2013)
Leaves	Methanol extraction; X-5 resin column solid phase purification; HPLC quantification; NMR identification	Cyanidin-3-glucoside; peonidin-3-glucoside; malvidin-3-glucoside; petunidin-3-glucoside; delphinidin-3-glucoside; pelargonidin-3-glucoside	– <i>In vitro</i> antioxidant activities: radical scavenging abilities (DPPH and ABTS); ferric-reducing antioxidant powder; oxygen radical absorbance capacity	Cui et al. (2013)
	Acetone extraction (crude extraction)	N/A*	– <i>In vitro</i> antioxidant activities: lipid peroxidation inhibitory; FRAP; chelating activity on ferrous ions – <i>In vivo</i> animal studies: thiobarbituric acid reacting substances; superoxide dismutase; catalase; glutathione peroxidase; nonenzymatic antioxidant	Lavanya et al. (2012)
Aerial part	Ethanol extraction; silica gel column chromatographic fractionation; NMR identification	Ciprofloxacin, clindamycin, erythromycin, gentamicin, oxacillin, penicillin, teicoplanin, tetracycline, trimethoprim-sulfamethoxazole, vancomycin	– <i>In vitro</i> antimicrobial activities against Staphylococci: agar disk diffusion; minimal inhibitory concentration; time-kill study	Saising et al. (2008)
	Ethanol extraction; medium pressure liquid chromatographic fractionation; crystal X-ray structure determination	Rhodomyrtone	– <i>In vitro</i> antibacterial activities against <i>B. cereus</i> , <i>B. subtilis</i> , <i>E. faecalis</i> , <i>S. aureus</i> , methicillin-resistant <i>S. aureus</i> , <i>S. epidermidis</i> , <i>S. gordonii</i> , <i>S. mutans</i> , <i>S. pneumoniae</i> , <i>S. pyogenes</i> , and <i>S. salivarius</i> : microdilution method; minimum inhibitory concentration; time-kill study; bacteriolysis; time-lapse microscopy	Salni et al. (2002) and Limsuwan et al. (2009)
N/A*	Distilled water and ethanol extraction (crude extraction)	N/A*	– <i>In vitro</i> prevention of DNA damage of osteoblastic cells: alkaline phosphatase activity; collagen synthesis; mineralisation	Tung et al. (2009)
N/A*	Methanol extraction; silica gel column chromatographic fractionation; UV quantification and NMR identification	4,8,9,10-tetrahydroxy-2,3,7-trimethoxyanthracene-6- β -D-glucopyranoside; 2,4,7,8,9,10-hexahydroxy-3-methoxyanthracene-6- α -L-rhamnopyranoside; quercetin; myricitrin; (3S, 5R, 6R, 7E, 9S)-megastiman-7-ene-3,5,6,9-tetrol	– <i>In vitro</i> prevention against DNA damage of lymphocytes – <i>In vivo</i> animal studies: glutathione; glutathione peroxidase; superoxide dismutase; catalase; 9-Oxo-2'-deoxyguanosine	Ke et al. (2013)
Skins	Acidified ethanol extraction; HPLC–ESI-MS identification	Dephinidin-3-glucoside; cyanidin-3-glucoside; peonidin-3-glucoside; petunidin-3-glucoside, malvidin-3-glucoside	N/A*	Liu et al. (2012)

N/A indicated that information was not investigated/disclosed in the literatures.

power and inhibition of lipid peroxidation activity, as well as high in superoxide anion radical and hydroxyl radical scavenging activities. The flavonoids rich extract also increased the activities of SOD and GSH-Px activities, and decreased the MDA content in serum of mice. Based on the study *in vitro* and *in vivo*, it is predicted that the flavonoids rich extract prepared in this study can be a potential resource of natural antioxidants to be used in functional foods and health products. Furthermore, based on the results of UPLC–TOF-MS/MS, six known flavonoid compounds having significant biological activities were putatively identified. *R. tomentosa* berries should be used as a new natural resource to prepare these health-promoting flavonoid compounds. However, some other unknown flavonoid compounds in *R. tomentosa* berries were not yet identified in this study, and the kind of flavonoids playing a dominant role in the antioxidant activities was not clear. Thus, further fractionation and identification of flavonoids are in progress in our team. Moreover, other constituents in *R. tomentosa* berries and their biological activities will be investigated in future studies.

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References

- Afnani, A., & Manaf, A. A. (2011). Antioxidant activity of *Rhodomyrtus tomentosa* (Ait.) Hassk. of Terengganu coastal area. *Planta Medica*, 77, 1322.
- Al-Gubory, K. H., Fowler, P. A., & Garrel, C. (2010). The roles of cellular reactive oxygen species, oxidative stress and antioxidants in pregnancy outcomes. *The International Journal of Biochemistry and Cell Biology*, 42, 1634–1650.
- Ames, B. N., Shigenaga, M. K., & Hagen, T. M. (1993). Oxidants, antioxidants, and the degenerative diseases of aging. *Proceedings of the National Academy of Sciences of the United States of America*, 90, 7915–7922.
- Benzie, I. F. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "Antioxidant Power": The FRAP assay. *Analytical Biochemistry*, 239, 70–76.
- Bonaccorsi, P., Caristi, C., Gargiulli, C., & Leuzzi, U. (2010). Flavonol glucoside profile of southern Italian red onion (*Allium cepa* L.). *Journal of Agricultural and Food Chemistry*, 53, 2733–2740.

- Chen, T., Yu, C. G., & Yang, B. L. (2011). Structure elucidation and NMR assignments for two new quinones from *Fructus rhodomyrti* of *Rhodomyrtus tomentosa*. *Chemistry of Natural Compounds*, 47, 524–526.
- Cui, C., Zhang, S. M., You, L. J., Ren, J. Y., Luo, W., Chen, W. F., et al. (2013). Antioxidant capacity of anthocyanins from *Rhodomyrtus tomentosa* (Ait.) and identification of the major anthocyanins. *Food Chemistry*, 139, 1–8.
- Dixon, R. A., & Paiva, N. L. (1995). Stress-induced phenylpropanoid metabolism. *The Plant Cell*, 7, 1085–1097.
- Geetha, K. M., Sridhar, C., & Murugan, V. (2010). Antioxidant and healing effect of aqueous alcoholic extract of *Rhodomyrtus tomentosa* (Ait.) Hassk on chronic gastric ulcers in rats. *Journal of Pharmacy Research*, 3, 2860–2862.
- Häkkinen, S. H., Kärenlampi, S. O., Heinonen, I. M., Mykkänen, H. M., & Törrönen, A. R. (1999). Content of the flavonols quercetin, myricetin, and kaempferol in 25 edible berries. *Journal of Agricultural and Food Chemistry*, 47, 2274–2279.
- Harborne, J. B., & Williams, C. A. (2000). Advances in flavonoid research since 1992. *Phytochemistry*, 55, 481–504.
- Harman, D. (1956). Aging: A theory based on free radical and radiation chemistry. *Journal of Gerontology*, 11, 298–300.
- Hiranrat, A., Chitbankloui, W., Mahabusarakam, W., Limsuwan, S., & Voravuthikunchai, S. P. (2011). A new flavellagic acid derivative and phloroglucinol from *Rhodomyrtus tomentosa*. *Natural Product Research*, 1, 1–6.
- Hsu, C. F., Zhang, L. J., Peng, H., Trivas-Sejdic, J., & Kilmartin, P. A. (2008). Scavenging of DPPH free radicals by polypyrrole powders of varying levels of overoxidation and/or reduction. *Synthetic Metals*, 158, 946–952.
- Hopps, E., Noto, D., Caimi, G., & Averna, M. R. (2010). A novel component of the metabolic syndrome: The oxidative stress. *Nutrition, Metabolism and Cardiovascular Diseases*, 20, 72–77.
- Jia, Z. S., Tang, M. C., & Wu, J. M. (1999). The determination of flavonoid content in mulberry and their scavenging effects on superoxide radicals. *Food Chemistry*, 64, 555–559.
- Julia, P., & Johanna, D. (1998). Flavonoids: Dietary occurrence and biochemical activity. *Nutrition Research*, 1995–2018.
- Ke, Y., Xu, X., Wu, S., Huang, J., Geng, Y., Misra, H., et al. (2013). Protective effects of extracts from *Fructus rhodomyrti* against oxidative DNA damage *in vitro* and *in vivo*. *Oxidative Medicine and Cellular Longevity*, 2013, 507407.
- Kumaran, A., & Karunakaran, R. J. (2007). In vitro antioxidant activities of methanol extracts of five *Phyllanthus* species from India. *LWT – Food Science and Technology*, 40, 344–352.
- Lai, T. N. H., Herent, M. F., Leclercq, J. Q., Nguyen, T. B. T., Rogez, H., Larondelle, Y., et al. (2013). Piceatannol, a potent bioactive stilbene, as major phenolic component in *Rhodomyrtus tomentosa*. *Food Chemistry*, 138, 1421–1430.
- Langeland, K. A., & Burks, K. C. (1998). *Identification and biology of non-native plants in Florida's natural areas*. Gainesville, FL: University Press of Florida.
- Lavanya, G., Voravuthikunchai, S. P., & Towatana, N. H. (2012). Acetone extract from *Rhodomyrtus tomentosa*: A potent natural antioxidant. *Evidence-Based Complementary and Alternative Medicine*.
- Li, J. L., Zhang, M., & Zheng, T. S. (2009). The *in vitro* antioxidant activity of lotus germ oil from supercritical fluid carbon dioxide extraction. *Food Chemistry*, 115, 939–944.
- Limsuwan, S., Trip, E. N., Kouwen, T. R., Piersma, S., Hiranrat, A., Mahabusarakam, W., et al. (2009). Rhodomirtone: A new candidate as natural antibacterial drug from *Rhodomyrtus tomentosa*. *Phytomedicine*, 16(6), 645–651.
- Liu, G. L., Guo, H. H., & Sun, Y. M. (2012). Optimization of the extraction of anthocyanins from the fruit skin of *Rhodomyrtus tomentosa* (Ait.) Hassk and identification of anthocyanins in the extract using high-performance liquid chromatography–electrospray ionization–mass spectrometry (HPLC–ESI–MS). *International Journal of Molecular Sciences*, 13, 6292–6302.
- Maskam, M. F. (2011). *Antioxidant effects of rhodomyrtus tomentosa* (Kemunting) extract on the development of experimental atherosclerotic-induced New Zealand white rabbit (Master's dissertation), University of Malaya.
- Mathew, S., & Abraham, T. M. (2006). Studies on the antioxidant activities of cinnamon (*Cinnamomum verum*) bark extracts, through various *in vitro* models. *Food Chemistry*, 94, 520–528.
- Miean, K. H., & Mohamed, S. (2001). Flavonoid (myricetin, quercetin, kaempferol, luteolin, and apigenin) content of edible tropical plants. *Journal of agricultural and food chemistry*, 49(6), 3106–3112.
- Mikkonen, T. P., Määttä, K. R., Hukkanen, A. T., Kokko, H. I., Törrönen, A. R., Kärenlampi, S. O., et al. (2001). Flavonol content varies among black currant cultivars. *Journal of Agricultural and Food Chemistry*, 49, 3274–3277.
- Najeeb, Q., Bhaskar, N., Masood, I., Wadhwa, S., Kaur, H., & Ishaq, S. (2012). Malondialdehyde (MDA) superoxide dismutase (SOD) levels-distinguishing parameters between benign malignant pleural effusions. *Free Radicals and Antioxidants*, 2, 8–11.
- Ohkoshi, E., Miyazaki, H., Shindo, K., Watanabe, H., Yoshida, A., & Yajima, H. (2007). Constituents from the leaves of *Nelumbo nucifera* stimulate lipolysis in the white adipose tissue of mice. *Plant Medica*, 73, 1255–1259.
- Oyaizu, M. (1986). Studies on products of browning reaction: antioxidative activities of products of browning reaction prepared from glucosamine. *Japanese Journal of Nutrition*, 44, 307–315.
- Ozyürek, M., Bektaşoğlu, B., Güçlü, K., & Apak, R. (2008). Hydroxyl radical scavenging assay of phenolics and flavonoids with a modified cupric reducing antioxidant capacity (CUPRAC) method using catalase for hydrogen peroxide degradation. *Analytica Chimica Acta*, 2, 196–206.
- Rio, D. D., Stewart, A. J., & Pellegrini, N. (2005). A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutrition, Metabolism and Cardiovascular Diseases*, 15, 316–328.
- Saising, J., Hiranrat, A., Mahabusarakam, W., Ongsakul, M., & Voravuthikunchai, S. P. (2008). Rhodomirtone from *Rhodomyrtus tomentosa* (Aiton) Hassk. as a natural antibiotic for staphylococcal cutaneous infections. *Journal of Health Science*, 54, 589–595.
- Saising, J., Ongsakul, M., & Voravuthikunchai, S. P. (2011). *Rhodomyrtus tomentosa* (Aiton) Hassk. ethanol extract and rhodomirtone: A potential strategy for the treatment of biofilm-forming staphylococci. *Journal of Medical Microbiology*, 60, 1793–1800.
- Salni, D., Sargent, M. V., Skelton, B. W., Soediro, I., Sutisna, M., White, A. H., et al. (2002). Rhodomirtone, an antibiotic from *Rhodomyrtus tomentosa*. *Australian Journal of Chemistry*, 55, 229–232.
- Sies, H. (1997). Oxidative stress: Oxidants and antioxidants. *Experimental Physiology*, 82, 291–295.
- Starr, F., Starr, K., & Loope, L. (2003). *Rhodomyrtus tomentosa*. Retrieved from: <http://www.starrenvironmental.com/publications/species_reports/pdf/rhodomyrtus_tomentosa.pdf>.
- Stefan, M., & Gudrun, M. (1974). Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *European Journal of Biochemistry*, 47, 469–474.
- Stevens, J. F., Hart, H., Elema, E. T., & Bolck, A. (1996). Flavonoid variation in eurasian sedum and sempervivum. *Phytochemistry*, 41, 503–512.
- Taleb-Senouci, D., Ghomari, H., Krouf, D., Bouderbala, S., Prost, J., Lacaille-Dubois, M. A., et al. (2009). Antioxidant effect of *Ajuga iva* aqueous extract in streptozotocin-induced diabetic rats. *Phytomedicine*, 16, 623–631.
- Tung, N. H., Ding, Y., Choi, E. M., Van Kiem, P., Van Minh, C., & Kim, Y. H. (2009). New anthracene glycosides from *Rhodomyrtus tomentosa* stimulate osteoblastic differentiation of MC3T3-E1 cells. *Archives of Pharmacological Research*, 32, 515–520.
- Verma, A. R., Vijayakumar, M., Rao, C. V., & Mathela, C. S. (2010). *In vitro* and *in vivo* antioxidant properties and DNA damage protective activity of green fruit of *Ficus glomerata*. *Food and Chemical Toxicology*, 48, 704–709.