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# An effective strategy to develop active cinnamic acid-directed antioxidants based on elongating the conjugated chains

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ABSTRACT

To optimize antioxidant activity and lipophilicity of cinnamic acid derivatives (CAs) including ferulic acid, sinapic acid, 3,4-dimethoxycinnamic acid, and *p*-hydroxycinnamic acid, four analogs bearing an additional double bond between their aromatic ring and propenoic acid moiety were designed and synthesized based on the conjugated chain elongation strategy. The antioxidant performance of the CAs were investigated by 2,2′-diphenyl-1-picrylhydrazyl (DPPH·)-scavenging, ferric reducing/antioxidant power, cyclic voltammetry, DNA strand breakage-inhibiting and anti-haemolysis activity assays. It was found that CAs with elongation of conjugated chains display increased DPPH·-scavenging, DNA strand breakage-inhibiting and anti-haemolysis activities as compared to their parent molecules, due to their improved hydrogen atom-donating ability and lipophilicity. Overall, this work highlights an effective strategy to develop potential CA-directed antioxidants by elongating their conjugated chain.



## 1. Introduction

Polyphenols are a large family of bioactive compounds widely distributed in plant kingdom, among which cinnamic acid (CA) and its derivatives occupy an important position as known antioxidants found in plant foodstuffs (Block, Patterson, & Subar, 1992; Clifford, 1999; Herrmann & Nagel, 1989; Shahidi & Chandrasekara, 2010). A sufficient source of phenolic acids in the daily diet of plant origin not only plays an essential role in the organism protection against deleterious oxidative damage for the human health, but also prevents certain chemotherapy-caused side effects and slows down the cancer progression, such as angiogenesis, invasion and metastasis for cancer patients (Conklin, 2000; Weng & Yen, 2012). Oxidative stress induced by excessive production of reactive oxygen species (ROS) or free radicals is related to the development of a wide range of diseases, mainly atherosclerosis, inflammatory injury, neurodegenerative diseases, cancer, and the accelerated ageing of organisms (Darvesh, Carroll, Bishayee, Geldenhuys, & Van der Schyf, 2010; Fresco, Borges, Diniz, & Marques, 2006; Thomasset et al., 2006). On another hand, although ROS play important roles in prooxidant cancer therapy (Trachootham, Lu, Ogasawara, Rivera-Del Valle, & Huang, 2008), they are also responsible for some adverse effects of many clinically used anticancer drugs, such as gastrointestinal toxicity and mutagenesis (Conklin, 2000; Deavall, Martin, Horner, & Roberts, 2012). The CA derivatives (CAs) have become attractive in medicinal research mainly due to their natural origin and their preventive and defensive effects against the above diseases based on the antioxidant capacity against the damaging free radicals and ROS (Darvesh et al., 2010), lacking adverse health effects in humans.

Therefore, the past two decades have witnessed much interest in investigating antioxidant mechanisms (Cheng, Dai, Zhou, Yang, & Liu, 2007; Foti, Daquino, & Geraci, 2004) of CAs and modifying their molecule structure to improve antioxidant activity. Structural modifications of CAs focus mainly on optimizing the aromatic ring substitution (Bakalbassis et al., 2001; Gaspar et al., 2009; Rice-Evans, Miller, & Paganga, 1996) and grafting alkyl ester side chains (Figueroa-Espinoza & Villeneuve, 2005; Gaspar et al., 2010; Laguerre et al., 2009; López-Giraldo et al., 2009; Nenadis, Zhang, & Tsimidou, 2003; Reis et al., 2010). The second strategy is to improve their lipophilicity since the hydrophilic nature of CAs results in some drawbacks: a limited application in oil-based industrial processes and the poor membrane permeability which greatly influences the antioxidant behaviour in biological systems. Additionally, this structure modification strategy has also been applied in other hydrophilic phenols including an important tea polyphenols, epigallocatechin gallate (Zhong & Shahidi, 2011).

Currently, there are few researches focusing on the structure modification of the middle part (double bond moiety) of CAs in improving antioxidant activity. We have previously found that a middle part modification in the stilbene scaffold of resveratrol by

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inserting additional double bonds between two aromatic rings, could significantly improve its antioxidant performance including the hydrogen atom- or electron-donating ability and its lipophilicity (Tang et al., 2011). Thus, we tried to mimic this strategy of the conjugated chain elongation to modifying the molecule structure of CAs including ferulic acid (FA, **A1**), sinapic acid (SA, **A2**), 3,4-dimethoxycinnamic acid (DMA, **A3**), and *p*-hydroxycinnamic acid (*p*-HCA, **A4**), where an additional double bond was inserted between the aromatic ring and the propenoic acid moiety to obtain more lipophilic derivatives (**B1**–**4**, Scheme 1). Furthermore, whether and how the conjugated chain elongation affects the antioxidant properties of CAs were also examined. The antioxidant activity of the eight CAs (**A1**–**4** and **B1**–**4**) was determined by employing five different and commonly used methods: the 2,2′-diphenyl-1-picrylhydrazyl (DPPH·)-scavenging, ferric reducing/antioxidant power (FRAP), cyclic voltammetry, DNA strand breakage-inhibiting and anti-haemolysis activity assays. The assays are indicative of their formal hydrogen-transfer and electron-donating abilities, inhibitory ability against DNA damage induced by free radicals and protective activity against lipid peroxidation in a heterogeneous environment, respectively.

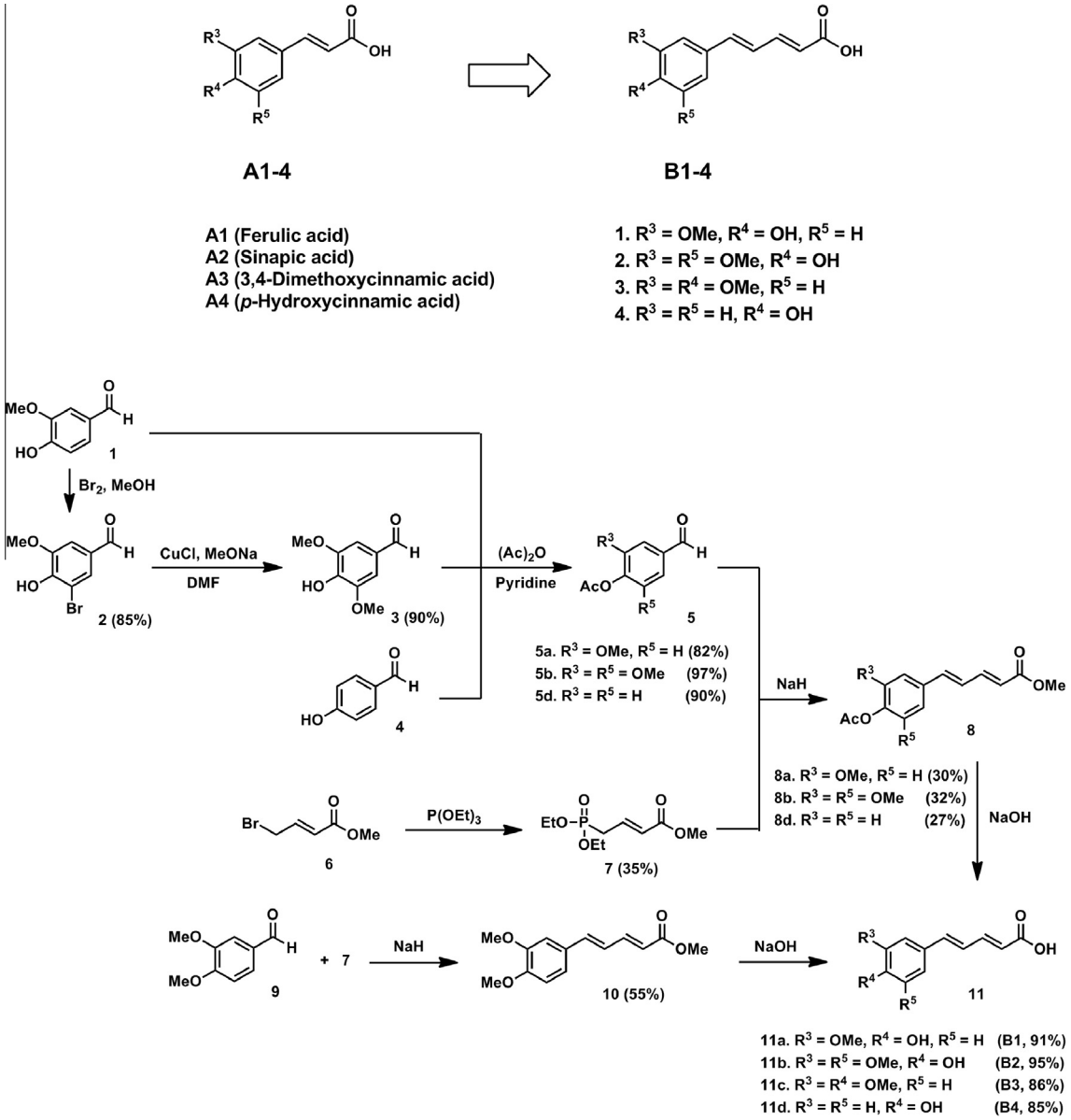
## 2. Materials and methods

### 2.1. Materials

2,2′-Diphenyl-1-picrylhydrazyl radical (DPPH·), pBR322 DNA and 2,2-azobis (2-amidinopropane hydrochloride) (AAPH), were obtained from Sigma–Aldrich Inc., (St. Louis, MO, USA). 2,4,6-Tri(2-pyridyl)-S-triazine (TPTZ) was from Alfa Aesar Co., Ltd. (MA, USA). The compounds (**A1**–**4**) were purchased from Adamas Reagent Co. Ltd., (Shanghai, China). Other chemicals used were of analytical grade.

### 2.2. Synthesis

The synthetic details and spectra ($^1$H, $^{13}$C NMR and MS) of the target compounds (**B1**–**4**) were described in the Supplementary




**Scheme 1.** Molecular structures and synthesis of cinnamic acid derivatives with the conjugated chain elongation.

material. $^1$H and $^{13}$C NMR spectra were recorded using a Bruker AV 400 (Bruker Biospin Co. Ltd., Switzerland) spectrometer with deuterated chloroform ($CDCl_3$) or deuterated dimethyl sulfoxide (DMSO-d6) as a solvent. Mass spectra were recorded on a Bruker Daltonics Esquire 6000 (Billerica, MA, USA) spectrometer (ESI-MS).

*2.2.1. (2E,4E)-5-(4-Hydroxy-3-methoxyphenyl)penta-2,4-dienoic acid (**11a, B1**)*

$^1$H NMR (400 MHz, DMSO-d6): $\delta$ = 3.81 (s, 3H), 5.90 (d, $J$ = 15 Hz 1H), 6.77 (d, $J$ = 8 Hz 1H), 6.92 – 6.98 (m, 3H), 7.16 (s, 1H), 7.31 (m, $J$ = 15 Hz, 1H), 9.43 (s, 1H), 12.12 (s, 1H); $^{13}$C NMR (100 MHz, DMSO-d6): $\delta$ = 55.6, 110.2, 115.6, 120.2, 121.5, 123.6, 127.6, 140.6, 145.0, 147.9, 148.0, 167.8; MS (EI): ($m/z$) = 219 $[M-H]^-$.

*2.2.2. (2E,4E)-5-(4-Hydroxy-3,5-dimethoxyphenyl)penta-2,4-dienoic acid (**11b, B2**)*

$^1$H NMR (400 MHz, DMSO-d6): $\delta$ = 3.79 (s, 6H), 5.91 (d, $J$ = 15 Hz, 1H), 6.85 (s, 2H), 6.92 (d, $J$ = 15 Hz, 1H), 7.00 (dd, $J$ = 15 Hz, $J$ = 10 Hz, 1H), 7.31 (dd, $J$ = 15 Hz, $J$ = 10 Hz, 1H), 8,78 (s, 1H), 12.16 (s, 1H); $^{13}$C NMR (100 MHz, DMSO-d6): $\delta$ = 56.0 (2C), 105.0 (2C), 120.3, 124.0, 126.4, 137.1, 140.9, 144.9, 148.1 (2C), 167.7; MS (EI): ($m/z$) = 251 $[M+H]^+$.

*2.2.3. (2E,4E)-5-(3,4-Dimethoxyphenyl)penta-2,4-dienoic acid (**11c, B3**)*

$^1$H NMR (400 MHz, DMSO-d6): $\delta$ = 3.77 (s, 3H), 3.80 (s, 3H), 5.94 (d, $J$ = 15 Hz, 1H), 6.95 (d, $J$ = 8 Hz, 1H), 6.92 – 7.01 (m, 2H), 7.07 (d, $J$ = 8 Hz, 1H), 7.20 (s, 1H), 7.33 (dd, $J$ = 15 Hz, $J$ = 10 Hz, 1H), 12.20 (s, 1H); $^{13}$C NMR (100 MHz, DMSO-d6): $\delta$ = 55.8 (2C), 109.8, 112.0, 121.0, 121.6, 124.8, 129.2, 140.5, 145.1, 149.3, 150.2, 168.0; MS (EI): ($m/z$) = 257 $[M+Na]^+$, 235 $[M+H]^+$.

*2.2.4. (2E,4E)-5-(4-Hydroxyphenyl)penta-2,4-dienoic acid (**11d, B4**)*

$^1$H NMR (400 MHz, DMSO-d6): $\delta$ = 5.91 (d, $J$ = 15 Hz, 1H), 6.77 (d, $J$ = 8 Hz, 2H), 6.87 (dd, $J$ = 15 Hz, $J$ = 10 Hz, 1H), 6.94 (d, $J$ = 15 Hz, 1H), 7.32 (dd, $J$ = 15 Hz, $J$ = 10 Hz, 1H), 7.39 (d, $J$ = 8 Hz, 2H), 9.82 (s, 1H), 12.12 (s, 1H); $^{13}$C NMR (100 MHz, DMSO-d6): $\delta$ = 115.8 (2C), 120.2, 123.3, 127.1, 128.9 (2C), 140.3, 145.1, 158.6, 167.8; MS (EI): ($m/z$) = 189 $[M-H]^-$.

*2.3. Assay for DPPH·-scavenging activity*

The $EC_{50}$ values of CAs (**A1**–**4** and **B1**–**4**) in the scavenging of DPPH· were determined by monitoring the absorbance change of DPPH· (100 μM) at 517 nm in methanol after 1 h incubation in the dark with different concentrations of compounds at 25 °C, using a TU-1901 UV/Vis spectrometer (Beijing Purkinje General Instrument Co. Ltd., Beijing, China). The percentage of radical scavenging activity was calculated as follows: radical scavenging rate (RSR, %) = $(A_0 - A_s)/A_0 \times 100$, where $A_0$ is the absorbance of 100 μM DPPH· only and $A_s$ is the absorbance of the reaction mixture after 1 h incubation. The stoichiometry (the equivalent of DPPH· scavenged by one equivalent of antioxidant) $n$ was calculated follow the equation: $n$ = 100 (μM)/($EC_{50}$ (μM) × 2) (Brand-Williams, Cuvelier, & Berset, 1995).

*2.4. Assay for ferric reducing/antioxidant power (FRAP)*

The FRAP assay (Benzie & Strain, 1996) was used to evaluate the reducing capability of CAs, and the procedure has been previously described in detail (Tang et al., 2011).

*2.5. Assay for the electrochemistry behaviour*

Cyclic voltammetry, as a widely used electrochemical technique, was performed using a computer controlled CHI-660C potentiostat (Chenhua Instruments Inc., Shanghai, China) following the procedure described previously with some minor modification (Yang et al., 2010). Briefly, the solutions of CA derivatives (1 mM) were prepared in supporting electrolyte (tetra-n-butylammonium perchlorate, TBAP, 0.1 M in methanol). The glassy carbon working electrode (CH Instruments Inc., 3700 Tennison Hill Drive, Austin, TX, USA) was polished with ultrafine alumina slurry and washed thoroughly with water under sonication before use. A Pt wire was employed as the counter electrode and an $Ag/Ag^+$ electrode (CH Instruments Inc., 3700 Tennison Hill Drive, Austin, TX, USA) served as the reference electrode. The potential difference between $Ag/Ag^+$ and a saturated calomel electrode (SCE) was determined ($\Delta E_{Ag/Ag^+-SCE} = 0.51$ V)), and all the reported potentials were with respect to SCE. All the CV measurements were carried out in the TBAP electrolyte under an argon atmosphere using Ferrocene as an internal standard for calibrating redox potentials against the ferricenium/ferrocene ($Fc^+/Fc$) couple ($E_p^a$ = 0.43 V vs. SCE).

*2.6. Assay for oxidative DNA strand breakage induced by AAPH*

The inhibition of AAPH-induced DNA strand breakage by CAs was assessed by measuring the conversion of supercoiled closed-circular pBR322 plasmid DNA to its open-circular and linear forms by gel electrophoresis following the procedure described previously (Qian et al., 2011).

*2.7. Assay for haemolysis of RBCs*

Human red blood cells (RBCs) were provided by the Red Cross Center for Blood (Gansu, China). The extent of haemolysis was determined spectrophotometrically at 540 nm and compared with that of complete haemolysis by treating with distilled water as described previously (Qian et al., 2011).

## 3. Results and discussion

*3.1. Synthesis of CAs with the conjugated chain elongation*

The overall strategy for synthesis of the CAs (**B1**–**4**) with the conjugated chain elongation is outlined in Scheme 1. Horner–Wadsworth–Emmons reactions of appropriate aldehydes with a prior-synthesized carbomethoxy-methylenetriphenylphosphorane were performed under a strong base (NaH) condition in absolute anhydrous THF to give the corresponding methyl ester **8a**–**d** and **10**. The desired CAs **B1**–**4** was obtained after an alkaline hydrolysis of the methyl esters with NaOH in methanol–water. Additionally, the acetyl protection groups can be removed by base in the last step to furnish **B1**–**2** and **B4** bearing a phenolic hydroxyl group.

*3.2. Antioxidant activity of CAs evaluated by the DPPH·-scavenging and FRAP assays*

The DPPH·-scavenging activity was measured at 25 °C in methanol as a commonly accepted characteristic responsible for antioxidant capability. The $EC_{50}$ (concentration for 50% radical scavenging) and stoichiometric factor $n$ values as well as radical scavenging rate (RSR) of CAs and Trolox C (a reference compound) are represented in Table 1. According to the $EC_{50}$ and $n$ values, the DPPH·-scavenging activity decreased in the order **B2** > **A2** > **B1** > **A1** > Trolox C > **B4** > **A4** > **B3** > **A3**. All the studied SA and FA derivatives presented higher activity than Trolox C, while **A3** and **B3** without hydroxyl group had the poorest activity. These results clearly indicate that *p*-hydroxyl group on the aromatic ring is not the only factor responsible for the DPPH·-scavenging activity, and the substitution of electron-donating methoxy group in

**Table 1**
Antioxidant performance, oxidation potentials, and *ClogP* values of CAs.

| Compounds | DPPH·-scavenging activity | | | $n_e$[a] | $E_p$ (V vs. SCE) | $t_{eff}$ (min)[d] | *ClogP*[e] |
|---|---|---|---|---|---|---|---|
| | $EC_{50}$ (μM)[a] | $n$[b] | RSR (%)[c] | | | | |
| **A1** | 44.63 ± 0.16 | 1.08 | 51.15 ± 0.42 | 1.659 ± 0.037 | 0.824 | 37 | 1.421 |
| **B1** | 41.18 ± 0.34 | 1.17 | 58.71 ± 1.28 | 1.969 ± 0.019 | 0.753, 1.518 | 57 | 1.875 |
| **A2** | 28.26 ± 0.27 | 1.87 | 71.32 ± 0.38 | 2.679 ± 0.059 | 0.752, 1.458 | 34 | 1.204 |
| **B2** | 23.30 ± 0.32 | 2.17 | 92.63 ± 0.82 | 1.709 ± 0.073 | 0.798, 1.500 | 47 | 1.658 |
| **A3** | >> 300 | – | 1.64 ± 0.64 | ≈ 0 | 1.498 | 14 | 1.897 |
| **B3** | >> 300 | – | 2.68 ± 1.08 | ≈ 0 | 1.419 | 20 | 2.351 |
| **A4** | >> 300 | – | 14.71 ± 0.69 | 0.130 ± 0.004 | 1.132 | 27 | 1.572 |
| **B4** | 136.44 ± 2.95 | 0.40 | 36.94 ± 0.89 | 1.382 ± 0.006 | 0.920 | 30 | 2.026 |
| Trolox C | 51.37 ± 0.67 | 1.05 | 52.28 ± 0.72 | 1.964 ± 0.005 | – | 38 | 3.089 |

[a] Data are expressed as the mean ± SD for three determinations.
[b] Calculated by the equation: $n$ = 100 (μM)/($EC_{50}$ (μM) × 2).
[c] RSR refers to the radical scavenging rate (%) after 1 h incubation of 50 μM compounds with 100 μM DPPH·.
[d] Data are the averages of three determinations which were reproducible with deviation less than ± 10%.
[e] Calculated using Bio-Loom software (Biobyte Corp. version 5).

*ortho*-positions to the hydroxyl group also contributes to the activity, in accordance with the previous observation (Graf, 1992). Kinetic results suggest that hydrogen atom abstraction is much easier from intramolecularly hydrogen bonded methoxyphenols than from intermolecularly hydrogen bonded molecules, and the small kinetic solvent effect of *o*-methoxyphenols renders them good antioxidants, even in a polar environment (de Heer, Mulder, Korth, Ingold, & Lusztyk, 2000). Theoretical calculation also indicates that the bond dissociation energy (BDE) of *o*-methoxyphenol is 1.4 kcal/mol lower than that of phenol (Wright, Johnson, & DiLabio, 2001). Noticeably, a comparison of $EC_{50}$, $n$ or RSR values of the corresponding compounds in series A with B clearly indicates that inserting an additional double bond between the aromatic ring and the propenoic acid moiety, results in an obvious or appreciable increase in the DPPH·-scavenging ability, especially in the case of RSR determination. For example, the RSR values of **B2** and **B4** reached 92.63% and 36.94%, 30% and 151% higher than that of the corresponding **A2** and **A4**, respectively. The data reflect that the conjugated chain elongation could stabilize resonantly the phenoxyl radical to decrease the BDE of the phenolic O–H bond and therefore improve the hydrogen atom-donating ability of CAs.

The reducing capacity of CAs was evaluated by the FRAP assay (Benzie & Strain, 1996) and the results are expressed as the number of electrons ($n_e$) donated by each antioxidant molecule and summarized in Table 1. Only SA derivative **A2** possessed a better reducing capacity than Trolox C. while **A3** and **B3** without hydroxyl group in their structure had scarcely reducing capacity in the assay, which are in accordance with the data from DPPH assay. Interestingly, inserting an additional double bond in the side chain had different effects on the $n_e$ values of CAs. Specifically, **B1** and **B4** exhibited approximately 1.2- and 10-fold increased $n_e$ values relative to **A1** and **A4**, respectively. However, in the case of SA derivatives, a clear reduction in the $n_e$ values was observed for **B2**, relative to **A2**. The above results suggest that the conjugated chain elongation strategy could enhance the electron-donating ability of **A1** and **A4**, but decrease that of **A2**. This can be understood by Eq. (1), which suggests that the FRAP activity of compounds is relevant to stability of the resulting phenoxyl radical cations ($ArOH^{\cdot+}$). Inserting an additional double bond could augment $p$ electron-delocalization of phenoxyl radicals, yet increase the electron-withdrawing ability of the entire side chain, leading to the instability of cations. This paradox effect on stability of the resulting $ArOH^{\cdot+}$ is in full accord with different influence of the conjugated chain elongation on the electron-donating ability of CAs. However, we have previously proven that the conjugated chain elongation significantly enhances the electron-donating ability of resveratrol and its hydroxystilbenoid analogs (Tang et al., 2011). Thus, the different results in the case of CAs and hydroxystilbenoid derivatives highlight that effect of the conjugated chain elongation strategy on the electron-donating ability of compounds depends on their structure types, and even their substituent groups.

$$ArOH + Fe^{3+} \rightarrow ArOH^{\cdot+} + Fe^{2+} \quad (1)$$

### 3.3. Electrochemistry behaviour of CAs

Cyclic voltammetry as a classical electrochemical analysis technology has been widely used to study the redox reaction process in biochemistry and many other related fields. Considering that many biological antioxidants function actually through inhibiting the oxidation process of biological macromolecules, thus usually possessing significant electrochemical activity, electrochemical technology is likely to provide powerful evidence in evaluation of antioxidant ability (Aguirre et al., 2010; Sochor et al., 2013).

Herein, we employed cyclic voltammetry to investigate the electrochemistry behaviour of CAs using a glassy C working electrode. Cyclic voltammetric scan of all the studied antioxidants showed the occurrence of irreversible oxidation peaks (Fig. 1), among which **A3** and **B3** lacking phenolic hydroxyl group had the most positive potentials (1.489 and 1.419 V vs. SCE, respectively) and thus hardly exhibited reducing capacity. **A1**, **A2** and **A4** exhibited more negative oxidation potentials (Table 1) which was associated with the oxidation of the phenolic hydroxyl (Galato et al., 2001). The installation of one or two methoxy into the *ortho*-position of 4-OH, to generate **A1** or **A2**, led to significant decrease of the oxidation potentials, relative to **A4**. Additionally, cyclic voltammograms of **A2** showed two convolved anodic peaks, at 0.752 and 1.458 V, respectively (Fig. 1; Table 1). An interpretation has been proposed that these two peaks are attributable to oxidation by electron transfer for both free and adsorbed forms of the species: the free form corresponding to the first peak while the stabilized adsorbed form to the more positive peak (Gaspar et al., 2009). The oxidative data of **B1**–**4** demonstrate that the conjugated chain elongation cause a negative shift for the oxidation potentials of **A1**, **A3** and **A4**, but a positive shift for that of **A2**, which are in good accordance with the FRAP assay. Furthermore, a good linear relationship ($r$ = 0.869) between the oxidative potentials ($E_p$) and the $n_e$ values determined from the FRAP method, was also observed (Fig. 2), which proves valuable to explore the electron-donating ability of compounds by the FRAP assay.

### 3.4. Inhibition of AAPH-induced DNA oxidative damage by CAs

An important biological function of antioxidants is to protect DNA as a primary target of ROS from oxidative damage, and thus

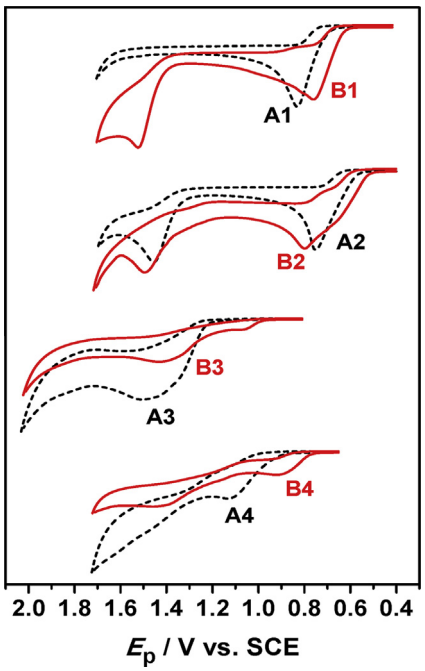



**Fig. 1.** Cyclic voltammetrys for the oxidation of 1 mM CAs in methanol with 0.1 M $Bu_4NClO_4$ at the scan rate of 50 mV $s^{-1}$. **A1**, $E_p^a$ = 0.824 V (vs. SCE); **B1**, $E_p^a$ = 0.753, 1.518 V; **A2**, $E_p^a$ = 0.752, 1.458 V; **B2**, $E_p^a$ = 0.798, 1.500 V; **A3**, $E_p^a$ = 1.498 V; **B3**, $E_p^a$ = 1.419 V; **A4**, $E_p^a$ = 1.132; **B4**, $E_p^a$ = 0.920 V.

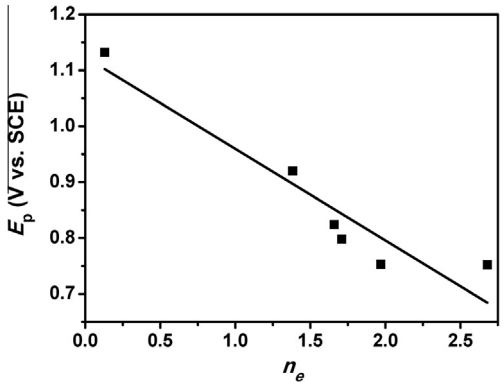



**Fig. 2.** Correlation analysis for the $n_e$ values of CAs obtained from FRAP assay and their oxidation potentials $E_p$ (V vs. SCE).

prevent occurrence of cancer in the initial stage (Pan & Ho, 2008). Therefore, the ability of CAs to inhibit AAPH-initiated DNA damage was assessed *in vitro* by the plasmid DNA nicking assay which has been accepted as a simple, yet sensitive and semi-quantitative assay based on the differential mobility of supercoiled, circular and linear forms of pBR322 plasmid DNA in agarose gel electrophoresis. Fig. 3A illustrates the change of the supercoiled DNA after incubation with different concentrations of AAPH. As shown in lanes 2–7 of Fig. 3A, the DNA supercoiled form decreased gradually and converted into the open circular (indicating a single-strand breakage) and further linear form (indicating a double-strand breakage) with the AAPH concentration increase from 1.25 to 40 mM. All the compounds except **A3** and **B3** showed clear protective effect against the DNA strand breakage induced by 5 mM AAPH (Fig. 3B) with the activity sequence of **B1** > **A1** – **B2** > **A2** > Trolox C > **B4** > **A4** > **B3** > **A3**, obtained by semi-quantitative percentage of intact supercoiled DNA (Fig. 3C). This sequence is similar to that obtained from the DPPH-scavenging assay with slight differences (a reverse order between **1** and **2**). Moreover, the appreciably positive role of the conjugated chain elongation on inhibiting the DNA strand breakage can be found by comparing **A1**–**4** with their corresponding **B1**–**4**, further suggesting that this modification strategy could effectively improve the hydrogen atom-donating or DNA-binding abilities of CAs.

### 3.5. Inhibition of AAPH-induced RBC haemolysis by CAs

Considering that lipids are another major target for free radical mediated injury and this injury has become a potential cancer risk factor (Niki, 2009), an anti-haemolysis assay was performed to evaluate the protective activities of the CAs against free radical-induced lipid oxidation. The RBCs model was chosen due to its heterogeneous biological system with high content of polyunsaturated fatty acids, and may provide valuable information for antioxidant protection in cellular system. Peroxyl radicals initiated by AAPH could attack erythrocytes, inducing the chain oxidation of lipid and protein, disturbing the membrane organization and eventually resulting in haemolysis (Niki et al., 1988).

As expected, no haemolysis occurred during 5 h in the absence of AAPH, (line a of Fig. 4), whereas 50 mM AAPH induced fast haemolysis after an intrinsic inhibition time ($t_{inh}$) of 96 min (line b) produced by endogenous antioxidants in RBC membranes (Esterbauer & Ramos, 1996). All of the CAs effectively prolonged the intrinsic inhibition time and delayed haemolysis occurrence (lines c–k), in contrast with the case absent of exogenous antioxidant (line b). The prolonged time portion relative to $t_{inh}$ can be defined as the effective inhibition time ($t_{eff}$), and comparing the $t_{eff}$ values of CAs (Table 1), gives the antioxidant efficacy order of **B1** > **B2** > Trolox C > **A1** > **A2** > **B4** > **A4** > **B3** > **A3**. It should be pointed out that in this model, **A3** and **B3** bearing no any phenolic hydroxyl groups are also effective antioxidants. The possible reason is that their methoxy groups can be demethylated by demethylase contained in erythrocytes and metabolized to produce phenolic hydroxyl groups as indicated by a study on erythrocyte haemolysis-inhibitory activity of schisandrin A and schisandrin B bearing numerous methoxy groups but no any phenolic hydroxyl groups (Zheng et al., 1997). Noticeably, among the compounds investigated, neither **B2** (as the strongest hydrogen atom donor) nor **A2** (as the best electron donor), instead, **B1** exhibited the longest $t_{eff}$ (57 min) and highest anti-haemolysis activity. This indicates that in addition to the hydrogen atom- and electron-donating abilities, the lipophilicity of compounds is also of great importance for antioxidant efficiency in heterogeneous media such as RBCs, and determines localization of compounds in the cells. Moderate lipophilicity increase of compounds could facilitate their penetration into the membrane and subsequent reaction with the propagating lipid peroxyl radicals within the membranes as indicated by our previous study on antioxidant activity of resveratrol and curcumin analogs (Li et al., 2012; Shang et al., 2010; Tang et al., 2011). Consequently, we acquired the calculated logarithms of octanol/water partition coefficients (*ClogP*) of CAs by using Bio-Loom software (Hansch & Leo, 1995; Selassie, Kapur, Verma, & Rosario, 2005). Not surprisingly, for each group of CAs with the same substituents, inserting an additional double bond in the conjugated chain all results in increase of the *ClogP* values. Intriguingly, there is a clear positive correlation between anti-haemolysis activity ($t_{eff}$) and lipophilicity (*ClogP*) of the four most active compounds (**A1**, **A2**, **B1** and **B2**). In addition, the most lipophilic compounds (**B3** and **B4**) are less active than the four aforementioned compounds due to their decreased hydrogen atom-donating ability. The above results reveal that both of the hydrogen atom-donating ability and lipophilicity of CAs contribute to their anti-haemolysis activity, and the conjugated chain elongation could improve their hydrogen atom-donating ability and

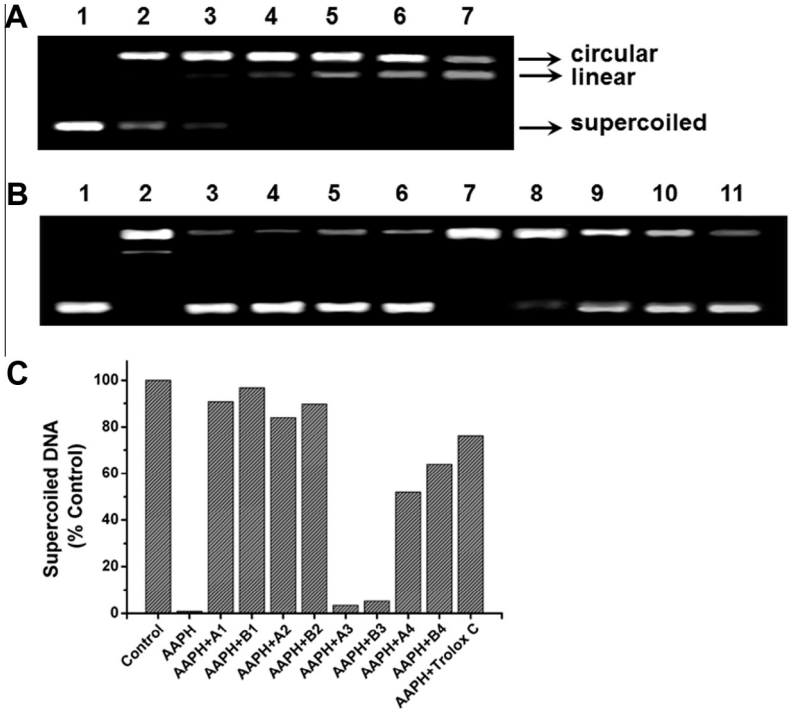



**Fig. 3.** Agarose gel electrophoresis pattern of pBR322 DNA strand breakage after incubation with AAPH in the absence or presence of CAs in PBS (pH 7.4) at 37 °C for 1 h. (A) DNA strand breakage induced by AAPH with different concentrations. Lane 1: control; Lanes 2–7: 1.25, 2.5, 5, 10, 20 and 40 mM AAPH, respectively. (B) Protective effect of CAs (40 μM) on 5 mM AAPH-induced DNA strand breakage. Lane 1: control; Lane 2, AAPH alone; Lanes 3–11: AAPH with **A1**, **B1**, **A2**, **B2**, **A3**, **B3**, **A4**, **B4** and Trolox C, respectively. (C) Semi-quantitative analysis for the protective effects of CAs from (B). DNA damage is represented by the percentage of supercoiled DNA relative to native DNA.

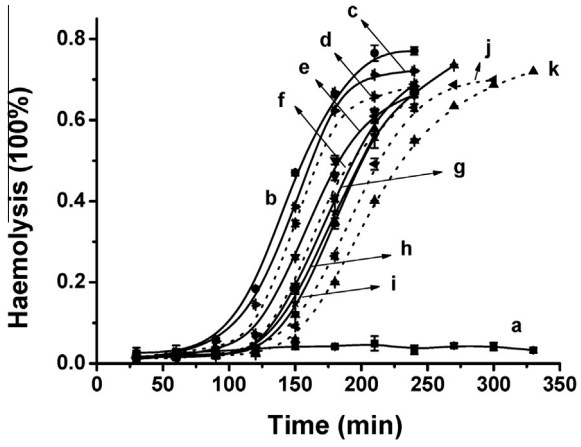



**Fig. 4.** Inhibition of CAs (50 μM) on 50 mM AAPH-induced haemolysis of 5% human RBCs in PBS (pH 7.4) under air atmosphere at 37 °C. (a) Blank (without AAPH); (b) AAPH alone; (c) AAPH + **A3**; (d) AAPH + **B3**; (e) AAPH + **A4**; (f) AAPH + **B4**; (g) AAPH + **A2**; (h) AAPH + Trolox C; (i) AAPH + **A1**; (j) AAPH + **B2**; and (k) AAPH + **B1**.

lipophilicity, resulting in the compounds in series B being more active than the corresponding compounds in series A, and **B1** being the most active among the compounds investigated.

## 4. Conclusion

Four CAs were tailored to improve their antioxidant activity based on the conjugated chain elongation strategy. This strategy could effectively increase the DPPH·-scavenging, DNA strand breakage-inhibiting and anti-haemolysis activities of CAs, due to their increased hydrogen atom-donating ability and lipophilicity. However, this is not a general rule for the electron-donating ability of CAs as exemplified by **B2** in the FRAP and cyclic voltammetry assay. Overall, the current work provides not only an effective strategy of the conjugated chain elongation in improving antioxidant activity of CAs, but also useful information in rationally designing CA-directed antioxidants.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.02.092.

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