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Antioxidant activities of chlorogenic acid derivatives with different acyl donor chain lengths and their stabilities during *in vitro* simulated gastrointestinal digestion

Shan Wang [a,b], Yue Li [a,c], Xiangyong Meng [d], Shangwei Chen [a], Dejian Huang [e], Yongmei Xia [a], Song Zhu [a,b,*]

[a] State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu 214122, China
[b] International Joint Laboratory on Food Safety, Jiangnan University, Wuxi, Jiangsu 214122, China
[c] School of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu 214122, China
[d] College of Life Science, Anhui Normal University, Wuhu, Anhui 241000, China
[e] Department of Food Science and Technology, National University of Singapore, Singapore 117543, Singapore

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ABSTRACT

In this study, chlorogenic acid (CA) was acylated with vinyl esters of different carbon chain lengths under the action of the lipase Lipozyme RM. Five CA derivatives (C2-CA, C4-CA, C6-CA, C8-CA, and C12-CA) with different lipophilicities were obtained, and their digestive stabilities and antioxidant activities were evaluated. The lipophilicities were positively correlated with the digestive stabilities of CA derivatives. The antioxidant activities of CA derivatives did not change with the reduction of phenolic hydroxyl groups, and their capacity to scavenge 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{+}\bullet$) and 1,1-diphenyl-2-picrylhydrazyl (DPPH•) were similar to those of CA. In cellular antioxidant activity (CAA) tests, it was found that the capacity of these derivates to cross cell membranes were enhanced upon enhancing lipophilicity, and their antioxidant activities were improved. C12-CA showed the best antioxidant activity with a median effective dose (EC_{50}) of 9.40 μg/mL, which was significantly lower than that of CA (i.e., 29.08 μg/mL).

1. Introduction

Chlorogenic acid (CA) is widely found in honeysuckle, coffee beans, and other plants, and is known to exhibit various positive biological activities (Araújo, de Paulo Farias, Neri-Numa, & Pastore, 2021; Sato et al., 2011). For example, CA exhibits good antioxidant activity owing to its polyphenolic structure (Zhong & Shahidi, 2011). However, the CA polar nature renders it relatively insoluble in a lipid matrix (Hernandez, Chen, Chang, & Huang, 2009). Moreover, its polyphenolic structure leads to its instability and poor penetration across the lipophilic membrane barrier, limiting its absolute bioavailability in the human body (Chen et al., 2017). This indicates that the biological activity of CA is closely related to its chemical structure. Lipophilic reactions were used to alter the molecular structure of CA and improve its hydrophobicity (López-Giraldo et al., 2009). One of these lipophilic reactions is acylation, which can effectively improve the hydrophobicity of CA by reducing the number of hydroxyl groups.

The structural differences between CA and its lipophilic derivatives result in different biological activities (Tang et al., 2016). López-Giraldo et al. (2009) reported that the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging pathways observed for CA were different from those of CA esters obtained by the reaction of CA with different fatty alcohols. These researchers confirmed that during the first two minutes of the reaction, only one unstable H atom could be transferred to the DPPH radical by the CA esters. While there were two unstable H atoms could be transferred to the DPPH radical by CA. Three new acylated quercetin analogs were also synthesized by the acylation of quercetin with cinnamic acid, whereby the acylated quercetins were more lipophilic than quercetin (Saik, Lim, Stanslas, & Choo, 2016). In addition, Nardi et al. (2017) reported that lipophilic oleuropein aglycone derivatives displayed higher lipophilicities than oleuropein, and their radical scavenging powers in organic media and biological environments were also strengthened.

The antioxidant activities determined by spectrophotometric

* Corresponding author.
E-mail address: zhusong@jiangnan.edu.cn (S. Zhu).

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methods strongly depends on the experimental conditions (Tomac, Šeruga, & Labuda, 2020). Various methods for determining antioxidant activities, based on different mechanisms, have been established to comprehensively evaluate the antioxidant activities of the substances. For example, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) scavenging is employed to determine the antioxidant activity of substances, whereas the ferric reducing power is mainly used to determine the reducing ability (Oh & Shahidi, 2017; Tomac et al., 2020). However, the radical scavenging effects observed in a chemical matrix are rarely observed in the human body (Schaich, Tian, & Xie, 2015). An antioxidant's effectiveness mainly depends on its molecular structure, hydrophobicity, and cellular uptake (Liao, Brock, Jackson, Greenspan, & Pegg, 2020). Notably, upon ingestion, an antioxidant initially undergoes digestion in the gastrointestinal tract, which is a complex system wherein many factors (e.g., pH and presence of enzymes) affect the stability of the antioxidant. In the context of this study, CA is known to be unstable and is easily affected by many factors, such as temperature and pH. Therefore, the digestive stabilities of acylated CA derivatives were examined in an *in vitro* simulated digestive system (Limwachiranon et al., 2020). Furthermore, the celluar antioxidant activity (CAA) experiment, established by Wolfe and Liu (2007), was employed to reflect the absorption and antioxidant capacities of the antioxidants at the cellular level.

Although several studies have been conducted on the antioxidative and biological activities of CA (Naveed et al., 2018; Nikpayam et al., 2020), related studies on the acylated derivatives of CA were limited, especially in terms of the structural effects of different chain lengths at the same substitution site. Since the chemical structure of an antioxidant determines its mode of action, the differences in the chemical structures of CA and its derivatives would lead to different antioxidant effects. However, the antioxidant results obtained using chemical media have been questioned in terms of their relevance in the context of an *in vivo* environment (Liao et al., 2020).

In this study, given the advantages of using a lipase, including good substrate specificity and mild reaction conditions (Villeneuve, 2007), in addition to the observed enhancement in the hydrophobicity of CA following acylation, the lipase Lipozyme RM was used to catalyze the acylation of CA to acquire CA derivatives. The CA derivatives were prepared from CA and vinyl esters with different acyl donor chain lengths using Lipozyme RM as the catalyst. The lipophilicities, digestive stabilities, and antioxidant properties (DPPH and ABTS radical scavenging, ferric reducing power, and CAA tests) of CA and its derivatives were then investigated.

2. Materials and methods

2.1. Materials

CA (purity > 98%), quercetin, and ABTS were purchased from Macklin Biochemical Technology Ltd. (Shanghai, China). Vinyl acetate (>99%), vinyl butyrate (>99%), vinyl hexanoate (>99%), vinyl octanoate (>99%), and vinyl laurate (>99%) were purchased from Tokyo Chemical Industry Ltd. (Tokyo, Japan). Pepsin, trypsin, DPPH, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,2′-azobis (2-amidinopropane) dihydrochloride (ABAP), and 2′,7′-dichlorofluorescin diacetate (DCFH-DA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Lipozyme *Rhizomucor miehei* (RM) was supplied by Novozymes (Bagsværd, Denmark). Analytical grade absolute ethanol, methyl *tert*-butyl ether (MTBE), potassium persulfate, dibasic sodium phosphate, sodium chloride, sodium dihydrogen phosphate, potassium ferricyanide, trichloroacetic acid solution, iron(III) chloride hexahydrate, octanol, hydrochloric acid, sodium bicarbonate, methanol, vitamin C (Vc), *tert*-butylhydroquinone (TBHQ), and dimethyl sulfoxide (DMSO) were provided by the Sinopharm Group Chemical Reagent Ltd. (Shanghai, China). Finally, Dulbecco's modified Eagle's medium (DMEM), phosphate buffer solution (PBS), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Thermo Fisher Scientific (Waltham, MA, USA). The water used in all experiments was purified using a Milli-Q purification system.

2.2. Preparation of the acylated CA derivatives

Five acylated CA derivatives (Fig. 1), namely 4-*O*-acetyl-chlorogenic acid (*C*2-CA), 4-*O*-butyryl-chlorogenic acid (C4-CA), 4-*O*-hexanoyl-chlorogenic acid (C6-CA), 4-*O*-octanoyl-chlorogenic acid (C8-CA), and 4-*O*-lauroyl-chlorogenic acid (C12-CA), were synthesized using Lipozyme RM, as reported previously (Zhu, Wang, Chen, Xia, & Li, 2020). CA (100 mg), Lipozyme RM (110 mg), MTBE (10 mL), and acyl donors (vinyl acetate, vinyl butyrate, ethenyl hexanoate, vinyl octanoate, and vinyl laurate; CA-to-acyl donor molar ratio = 1:10) were mixed at 55 °C and 400 rpm for 7 d. The acylated CA derivatives were then separated and purified using a Waters 2545 preparatory HPLC instrument (Waters, Milford, MA, USA) and subsequently identified according to the method reported by Zhu et al. (2020).

2.3. Analysis of the CA derivatives

The method used to analyze CA and its derivatives was adapted from a previously reported method with some modifications (Zhu et al., 2020). CA and its derivatives were monitored at 320 nm using an Agilent 1260 HPLC system (Santa Clara, CA, USA) equipped with a C18 column (250 mm × 4.6 mm; i.d., 5 μm, Waters, Milford, MA, USA) at 320 nm and 30 °C. The flow rate was 0.8 mL/min, and the injection volume was 10 μL. Mobile phases A and B comprised water:methanol:phosphoric acid in ratios of 90:10:0.05 and 20:80:0.05 (v/v/v), respectively. The gradient elution process was as follows: 0.00–20.00 min, 20–100% B; 20.00–30.00 min, 100% B, 30.00–35.00 min, 100–20% B, and 35.00–45.00 min, 20% B.

2.4. Octanol-water partition coefficient (log P)

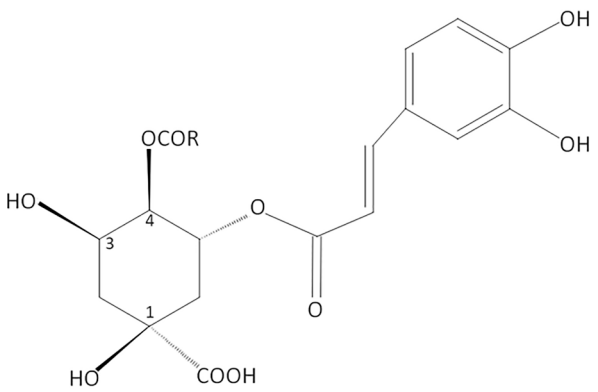
To evaluate the lipophilicities of CA and its acylated derivatives, the log *P* values were determined using a method previously reported by Yang, Kortesniemi, Yang, and Zheng (2018), with some modifications. Briefly, a saturated mixture of *n*-octanol and water (1:1, v/v) was prepared in a shaking water bath at 100 rpm and 37 °C for over 24 h. To separate the water-saturated *n*-octanol from the *n*-octanol-saturated water, the mixture was allowed to stand overnight. Subsequently, the CA and acylated CA samples (2 mg) were dissolved in water-saturated *n*-octanol (5 mL). An aliquot of each sample (0.4 mg/mL, 1 mL) and *n*-octanol-saturated water (1 mL) were then mixed in a shaking water bath at 100 rpm and 37 °C for 24 h. After allowing standing overnight, the sample solution (200 μL) was drawn from the upper (*n*-octanol) and lower (water) layers and subsequently measured using an Agilent 1260 HPLC system (Santa Clara, CA, USA). The CA and CA derivative contents were quantified from the HPLC peak area using the method described in section 2.3. The log *P* values were calculated using the following equation:

$$\mathrm{Log}P = \log \frac{A_1}{A_2} \quad (1)$$

where A_1 and A_2 are the peak areas of the sample in *n*-octanol and water, respectively.

2.5. In vitro simulated digestion

The digestive stabilities of CA and its acylated derivatives were evaluated by an *in vitro* simulated digestion method that involved a two-stage process, as adapted from a previous study with some modifications (Celep, Charehsaz, Akyüz, Acar, & Yesilada, 2015). More specifically, in a conical flask with a stopper, an aqueous NaCl solution (4 mL, 9 mg/



R	Compound	Abbreviation
CH_3	4-*O*-Acetyl-chlorogenic acid	C2-CA
$(CH_2)_2CH_3$	4-*O*-butyryl-chlorogenic acid	C4-CA
$(CH_2)_4CH_3$	4-*O*-hexanoyl-chlorogenic acid	C6-CA
$(CH_2)_6CH_3$	4-*O*-octanoyl-chlorogenic acid	C8-CA
$(CH_2)_{10}CH_3$	4-*O*-lauroyl-chlorogenic acid	C12-CA

Fig. 1. Structures of the acylated CA derivatives.

mL) was mixed with a pepsin solution (4 mL, 1 mg/mL), prepared using a 0.1 mol/L aqueous HCl solution. The pH of the resulting solution was adjusted to 2.0–2.5 using 0.1 mol/L HCl to afford the simulated stomach solution. Solutions of CA and the acylated CA samples (1 mL, 3 mg/mL) prepared with anhydrous ethanol and the simulated stomach solution were then incubated in a shaking water bath at 100 rpm and 37 °C for 2 h. Aliquots of the mixture (200 μL) were drawn from the conical flask at 0 and 2 h.

The pH of the remaining solution was then adjusted to 6.5–7.0 using a 1 mol/L aqueous $NaHCO_3$ solution. After shaking at 100 rpm and 37 °C for 45 min, a trypsin solution (1 mL, 0.9 mg/mL) prepared using 0.1 mol/L $NaHCO_3$ was added, and the pH of the mixture solution was adjusted to 7.0–7.5 using 1 mol/L $NaHCO_3$ to afford the simulated intestinal solution. This latter solution was shaken for 3 h to simulate intestinal digestion. Aliquots of the mixture (200 μL) were also drawn from the conical flask at 0 and 3 h.

The CA and CA derivative contents in the overall system before and after digestion were quantified from the HPLC peak area using the method described in section 2.3. The recovery index (RI; digested-to-nondigested sample concentration ratio) values, which were used to evaluate the stability of the samples, were calculated using the following equation:

$$\mathrm{RI}(\%) = \frac{A_1}{A_2} \times 100 \tag{2}$$

where A_1 and A_2 are the peak areas of the digested and nondigested samples, respectively.

2.6. Antioxidant capacity

The DPPH and ABTS radical scavenging activities and ferric reducing capacities were used to evaluate the antioxidant capacities of CA and its acylated derivatives according to previously reported methods (Liu & Yan, 2019; Sun et al., 2020) with some modifications. Vc and TBHQ were used as the controls.

The samples (CA and its acylated derivatives, 1 mL), with derivative concentrations ranging from 0 to 300 μmol/L, were mixed well with an ethanolic solution of DPPH (1 mL, 0.2 mmol/L) and incubated in the dark for 30 min at 30 °C. Subsequently, the absorbance, A, of the sample was determined at 517 nm using a UV-1206 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The DPPH radical scavenging activities of CA and its acylated derivatives were then calculated using the following equation:

$$\textit{DPPH radical scavenging activity}\ (\%) = \frac{A_0 - (A_1 - A_2)}{A_0} \times 100 \tag{3}$$

where A_0, A_1, and A_2 are the absorbance values of the ethanol/DPPH, the sample/DPPH, and the sample/ethanol mixtures, respectively.

Subsequently, the ABTS radical solution was prepared using an aqueous ABTS solution (7 mmol/L) and an equal volume of aqueous potassium persulfate solution (2.45 mmol/L). After incubation at 30 °C in the dark for 12–16 h, the mixture was diluted using anhydrous ethanol to form the final ABTS radical test solution. This test solution (1.9 mL) was then mixed with different concentrations of the sample (100 μL, 0–100 μmol/L) at 30 °C for 6 min, after which the absorbance was determined at 734 nm. The ABTS radical scavenging activities of CA and its acylated derivatives were calculated using the following equation:

$$\text{ABTS radical scavenging activity}(\%) = \frac{A_0 - (A_1 - A_2)}{A_0} \times 100 \tag{4}$$

where A_0, A_1, and A_2 are the absorbance values of the ethanol/ABTS radical test solutions, sample/ABTS radical test solutions, and sample/

ethanol solutions, respectively.

Finally, the sample (CA and its acylated derivatives, 100 μL, 0–100 μmol/L), phosphate buffer solution (2.5 mL, 2 mol/L, pH 6.6), and potassium ferricyanide (2.5 mL, 1%, w/w) were incubated at 50 °C for 20 min. After rapid cooling of the mixture, trichloroacetic acid (2.5 mL, 10%, w/w) was added, and each mixture was deposited for 10 min. Subsequently, the supernatant (2.5 mL), water (2.5 mL), and ferric chloride (0.5 mL, 0.5%, w/w) were mixed at 30 °C for 10 min. Using an absorbance wavelength of 700 nm, the ferric reducing capacities of CA and its acylated derivatives were calculated using the following equation:

$$\text{Reducing activity} = A_t - A_0 \quad (5)$$

where A_0 and A_t are the absorbance of the blank and sample, respectively.

2.7. Cell culture

HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in a growth medium prepared with DMEM, FBS (10%, v/v), and antimycotic solution (1%, v/v, 100 U/mL penicillin, and 100 μg/mL streptomycin) at 37 °C and 5% CO_2, as previously reported (Wolfe & Liu, 2007). Cells between passages 27 and 36 were used in this study.

2.8. Cytotoxicity analysis

HepG2 cells were seeded on 96-well plates (7×10^3/well) in a growth medium (100 μL) and cultured at 37 °C and 5% CO_2 for 24 h. The growth medium was then removed, and the cells were washed once with PBS. The CA and acylated CA derivatives were then dissolved in DMSO and diluted to different concentrations (0–100 μg/mL) using DMEM containing 1% antimycotic solution before their addition to the wells. After 24 h, the DMEM was removed, and the wells were fortified with 5 mg/mL MTT solution (50 μL) and incubated for 4 h. Subsequently, the MTT was removed, and the produced formazan was dissolved in DMSO with shaking for 15 min. The absorbance of the plate was measured at 570 nm using a Multiskan GO spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The cell survival rate, which represents the sample cytotoxicity, was calculated using the equation:

$$\text{Cell survival rate}(\%) = \frac{A_{sample} - A_{blank}}{A_{control} - A_{blank}} \times 100 \quad (6)$$

where A is the absorbance at 570 nm. The concentrations of CA and its acylated derivatives with absorbance values > 90% compared to that of the control were considered nontoxic.

2.9. Cellular antioxidant activity (CAA)

HepG2 cells were seeded on 96-well black plates (6×10^4/well) in a growth medium (100 μL) and cultured at 37 °C and 5% CO_2 for 24 h. Then, the growth medium was removed, and the cells were washed once with PBS. DMEM (100 μL) containing 1% antimycotic solution, the sample, and DCFH-DA (25 μmol/L) was used to treat the wells for 1 h. Subsequently, ABAP (100 μL, 600 μmol/L) was applied to the cells after either a PBS wash or no PBS wash. The 96-well black plate was measured using a Thermo Scientific Fluoroskan Ascent FL (Thermo Fisher Scientific, Waltham, MA, USA) every 5 min for 1 h with an emission of 538 nm and excitation of 485 nm. The CAA value was calculated using the equation:

$$\text{CAA unit} = \left(1 - \frac{\int \text{SA}}{\int \text{CA}}\right) \times 100 \quad (7)$$

where ∫SA and ∫CA equal the integrated areas under the sample fluorescence intensity versus time curve and the control fluorescence intensity versus time curve, respectively. The median effective dose (EC_{50}) was calculated using a previously described method (Wolfe & Liu, 2007).

2.10. Statistical analysis

All data obtained in this study were analyzed using IBM SPSS Statistics 21.0 (SPSS Inc., Chicago, IL, USA) and Origin 8.6.0 software (OriginLab Corp., Northampton, MA, USA). The results are presented as the mean ± standard deviation (SD), and differences of $p < 0.05$ were considered significant.

3. Results and discussion

3.1. Lipophilicity

The log *P* value, which was used to evaluate lipophilicity, was positively correlated with lipophilicity (Yang et al., 2018). According to Fig. 2(A), the lipophilicity of the acylated CA ($p < 0.05$) increased with the increasing carbon chain length of the acyl donor. More specifically, the log *P* value was − 0.25 ± 0.04 for CA and 1.44 ± 0.05 for C12-CA. The lipophilicities of resveratrol derivatives were also reported to increase with increasing chain length (Oh & Shahidi, 2017). However, according to the report of Oh and Shahidi (2017), when unsaturated fatty acids were used to acylate resveratrol, the lipophilicity of resveratrol decreased, regardless of the extension in the chain length. This was attributed to the properties of the carbon–carbon double bond. Therefore, we assumed that in addition to the chain length, the properties of the nonpolar groups also increase the lipophilicity of CA. Moreover, the lipophilicities of acylated epigallocatechin gallate, hydrophilic cyanidin, and delphinidin rutinosides were reported to be higher than those of their nonacylated equivalents (Wang, Zhang, Zhong, Perera, & Shahidi, 2016; Yang, Kortesniemi, Ma, Zheng, & Yang, 2019), thereby expanding the range of applications of such compounds. Viskupicova, Danihelova, Ondrejovic, Liptaj, and Sturdik (2010) also reported that more lipophilic of rutin derivatives, the superior antioxidant effect of it in an oil matrix.

3.2. In vitro simulated digestion analysis

As a member of the phenol family, CA is also unstable and poorly absorbed in the small intestine. However, the metabolism and bioavailability of this compound in the body directly influence its biological activities. Therefore, improvement in the stability and effectiveness of CA, previously achieved by the addition of wheat gluten hydrolysate to simulated intestinal juice (He et al., 2020), is considered highly important. In this study, we aimed to improve the stability of CA by forming CA derivatives. Therefore, the digestive stabilities of CA and its acylated derivatives were evaluated, and the effect of the chain length on the stability of CA was also examined by *in vitro* stimulated digestion.

As shown in Fig. 2(B), in the gastric digestion simulation stage, CA was relatively stable, and its RI approached 100%. Although most of the CA derivatives remained relatively stable, their RI values decreased. The RI values of *C*2-CA, C4-CA, C6-CA, and C8-CA were ˃90%. In addition, with an increase in the chain length of the acyl donor, the RI value initially increased and then decreased after C4-CA ($p < 0.05$). Notably, C4-CA showed the highest RI value among the acylated CA derivatives, which was closed to that of CA ($p > 0.05$). On the other hand, in the intestinal digestion stage, the RI value of CA was minimal and only slightly higher than that of *C*2-CA ($p > 0.05$). The regularity of the CA derivative chain length and RI trend was unclear during the intestinal digestion stage. However, the RI of C4-CA was higher than that of the other acylated CA derivatives and 2.11 times higher than that of CA. Previous studies have also shown that the amount of CA remained relatively unchanged during the gastric digestion stage and decreased significantly during the intestinal digestion (Bermúdez-Soto, Tomás-

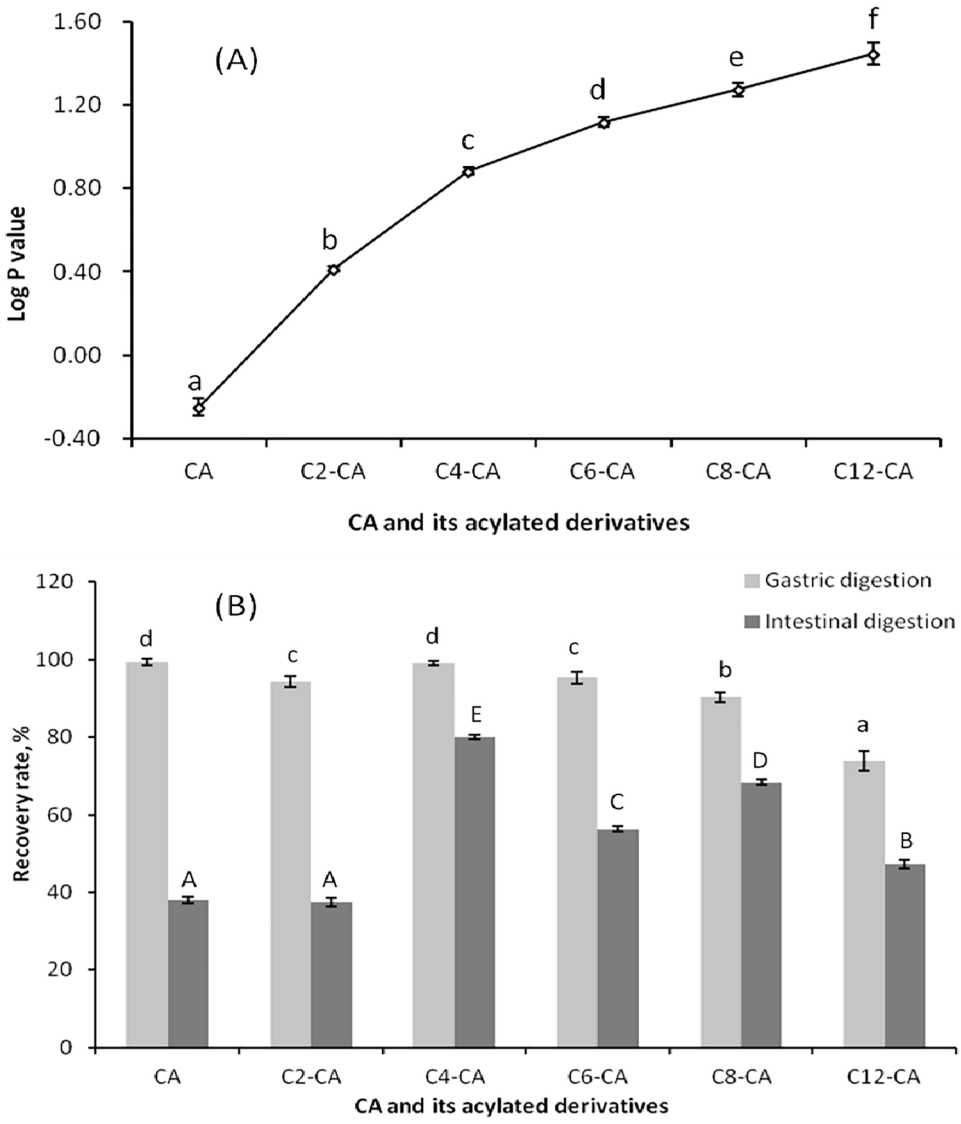


Fig. 2. Lipophilicities and simulated digestion stabilities of CA and its acylated derivatives: (A): Lipophilicities of CA and its acylated derivatives; lipid-water partition coefficient (log *P*). Significant differences ($p < 0.05$) between the samples are marked as a, b, c, d, e, and f. (B): Simulated digestion stabilities of CA and its acylated derivatives; gastric digestion at 2 h, and intestinal digestion at 3 h. Significant differences ($p < 0.05$) between the samples in the gastric digestion experiments are marked as a, b, c, d, e, and f, and in the intestinal digestion experiments these are marked as A, B, C, D, E, and F.

Barberán, & García-Conesa, 2007; Narita & Inouye, 2013). It indicated that during gastrointestinal digestion, the stability of CA improved after acylation. Under neutral or alkaline conditions, CA is easily digested by mucosal esterases or oxidized to other substances (Xiong et al., 2020). However, the RI value of CA increased when CA was acylated, especially when acylation was conducted using an acyl donor containing four carbon atoms.

3.3. Antioxidant assay

The biological activities of polyphenols were reported to be positively correlated with the number of hydroxyl groups in their molecular structures. However, when the hydroxyl group was replaced with an acyl group, the stability and lipophilicity improved, which was conducive to the dissolution of such compounds in lipid substances. In the DPPH and ABTS radical scavenging experiments, most lipophilized EGCG derivatives possessed better scavenging capacities than EGCG (Wang et al., 2016). On the other hand, the scavenging capacities of the resveratrol derivatives were lower than that of resveratrol (Oh & Shahidi, 2017), while those of the EGCG derivatives acylated with palmitoyl chloride slightly decreased in the ABTS assay (Liu & Yan, 2019). Yang et al. (2019) also reported that the reducing powers of acylated delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, and cyanidin-3-*O*-rutinoside were significantly lower than the corresponding values for unmodified anthocyanins. Chlorogenic laurate, a lipophilized CA derivative, exhibits relatively strong antioxidant activity, which is higher than that of CA in the DPPH assay (Xiang & Ning, 2008); however, the DPPH radical scavenging activities of the acylated CA prepared using palmitoyl chloride were 70–80% that of CA (Lorentz et al., 2010). These contradictory results were attributed to the different numbers of carbon atoms in the substances used to improve the polyphenol lipophilicities. In this study, the antioxidant activities of CA and its derivatives prepared using vinyl esters of different chain lengths were evaluated using DPPH and ABTS radical scavenging and ferric reducing capacity assays. These assays are based on the same electron transfer mechanism, and thus, the relationship between lipophilicity and antioxidant activity based on electron transfer is easier to elucidate. However, there were also some differences between the three methods. Indeed, the medium of the DPPH radical scavenging test comprised an organic system, the ABTS radical scavenging test comprised a mixed system of ethanol and water, and the ferric reducing capacity assay comprised an aqueous solution.

In the DPPH assay, CA exhibited a weaker antioxidant capacity than the controls (hydrophilic Vc and lipophilic TBHQ; $p < 0.05$) but a superior capacity than its acylated derivatives, except C4-CA (Fig. 3(A) and Table 1). Indeed, C4-CA may have a greater affinity for the lipophilic DPPH radical than CA ($p < 0.05$), owing to its superior

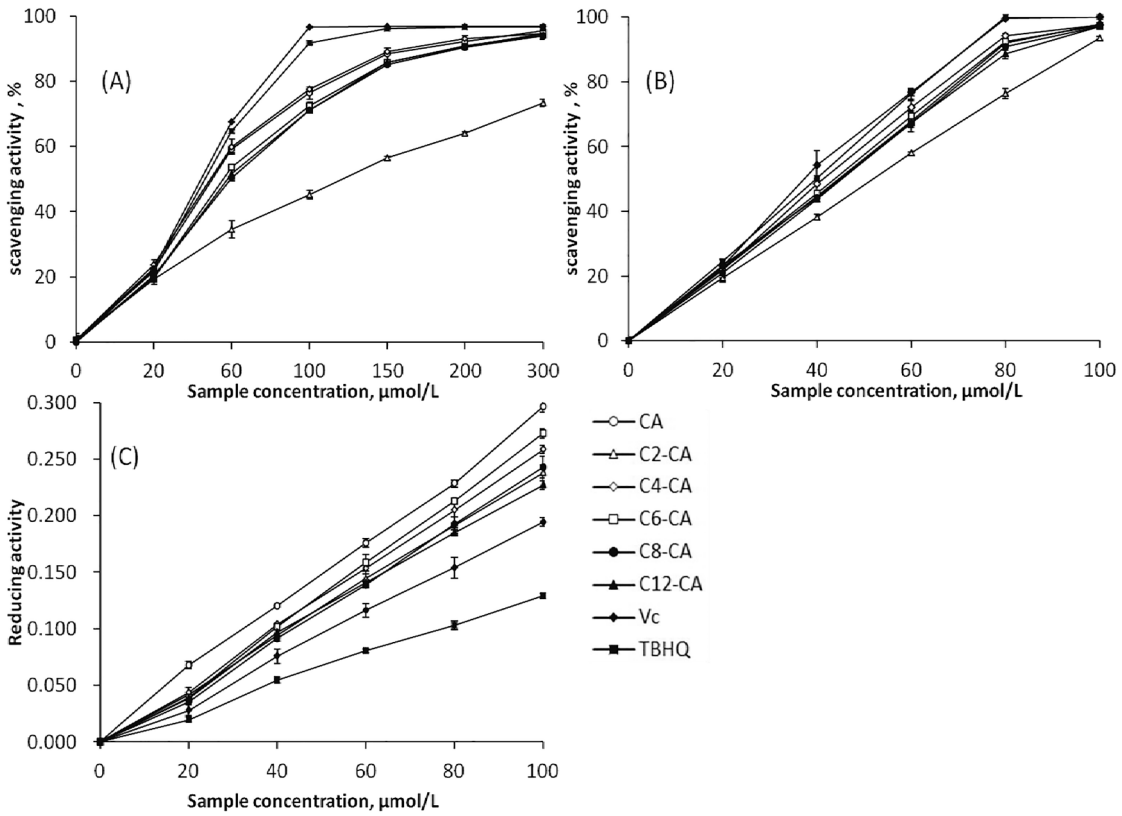


Fig. 3. Antioxidant activities of CA, its acylated derivatives, Vc, and TBHQ in chemical media: (A): DPPH radical scavenging capacity, (B): ABTS radical scavenging capacity, and (C): ferric reducing capacity.

Table 1
IC_{50} values for of samples in DPPH and ABTS radical scavenging tests and the EC_{50} values of samples in CAA test.

Samples	IC_{50}, μmol/L		EC_{50}, μg/mL	
	DPPH	ABTS	CAA unit	
			No wash	Wash
CA	45.61 ± 2.30^{b}	44.55 ± 1.35^{b}	12.05 ± 0.31^{dA}	29.08 ± 0.30^{eB}
*C*2-CA	110.67 ± 2.30^{d}	53.21 ± 0.70^{c}	20.79 ± 0.40^{fA}	33.12 ± 1.40^{gB}
C4-CA	43.80 ± 0.90^{b}	42.09 ± 1.14^{b}	15.44 ± 0.24^{eA}	30.92 ± 0.65^{fB}
C6-CA	51.73 ± 0.68^{c}	43.31 ± 1.27^{b}	11.38 ± 0.31^{cA}	25.96 ± 0.40^{dB}
C8-CA	53.13 ± 0.66^{c}	44.11 ± 1.88^{b}	11.28 ± 0.56^{cA}	24.28 ± 0.73^{cB}
C12-CA	52.87 ± 2.63^{c}	45.38 ± 1.43^{b}	10.04 ± 0.40^{bA}	9.40 ± 0.96^{bA}
Vc	36.48 ± 0.50^{a}	38.27 ± 1.42^{a}	–	–
TBHQ	38.97 ± 0.18^{a}	39.32 ± 1.03^{a}	–	–
Quercetin	–	–	8.03 ± 0.30^{aA}	6.86 ± 0.34^{aB}

lipophilicity to that of CA. Moreover, the hydrogen atom donation capability of CA may be affected by its electron density, which in turn may be altered by acylation (Zhong & Shahidi, 2011). Among these derivatives, the DPPH radical scavenging ability of *C*2-CA was the poorest, and its half-maximal inhibitory concentration (IC_{50}) was 2.43 times that of CA ($p > 0.05$). In the ABTS test (Fig. 3(B) and Table 1), the antioxidant abilities of CA and its derivatives were also weaker than those of Vc and TBHQ ($p < 0.05$). In addition, the IC_{50} value of *C*2-CA was 1.18 times higher than that of CA, while the radical scavenging ability of C4-CA was superior to that of CA. Moreover, with increasing acyl donor chain length, the ABTS radical scavenging abilities of the CA derivatives initially increased ($p < 0.05$) before decreasing again after C4-CA ($p > 0.05$). This was attributed to the lower lipophilicity of the ABTS radical. Thus, upon increasing the derivative lipophilicity, the affinity to the ABTS radical decreased, resulting in reduced ABTS radical scavenging abilities for the various derivatives (Wang et al., 2016). It was also observed that the scavenging effects of CA and its acylated derivatives on the ABTS radical were higher than the equivalent effects on the DPPH radical. In the ferric reducing capacity test, the ferric ion in potassium ferricyanide was reduced to the ferrous ion by CA and its acylated derivatives. Prussian blue, which exhibits a strong absorption at 700 nm, was formed by the reaction of the ferrous ions produced from the ferric ion in ferric chloride. These results indicated that a greater absorbance corresponded to a superior reducing capacity. Fig. 3(C) illustrates that the reducing capacities of the acylated CA derivatives were slightly lower than those of CA but higher than those of Vc and TBHQ. Using a sample concentration of 100 μmol/L, C6-CA exhibited the highest reducing capacity, followed by C4-CA. The reducing ability, therefore, decreased in the following order: CA > C6-CA > C4-CA > C8-CA > *C*2-CA > C12-CA > TBHQ > Vc, thereby indicating that the ferric reducing capacity test was more sensitive to CA and its derivatives than to Vc and TBHQ.

In this study, the CA derivatives acylated with medium-length chains presented stronger antioxidant capacities in all tests employed compared to the capacities observed for the other CA derivatives. Indeed, as previously reported, the antioxidant capacity of a sample is related not only to the number of hydroxyl groups, but also to the lipophilicity and electronic distribution (Wang et al., 2016). Thus, the lower reducing capacity of the acylated derivatives, compared to that of CA itself was likely due to the loss of a hydroxyl group, and the number of hydroxyl groups was related to the establishment of stable antioxidant properties (Naveed et al., 2018). Furthermore, the lipophilicities of the CA derivatives were superior to that of CA where the ferric reducing capacity assay was carried out in an aqueous solution. Thus, because the

solubilities of the CA derivatives were lower than that of CA in the ferric reducing capacity assay medium (i.e., water), a decrease in the reducing capacity was observed for the CA derivatives.

3.4. Caa

Despite the wide usage of DPPH and ABTS radical scavenging and ferric reducing capacity assays, their accuracy in predicting *in vivo* antioxidant activities has been questioned owing to their simplistic media (Kellett, Greenspan, & Pegg, 2018). Biological systems are more complex than chemical systems, and antioxidants that enter an organism are affected by a range of mechanisms, such as uptake, distribution, and metabolism. In addition, because the results obtained from the different antioxidant tests were different, it was necessary to study the antioxidant activities of CA and its acylated derivatives by CAA testing to reflect the complexity of the biological medium.

Prior to CAA testing, cell cytotoxicity was analyzed to determine the sample concentrations that had no effect on cell viability (i.e., cell survival rate > 90%). As shown in Fig. 4, with a sample concentration of ≤ 50 μg/mL, the cell survival rate was > 90%. However, with a sample concentration of 100 μg/mL, only C8-CA and C12-CA met the experimental requirements. Thus, concentrations of ≤ 50 μg/mL were used in the subsequent experiments. It was also found that the cytotoxicity of CA and its derivatives was reduced upon increasing the chain length. This was attributed to the loss of a hydroxyl group in the CA structure, which led to reduced biological activity. Moreover, the increase in the lipophilicity of the CA derivatives allowed them to enter the cells more easily and play a protective role, which is consistent with the CAA test results.

As shown in Fig. 5, the CAA unit of the samples and that of quercetin increased in a dose-dependent manner. More specifically, when the sample concentration was 50 μg/mL, the CAA unit of C12-CA was > 85, which well exceeds the corresponding values of CA and the other CA derivatives. The EC_{50} value was also calculated to evaluate the antioxidant capacities of CA and its acylated derivatives (Table 1). Interestingly, we observed that the EC_{50} value, which corresponds to stronger antioxidant activity in HepG2 cells ($p < 0.05$), decreased with increasing chain length. Moreover, the cellular antioxidant activities of C6-CA, C8-CA, and C12-CA were higher than that of CA ($p < 0.05$), regardless of whether a PBS wash was carried out. The results also indicated that the antioxidant activities of the CA derivatives were enhanced with increasing chain length in the order C12-CA > C8-CA > C6-CA > CA > C4-CA > C2-CA. Thus, upon increasing the chain length, the lipophilicity advantage of the CA derivatives gradually appeared and compensated for the loss of one hydroxyl group.

It has been previously reported that the surface ultrastructure of the cell membrane affects the entry of antioxidants into cells (Lu et al., 2020). Thus, the antioxidant activities of the samples with and without a PBS wash were compared to evaluate the absorption of the antioxidants and the binding degree between the membrane and the antioxidants (Wolfe & Liu, 2007). More specifically, when the cells were washed with PBS, the EC_{50} values of CA and its derivatives, except C12-CA, were almost twice those recorded in the absence of a PBS wash. On the other hand, similar to quercetin, C12-CA did not exhibit any significant differences in its EC_{50} values. Following cell washing with PBS, the antioxidants present on the cell surface that were either not bound or were loosely bound to the cell membrane were removed, and the antioxidants that were absorbed by the cells or tightly bound to the cell membrane were retained. As a result, the CAA unit decreased when PBS washing was employed. We also observed that an increase in the CA derivative hydrophobicity resulted in a decrease in the EC_{50} value. However, in the case of C12-CA, no significant differences in the EC_{50} values for the two test methods were observed. This suggests that C12-CA effectively enters or combines with the cell membrane to improve its antioxidant capacity, thereby playing a more important role than that of CA *in vivo*.

4. Conclusions

In this study, we evaluated the differences in the digestive stabilities and antioxidant activities of CA and its derivatives, which were synthesized using different vinyl esters. The relationship between lipophilicity and digestive stability as well as antioxidant capacity was

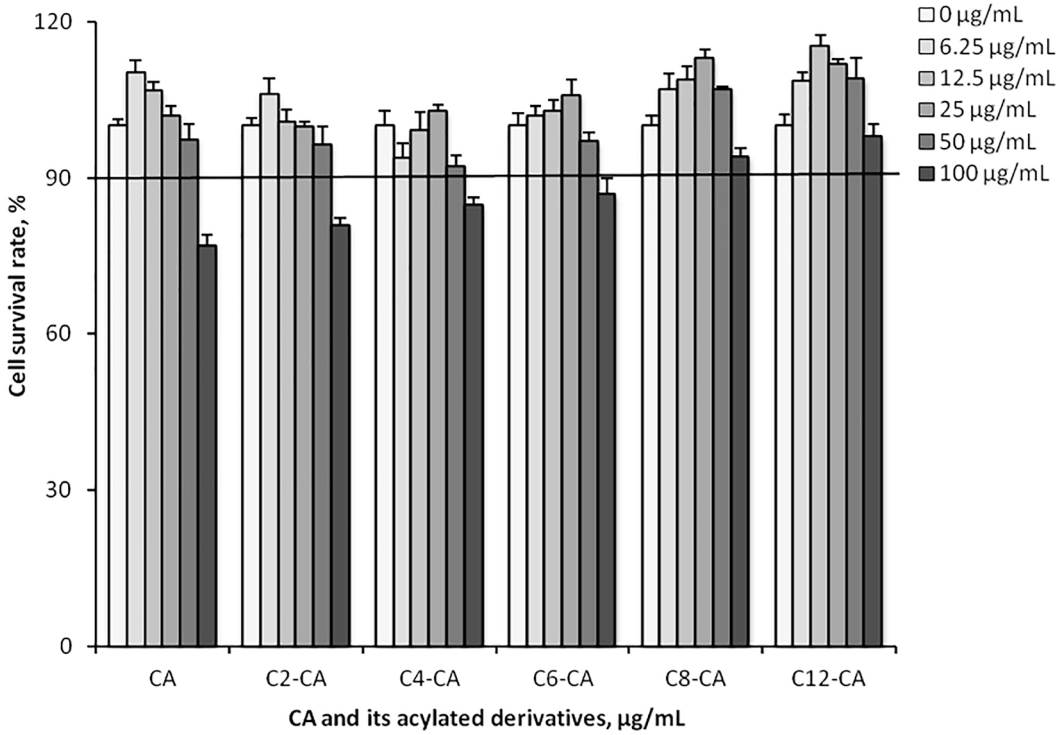


Fig. 4. Cytotoxicity of CA and its acylated derivatives toward HepG2 cells.

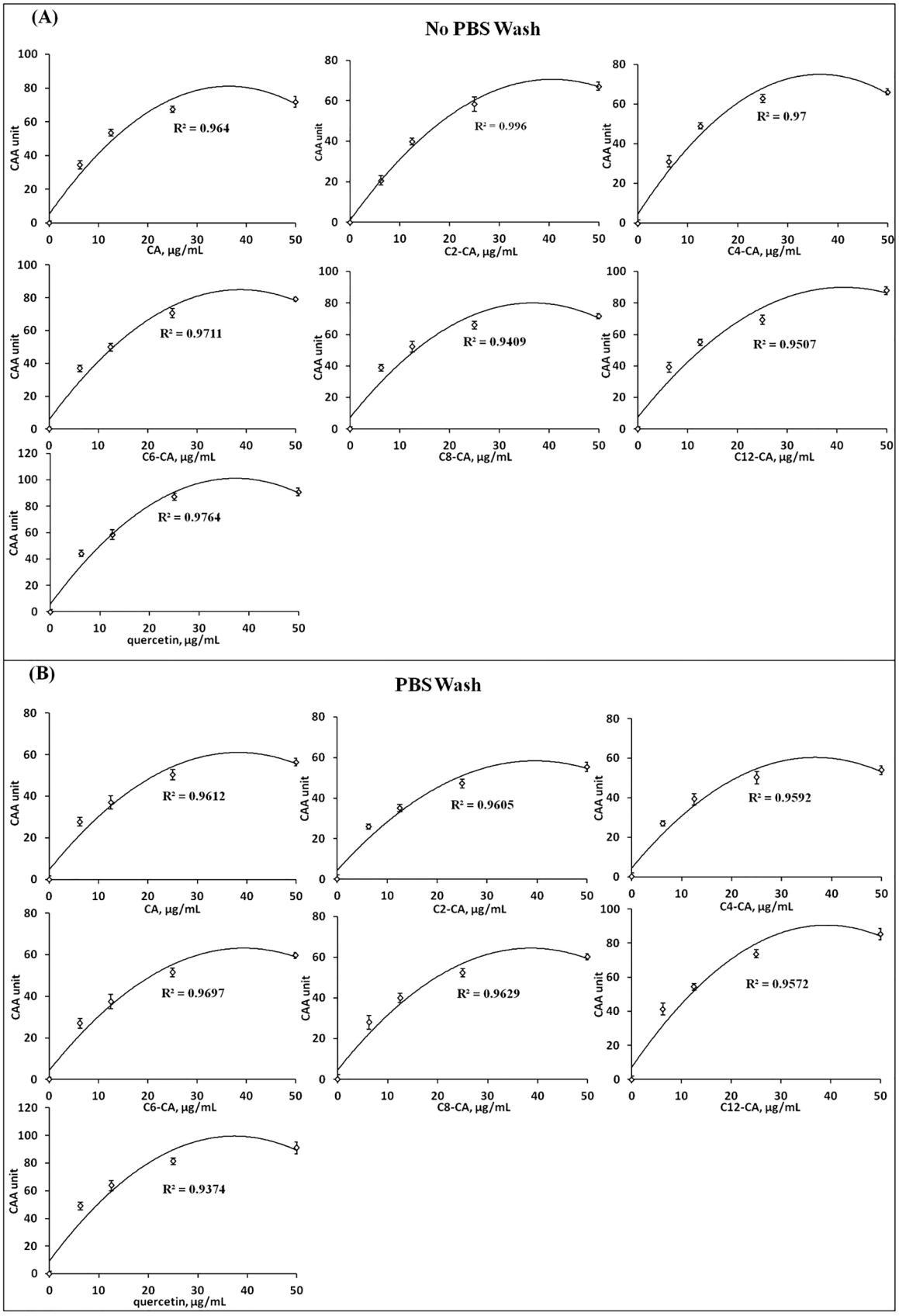


Fig. 5. Dose-response curves for inhibition by CA, its acylated derivatives and quercetin: (A): No PBS wash between the antioxidant and ABAP treatments, and (B): PBS wash between the antioxidant and ABAP treatments.

investigated. The results showed that the lipophilicity of CA was improved by acylation using CA and vinyl esters (vinyl acetate, vinyl butyrate, vinyl hexanoate, vinyl octanoate, and vinyl laurate). Following acylation, the antioxidant activity was found to be retained among the CA derivatives (with the exception of *C*2-CA), and their digestive stabilities were found to be improved. In the CAA test, the lipophilicities of the CA derivatives were found to be positively correlated with their antioxidant activities. Furthermore, the EC_{50} value of C12-CA, which possessed the longest-chain length of the derivatives examined herein, was significantly lower than those of CA and the other derivatives. These results indicate that lipophilic CA derivatives with good antioxidant activities exhibit great potential for application in lipid-based foods.

CRediT authorship contribution statement

Shan Wang: Formal analysis, Methodology, Writing - original draft. **Yue Li:** Formal analysis, Methodology. **Xiangyong Meng:** Data curation, Formal analysis, Methodology. **Shangwei Chen:** Data curation, Formal analysis, Methodology. **Dejian Huang:** Writing - review & editing. **Yongmei Xia:** Conceptualization, Formal analysis, Methodology. **Song Zhu:** Conceptualization, Data curation, Investigation, Resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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