JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Enzymatic Acylation of Isoorientin and Isovitexin from Bamboo-Leaf Extracts with Fatty Acids and Antiradical Activity of the Acylated Derivatives

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ABSTRACT: This study enzymatically acrylates two flavonoids from bamboo-leaf extracts, isoorientin and isovitexin, with different fatty acids as acyl donors using *Candida antarctica* lipase B (CALB). The conversion yield ranged from 35 to 80% for fatty acids with different chain lengths. Higher isoorientin and isovitexin conversion yields (>75%) were obtained using lauric acid in *tert*-amyl-alcohol as the reaction medium. ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy analysis showed that, in the presence of CALB, acylation occurred at the isoorientin and isovitexin primary hydroxyl group of glucose moiety and only monoesters were detected. Introducing an acyl group into isoorientin and isovitexin significantly improved their lipophilicity but reduced their antiradical activity.

KEYWORDS: *Isoorientin, isovitexin, Candida antarctica lipase B, fatty acids, enzymatic acylation, lipophilicity, antiradical activity*

1. INTRODUCTION

Flavonoids are a class of secondary phenolic metabolites and are found in almost all plants. They have received considerable attention because of their broad range of activities, including antioxidant, antimicrobial, anti-inflammatory, antitumor, and antiangiogenic activities.[1−7] Recently, bamboo-leaf, berry, grape, and pine-bark extracts, containing mainly flavonoids and anthocyans, have become available on the market. Food companies are increasingly becoming interested in flavonoids to meet consumer demands for healthier foods without artificial food additives, which may carry potential risks. Thus, flavonoids may provide many opportunities for food ingredient innovation and business developments. However, dependent upon their structure, their use is limited by their weak solubility in lipidic matrices or lipophilic media.[8−10] Studies have investigated chemical and enzymatic acylation to improve flavonoid properties.[11−14] In comparison to chemical methods, the enzymatic approach is more suitable for these modifications because enzymes are regioselective and the process can be conducted at mild temperatures and pressures. Therefore, the enzymatic approach is a promising method. Previous studies have used *Candida antarctica* lipase B (CALB) as a biocatalyst, and results showed that, for glycosylated flavonoids, the acylation mainly occurs at the primary hydroxyl group of the glucose part.[11−18]

The antioxidant of bamboo leaves (AOB), which is the trade name of bamboo-leaf extracts, has been approved as a natural food additive for puffed foods, aquatic products, meat products, edible oils, cereals, bakery products, fruit and vegetable juices, tea, and fried foods by the Chinese Ministry of Health. The functional components of AOB include flavonoids, lactones, and phenolic acids, but it is mainly composed of four main flavonoids, which are orientin, isoorientin, vitexin, and isovitexin.[19] However, its use is also limited by their weak solubility in lipidic matrices or lipophilic media. To our knowledge, the ability of CALB to catalyze acylation of flavonoids from AOB has never been reported.

Isoorientin and orientin and isovitexin and vitexin are 2 pairs of position isomers with almost identical properties.[20] Therefore, this paper focuses on the enzymatic acylation of two flavonoids, isoorientin and isovitexin, from AOB by CALB with different fatty acids as acyl donors. Their lipophilicity was compared by determining their 1-octanol/water partition coefficients. The second section of the paper provides an evaluation and a comparison of the antiradical activity of isoorientin, isovitexin, and their acylated derivatives using *in vitro* assays, including 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging and superoxide radical scavenging.

2. MATERIALS AND METHODS

2.1. Enzyme and Chemicals. Immobilized CALB (Novozyme 435) from Novozym (Tianjing, China) was used as a catalyst. The AOB was obtained from Golden Plate Bio-Tech Co., Ltd. (Hunan, China). The manufacturer stated that the antioxidant consisted of 80% (w/w) flavonoids. Standard isoorientin (5,7,3′,4′-tetrahydroxyflavone-6-glucoside) and isovitexin (5,7,4′-trihydroxyflavone-6-glucoside) were purchased from Aladdin (Shanghai, China). Ultrapure water, purified using the Milli-Q system (Millipore, Bedford, MA), was used for analytical high-performance liquid chromatography (HPLC) analysis. Silica gel C18-RP 60A and silica gel GF_{254} 60A plates were purchased from Merck (Germany). Molecular sieves for drying the reaction medium (10−20 mesh beads, with a pore diameter of 4 Å) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). DPPH, nitrobluetetrazolium (NBT), xanthine oxidase (XO), hypoxanthine, and adenosine triphosphate (ATP) were supplied by Sigma (Guangzhou, China). Other reaction media and chemicals were purchased from local suppliers.

Received: August 21, 2012
Revised: October 11, 2012
Accepted: October 12, 2012
Published: October 12, 2012

2.2. Isolating Isoorientin and Isovitexin. Silica-based column chromatography was performed on a low-pressure glass chromatographic column (80 cm × 70 mm inner diameter, Yintebeier Experiment Instrument Plant, Guangzhou, China) filled with silica gel C18-RP 60A. The AOB was loaded onto the column at a linear gradient of 10−100% acetonitrile in water (containing 0.1% formic acid, v/v). Qualitative analysis of isoorientin and isovitexin was conducted using thin-layer chromatography (TLC) on silica gel GF_{254} 60A plates with a solvent mixture of chloroform, methanol, acetic acid, and water (at a ratio of 6.5:1:0.15:0.15, v/v/v/v). Standard isoorientin and isovitexin were used as controls. The plates were observed and detected under ultraviolet (UV) light (254 nm). Studies have identified the chemical structure (Figure 1) of isoorientin and isovitexin.[20,21]

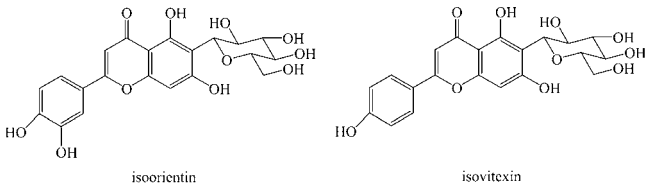

Figure 1. Chemical structures of isoorientin and isovitexin

Figure 1. Chemical structures of isoorientin and isovitexin.

2.3. Synthesizing the Flavonoid Esters. Enzymatic acylation of isoorientin and isovitexin (Figure 2) was conducted in an automated synthesis workstation (ASW1000, Chemspeed, Augst, Switzerland) equipped with 27 mL microreactors. The working volume was maintained at a constant 20 mL. Before each experiment, the reaction medium and acyl donor were dried on 4 Å molecular sieves and added to the flavonoids (10 g/L). The acyl donor/flavonoid molar ratio varied from 1 to 10. After complete dissolution of the substrates (following overnight stirring at 60 °C), the acylation was started by adding 10 g/L CALB at 65 °C. The water content of the reaction medium was maintained at less than 0.1 water activity with molecular sieves (100 mg/mL) added to the reaction medium.[22] Control experiments were also performed without incorporating the enzyme. Removing the enzyme by centrifugation stopped the reactions after 48 h.

2.4. HPLC Analytical Process. Reactions were quantitatively monitored using HPLC. Analyses were conducted with an Essentia LGE−UV system (Shimadzu, Kyoto, Japan) equipped with a column (Syncronis C18, 250 × 4.6 mm, Thermo, Guangzhou, China), a column oven (CTO-20A, Shimadzu, Kyoto, Japan), an autoinjector (SIL-10A, Shimadzu, Kyoto, Japan), an evaporating light-scattering detector (ELSD-LT II, Shimadzu, Kyoto, Japan), and an UV detector (SPD-10A, Shimadzu, Kyoto, Japan). Different reaction medium components were sperarated using a linear gradient of acetonitrile (A) and water (B) (both containing 0.1% formic acid, v/v) at a flow rate of 1 mL/min for 0 min (10:90, v/v), 25 min (100:0, v/v), and 35 min (10:90, v/v). The injection volume was 20 μL, and the column temperature was maintained at 40 °C. Different compounds were quantified at 330 nm with the UV detector.

2.5. Purification of Acylated Derivatives. Acylated derivatives were purified using liquid−liquid extraction. The unreacted acyl donor was extracted by agitating for 20 min with acetonitrile/*n*-heptane (2:5, v/v) at 60 °C. The residual flavonoids were removed using a mixture of ethyl acetate/water (3:5, v/v) for 20 min at 60 °C. Highly purified acylated derivatives were obtained by semi-preparative HPLC. An Agilent 1260 infinity preparative chromatography system performed chromatographic separation. It was equipped with an Agilent Prep LC controller and G1365B MWD detector (Agilent, Santa Clara, CA). A Bondapak C18 preparative column (300 × 7.8 mm inner diameter, 10 μm; Waters, Milford, MA) used a linear gradient of acetonitrile (A) and water (B) (both containing 0.1% formic acid, v/v) at a flow rate of 4 mL/min for 0 min (10:90, v/v), 50 min (100:0, v/v), and 60 min (10:90, v/v). Elution was performed at an ambient temperature at 330 nm with UV detection. Purified acylated derivatives were re-analyzed using the HPLC analytical process.

2.6. Nuclear Magnetic Resonance (NMR) Analytical Procedure. The chemical structures of the acylated derivatives were determined by ^{1}H NMR (500 MHz) and ^{13}C NMR (126 MHz) in DMSO-d_6 using a Bruker AC500 spectrometer (Bruker, Courtaboeuf, France).

2.7. 1-Octanol/Water Partition Coefficients. The partition coefficients of isoorientin, isovitexin, and their acylated derivatives were measured using methods from previous studies.[23] Each compound solution in 1-octanol (100 μM, 2 mL) was mixed vigorously with 2 mL of water. After centrifugation at 200*g* for 10 min, the derivative in each layer was measured and its amount was assessed using the HPLC analytical process. The results are shown as common logarithms (log *P*).

2.8. DPPH Scavenging Activity. The free radical scavenging effect of isoorientin, isovitexin, and their acylated derivatives was assessed using the DPPH radical scavenging method.[20,24] Isoorientin, isovitexin, and their derivatives were assayed at 33 μg/mL. The scavenging of free radicals by all compounds was evaluated spectrophotometrically at 517 nm against the absorbance of the DPPH radical. The activity of the compounds is presented as the mean half maximal inhibitory concentration (IC_{50}), calculated by linear regression analysis. All analyses were conducted in triplicate, and the results represented the mean values with standard deviation.

The decoloration percentage was calculated as follows:

$$\text{percentage of decoloration} = 1 - \frac{\text{absorbance of compound}}{\text{absorbance of blank}} \times 100$$

2.9. Superoxide Scavenging Activity. The enzyme XO is able to generate the superoxide anion by oxidation of reduced products

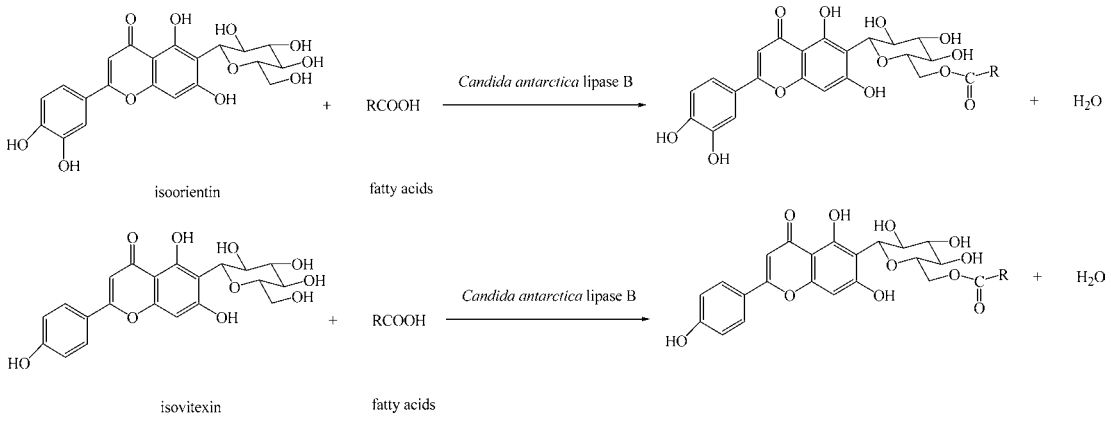

Figure 2. Schematic representation of the enzymatic acylation of isoorientin and isovitexin.

from intracellular ATP metabolism. In this reaction, the XO oxidizes the substrate hypoxanthine generating the superoxide anion, which reduces the NBT, leading to a chromophore with absorption maxima at 560 nm. Superoxide anion scavengers reduce the generation speed of the chromophore. The activity was measured spectrophotometrically as reported previously using a UV-1601 scanning spectrophotometer.[20,24] Compounds were evaluated at 50 μg/mL. The activity of the compounds is presented as IC_{50}, calculated by linear regression analysis. All analyses were conducted in triplicate, and the results represented the mean values with standard deviation.

The percentage of the superoxide anion scavenging effect was calculated as follows:

$$\text{percentage of scavenging activity} = \frac{E - S}{E} \times 100$$

where $E = A - B$ and $S = C - (B + D)$. A is the optical density of the control. B is the optical density of the control blank. C is the optical density of the sample. D is the optical density of the sample blank.

3. RESULTS AND DISCUSSION

3.1. Effect of the Reaction Medium Used. This comparison was conducted by studying the effect of different reaction media on isoorientin and isovitexin conversion yields. The acyl donor/flavonoid molar ratio was 2. Figure 3 shows that the highest isoorientin and isovitexin conversion yields were obtained in a *tert*-amyl-alcohol reaction medium. In contrast, acetone and *tert*-butanol reaction media produced conversion yields that were 10−35% lower. These results confirm those obtained by Ardhaoui et al.[22] and Gayot et al.,[25] who found that *tert*-amyl-alcohol was the most suitable reaction medium for obtaining the highest initial rates and conversion yields for flavonoid ester synthesis.

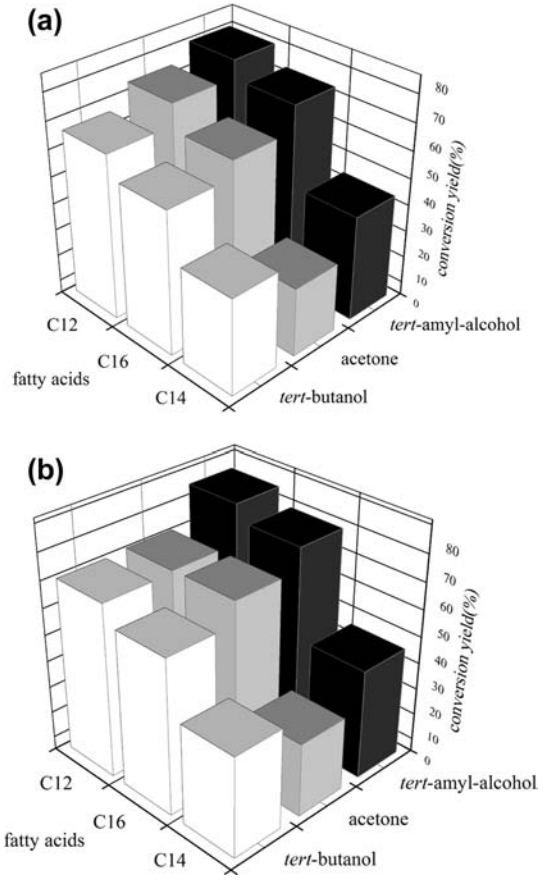

Figure 3. Conversion yield of (a) isoorientin and (b) isovitexin acylation with different fatty acids.

This may be explained by the log P difference of the reaction media. The log P of *tert*-amyl-alcohol, *tert*-butanol, and acetone is 1.15, 0.8, and −0.23, respectively. A study found that a low log P is conducive to destroying the water layer that stabilizes the enzymes.[26] However, Chebil et al.[11] indicated that a high conversion yield was correlated with solvents with a low log P.

3.2. Comparison of Isoorientin and Isovitexin Acylation. Acylation was compared by studying the effect of the chain length and fatty acid substitution on isoorientin and isovitexin conversion yields and initial conversion rates in a *tert*-amyl-alcohol reaction medium with an acyl donor/flavonoid molar ratio of 2. Results indicate that the conversion yield was higher when isovitexin was used as an acyl acceptor (Figure 3). For all fatty acids, conversion yields obtained with isovitexin were on average 5−10% higher than those obtained with isoorientin. Although initial conversion rate differences existed (Figure 4), they were not large. Because they both have a primary hydroxyl group of the glucose moiety and share a similar structure, their reactivity and solubility in the reaction media are almost identical. These differences can be explained by the slightly smaller size and polarity of isovitexin than isoorientin. Similar observations have been described during acylations catalyzed by CALB.[22,27,28]

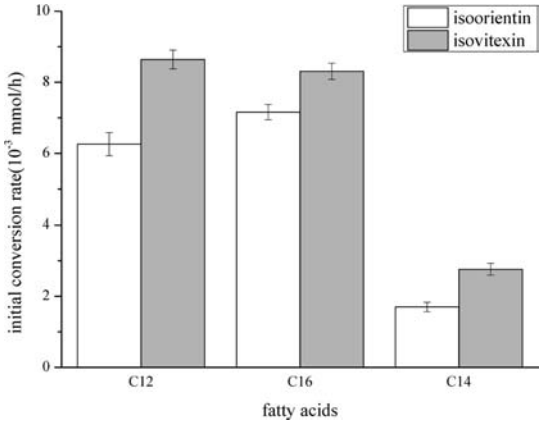

Figure 4. Initial conversion rates of isoorientin and isovitexin acylation with different fatty acids.

3.3. Effect of the Fatty Acids Pattern. The effect of the fatty acid carbon-chain length on the performance of isoorientin and isovitexin (20 mM) acylation was demonstrated using fatty acids with carbon numbers from 12 to 16 as acyl donors in a *tert*-amyl-alcohol reaction medium with an acyl donor/flavonoid molar ratio of 2. Figures 3 and 4 show that fatty acid patterns affect conversion yields and initial reaction rates. Conversion yields and initial conversion rates for lauric and palmitic acids were higher than those for myristic acid, which had a relatively low conversion yield and initial rate. Studies have reported similar behavior for this acid.[29,30] Isoorientin and isovitexin showed similar results, with the only difference occurring at the highest initial conversion rates (7.16×10^{-3} mM/h for isoorientin with palmitic acid as the acyl donor and 8.64×10^{-3} mM/h for isovitexin with lauric acid as the acyl donor). The conversion yields and initial rates obtained in this study confirm those found by Ardhaoui et al.[29] The effect of the fatty acid carbon-chain length can be

explained by the specific lipase used, the hydrophobicity of the medium,[31] and the volume of long fatty acids.[32]

3.4. Effect of the Acyl Donor/Flavonoid Molar Ratio. The effect of the acyl donor/flavonoid molar ratio on isoorientin and isovitexin acylation was demonstrated using lauric acid as an acyl donor in a *tert*-amyl-alcohol reaction medium. Figure 5 shows that the initial isoorientin and

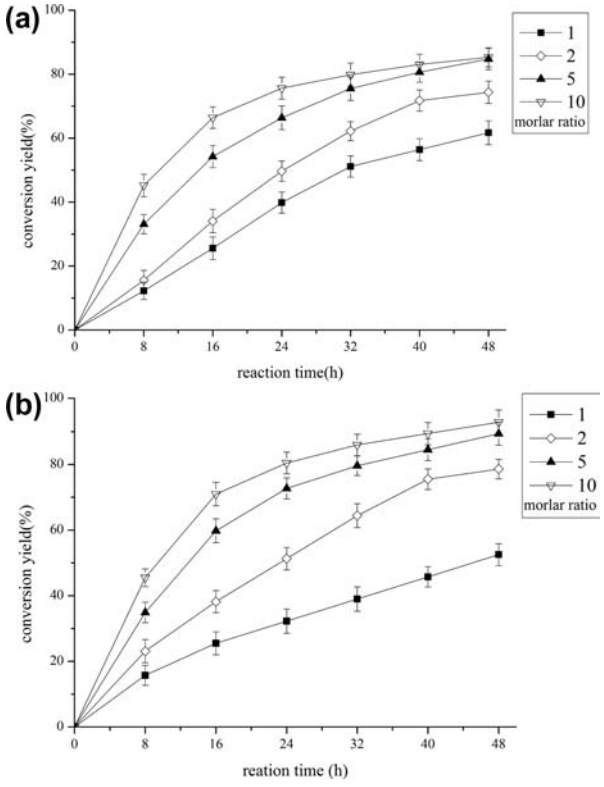

Figure 5. Reaction progress for the acylation of (a) isoorienin and (b) isovitexin with different acyl donor/flavonoid molar ratios.

isovitexin conversion rates and conversion yields increase with the acyl donor/flavonoid molar ratio. The highest conversion yields were obtained when the acyl donor/flavonoid molar ratio reached 5−10. The substrate molar ratio effect on the conversion yield may be caused by a thermodynamic shift in equilibrium in favor of isoorientin and isovitexin ester synthesis because of excess acyl donors. Chebil et al.[11] also produced similar results. Only monoesters were detected for all molar ratios. This may be caused by the presence of primary hydroxyl groups. Because of its high reactivity and accessibility, acylation at the secondary hydroxyl group may be inhibited.

3.5. Regioselectivity of Acylation. Only monoesters were detected for isoorientin, isovitexin, and the tested fatty acids. Isoorientin and isovitexin laurate esters were purified and characterized by ^{1}H and ^{13}C NMR.

For isoorientin and isovitexin with a glucose moiety, acylation occurred at the 6″-OH of the moiety because the primary hydroxyl group is highly reactive and accessible. Gao et al.[27] and Ardhaoui et al.[29] reported similar results for other glycoside flavonoids and showed that, in the presence of a primary hydroxyl group and CALB, acylation of aromatic acid vinyl esters occurs at the primary hydroxyl group of the glucose. However, Danieli et al.[33] indicated that this enzyme preferred both hydroxyl groups (6″-OH and 3″-OH) of the moiety.

3.6. Acylated Derivative Characterization by ^{1}H and ^{13}C NMR Analyses. The number of fatty chains linked to the flavonoids and their position on the glucose moiety were determined by comparing the ^{1}H and ^{13}C NMR spectra. A shift was observed in the glucose moiety spectra (downfield dd at 3.97 ppm and m at 3.94 ppm for $H_{6''a}$ and $H_{6''b}$ for isoorientin-6″-laurate and downfield s at 3.98 ppm and s at 3.90 ppm for $H_{6''a}$ and $H_{6''b}$ for isovitexin-6″-laurate).

^{1}H chemical shifts for isoorientin-6″-laurate were as follows: δ (ppm) 13.58 (s, 1H, OH5), 7.41 (s, 1H, H2′), 7.41−7.36 (m, 1H, 6′), 6.89 (s, 1H, H5′), 6.65 (d, 1H, H3), 6.48 (s, 1H, H8), 4.60 (d, 1H, H1″), 4.04 (m, 1H, H4), 3.97 (dd, 1H, H6″), 3.94 (m, 1H, H6″), 3.35 (m, 1H, H5″), 3.21 (d, 1H, H3″), 3.15 (m, 1H, H4″), 2.25 (m, 2H, $CH_2\alpha$ fatty chain), 1.45 (dd, 2H, $CH_2\beta$ fatty chain), 1.19 (d, 16H, CH_2 fatty chain), and 0.82 (d, 3H, CH_3 fatty chain).

^{13}C chemical shifts for isoorientin-6″-laurate were as follows: δ (ppm) 182.29 (C4), 173.43 (C═O), 164.09 (C2), 163.88 (C5), 161.26 (C7), 156.70 (C9), 150.27 (C4′), 146.25 (C3′), 121.80 (C1′), 119.38 (C6′), 116.50 (C5′), 113.69 (C2′), 109.04 (C6), 103.80 (C10), 103.17 (C), 93.91 (C8), 79.20 (C5″), 78.57 (C3″), 73.55 (C1″), 70.98 (C2″), 70.38 (C4″), 64.65 (C6″), 33.95 (aliphatic chain), 31.76 (aliphatic chain), 29.52 (aliphatic chain), 29.49 (aliphatic chain), 29.36 (aliphatic chain), 29.33 (aliphatic chain), 29.16 (aliphatic chain), 28.80 (aliphatic chain), 24.92 (aliphatic chain), 22.56 (aliphatic chain), and 14.40 (CH_3, aliphatic chain).

^{1}H chemical shifts for isovitexin-6″-laurate were as follows: δ (ppm) 13.56 (s, 1H, OH5), 7.92 (s, 2H, H2′, H6′), 6.93 (s, 2H, H3′, H5′), 6.77 (s, 1H, H3), 6.53 (s, 1H, H8), 4.60 (s, 1H, H1″), 4.03 (s, 1H, H2″), 3.98 (s, 1H, H6a″), 3.90 (s, 1H, H6b″), 3.28 (s, 1H, H3″), 3.18 (s, 1H, H5″), 3.06 (s, 1H, H4″), 2.25 (s, 2H, $CH_2\alpha$ fatty chain), 1.47 (s, 2H, $CH_2\beta$ fatty chain), 1.22−1.20 (m, 16H, CH_2 fatty chain), and 0.82 (s, 3H, CH_3 fatty chain).

^{13}C chemical shifts for isovitexin-6″-laurate were as follows: δ (ppm) 182.35 (C4), 173.42 (C═O), 164.18 (C2), 163.91 (C7), 161.71 (C5), 161.28 (C4′), 156.75 (C9), 128.86 (C2′, C6′), 121.52 (C1′), 116.47 (C3′, C5′), 109.09 (C6), 103.72 (C10), 103.16 (C3), 94.09 (C8), 79.21 (C5″), 78.56 (C3″), 73.58 (C1″), 70.99 (C4″), 70.41 (C2″), 64.66 (C6″), 33.96 (aliphatic chain), 31.76 (aliphatic chain), 29.53 (aliphatic chain), 29.49 (aliphatic chain), 29.47 (aliphatic chain), 29.21 (aliphatic chain), 29.17 (aliphatic chain), 28.80 (aliphatic chain), 24.93 (aliphatic chain), 22.56 (aliphatic chain), and 14.42 (CH_3, aliphatic chain).

3.7. Effect of Acylation on Lipophilicity. Figure 6 shows the partition coefficients of isoorientin, isovitexin, and their acylated derivatives. The partition coefficients of acylated derivatives increased with elongation of the acyl chain length. Specifically, the isoorientin partition coefficient was 0.21, and after acylation with lauric, myristic, and palmitic acids, partition coefficients increased significantly to 5.75, 6.8, and 7.86, respectively. Isovitexin exhibited a similar behavior. The partition coefficient order is closely correlated with the amount of derivatives incorporated into the lipidic matrices or lipophilic media, which is attributable to their lipophilicity.

3.8. Antiradical Activity. The antioxidant activity of isoorientin- and isovitexin-acylated derivatives was determined and compared to that of unacylated flavonoids (Figure 7). Generally, the acyalted derivatives exhibited less radical scavenging activity than unacyalted isoorientin and isovitexin. Specifically, the esters exhibited less DPPH radical scavenging

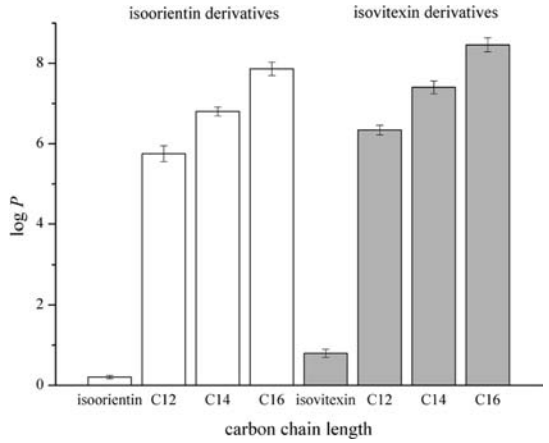

Figure 6. Log *P* of isoorientin, isovitexin, and their acrylated derivatives.

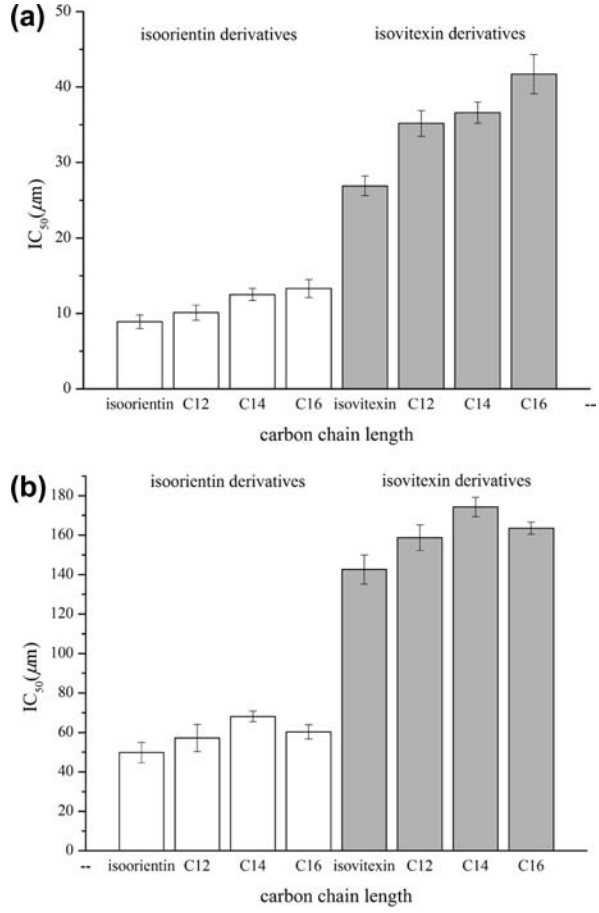

Figure 7. IC_{50} values of isoorientin, isovitexin, and their acylated derivatives for (a) DPPH scavenging and (b) superoxide radical scavenging.

activity, with IC_{50} values higher than for isoorientin and isovitexin. For isoorientin, acylation reduced ester superoxide radical scavenging activity. Isovitexin and its esters cannot be considered superoxide radical scavengers because their lowest IC_{50} value was 142.6 μM. This may be explained by the increased volume and steric hindrance after acylation, making it more difficult for acylated derivatives to reach radical active sites. These results confirm those described by Salem et al.,[34] who found that acylation of flavonoids with fatty acids decreased with their antioxidant activity. However, researchers have examined the radical scavenging activity of flavonoids and showed that acylation does not affect free radical scavenging potential.[35,36]

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Yintebeier Experiment Instrument Plant for timely delivery of the chemicals used and Guangwen Zhang, Aijun Li, and Yong Wang for excellent writing and experimental assistance.

ABBREVIATIONS USED

NMR, nuclear magnetic resonance; CALB, *Candida antarctica* lipase B; AOB, antioxidants of bamboo leaves; TLC, thin-layer chromatography; UV, ultraviolet; HPLC, high-performance liquid chromatography; log *P*, logarithm of the 1-octanol/water partition coefficient; DPPH, 1,1-diphenyl-2-picrylhydrazyl; XO, xanthine oxidase; ATP, adenosine triphosphate; NBT, nitro-bluetetrazolium; IC_{50}, half maximal inhibitory concentration

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