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Antioxidant Synergisms between *Cymbopogon citratus* Polyphenols and α-Tocopherol in DPPH Radical-Scavenging Assay

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The synergistic antioxidant effects of polyphenols obtained from *Cymbopogon citratus* Stapf (Gramineae) by bioassay-guided fractionation, including caffeic acid, chlorogenic acid, isoorientin 2''-*O*-rhamnoside and isoorientin, with α-tocopherol were studied using DPPH (1,1-diphenyl-2-picrylhydrazyl) radical-scavenging assay. Combination index (CI) and isobolographic analyses were employed to detect any inducement of the antioxidant activity compared with the individual activities of polyphenols and α-tocopherol. The reaction kinetics were studied by monitoring the consumption of DPPH•, α-tocopherol and *C. citratus* polyphenols. It was found that synergisms could occur between the four respective *C. citratus* polyphenols and α-tocopherol in the radical-scavenging assay, which were dependent on the antioxidants involved and their ratios in the mixtures. Kinetic studies on the antioxidation process revealed that these synergistic effects were associated with the sparing effects of *C. citratus* polyphenols on α-tocopherol, which have been ascribed to the regeneration of α-tocopherol by the polyphenols.

Keywords: *Cymbopogon citratus*, Polyphenols, α-Tocopherol, Radical-scavenging activity, Synergism.

INTRODUCTION

In the past decades, the use of synthetic antioxidants has been restricted owing to their toxicity and undesirable negative effects on human health[1]. As a consequence, growing attention has been focused on searching for alternative antioxidants, especially those antioxidants from natural sources such as foods and traditional herbal medicines as potential nontoxic antioxidants with healthy effects[2-4]. In fact, polyphenols from herbs and spices have shown a defence against oxidative stress from endogenous reactive oxygen species (ROS) and free radicals, which have been a promising source of compounds scrutinized to reduce or substitute butylated derivatives used as antioxidants in food, animal feed, pharmaceutical preparation and cosmetic formulation[5-7].

Antioxidants are present naturally in multiple combinations. For some combinations of natural antioxidants, the total effect is found to be more pronounced than the effect expected from a simple addition of the effects of the individual antioxidants entailing what has been termed antioxidant synergism[8,9]. This synergism is probably due to coadjuvant effects from antioxidants that potentiate each other in multi-component systems. α-Tocopherol, the most common and bioactive form of vitamin E, is one of the most widely used natural antioxidants[10,11]. The synergistic antioxidant effects between plant polyphenols and α-tocopherol have been well documented[1,11-14]. Several of these studies have shown that the regeneration of α-tocopherol by quercetin, catechin and other plant polyphenols produces persistent tocopherol, which is important for the antioxidant synergisms[15].

Cymbopogon citratus (DC.) Stapf (Gramineae) is a native herb from Sri Lanka and South India and also cultivated in other tropical and subtropical countries[16-18]. It is well known for its lemon flavor and has been widely used in food, perfumery, soap, cosmetic, pharmacy and insecticide industries all around its distribution areas[19,20]. Studies on extracts from this plant have proven its anti-inflammatory, hypotensive, vasorelaxting and diuretic activities[21], efficacies against oxidative damage[16,22], as well as cancer chemopreventive capacities[23,24]. Volatile compounds and polyphenols are reputed to be its main biologically active ingredients[17,23,25-27]. As part of our undergoing project towards the discovery of bioactive metabolites from *C. citratus*, four phenolic antioxidants, including caffeic acid, chlorogenic acid, isoorientin 2''-*O*-rhamnoside and isoorientin, were obtained by bioassay-guided fractionation. The aim of this study was to determine whether

the combinations of the four respective phenolic compounds with α-tocopherol would synergistically affect the antioxidant capacity using DPPH radical-scavenging assay. Based on the establishment of systems for which combinations of radical-scavenging antioxidants show significant synergisms, preliminary mechanisms for the synergisms were also discussed.

EXPERIMENTAL

The aerial part of *C. citratus* collected from Yunnan province was purchased from the Yunnan specialty store of Yunxiang (China). The voucher specimen was authenticated by Prof. Yongchuan Zhou and deposited in the Herbarium of Research Centre of Analysis and Test, East China University of Science and Technology. Folin-Ciocalteu reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) and α-tocopherol were purchased from Sigma (USA). HPLC-grade methanol was obtained from J&K Scientific (China). All other chemicals and solvents employed were of analytical grade and used as received. Water was purified through a Master-D UVF laboratory water purification system (Hitech, Shanghai).

Sample preparation: Dried *C. citratus* was pulverized with a grinder (XY-500A, Xingyu, Zhejiang, China). The powder sieved through a 60-mesh sieve was collected and vacuum-sealed in nylon-linear low-density polyethylene zipper pouches and then stored at -20 °C in a refrigerator until use.

Bioassay-guided fractionation of polyphenols from *C. citratus*: Three kilograms of dried *C. citratus* powder was extracted thrice with 95 % ethanol (15 L) under reflux for 3 h. The extract was filtered and evaporated on a rotary evaporator at 40 °C under reduced pressure to remove the ethanol. The aqueous solution was successively partitioned with petroleum ether, dichloromethane, ethyl acetate and water-saturated *n*-butanol, using liquid-liquid extraction method, to obtain five fractions termed petroleum ether fraction (PF), dichloromethane fraction (DF), ethyl acetate fraction (EF), *n*-butanol fraction (BF) and water layer fraction (WF), respectively. Each fraction was then evaporated under vacuum until dryness and weighed to determine the yields, total phenolic contents (TPCs) and antioxidant activities by means of the selected assays.

Two grams of dried *n*-butanol fraction was applied to a column (4 cm × 40 cm) of ADS-7 macroporous absorption resin and the column was sequentially eluted with 10, 30, 70 and 90 % ethanol to yield four sub-fractions (FA: the 10 % ethanol elution; FB: the 30 % ethanol elution; FC: the 70 % ethanol elution; FD: the 90 % ethanol elution). Purification of FC was carried out with a preparative TBE-300B high speed counter-current chromatography (HSCCC, auto Biotech Co., Ltd., Shanghai, China) equipped with a Model TBP5002 constant-flow pump, a Model UV500 monitor and a Model 3057 recorder. The solvent system for the HSCCC separation was ethyl acetate/*n*-butanol/water (1:2:3, v/v/v). It was completely equilibrated in a separatory funnel under room temperature and the two phases were separated and degassed by sonication 40 min prior to use. First, the upper phase (the stationary phase) was pumped into the multilayer-coiled column with an AKTA prime plus system. When the column was totally filled with the upper phase, only the lower phase (the mobile phase) was pumped at a flow rate of 3 mL/min, meanwhile, the HSCCC apparatus was run at a revolution speed of 900 rpm in forward rotation model. After the mobile phase front emerged and the hydrodynamic equilibrium was reached, about 45 % of the stationary phase was retained in the coil and 10 mL of filtered sample solution (500 mg FC, dissolved in 5 mL of the upper phase and 5 mL of the lower phase) was injected into the separation column. After 230 min, the forward rotation mode was converted to the reverse rotation mode. All through the experiment, the separation temperature was controlled at 25 °C. The effluent from the outlet of the column was continuously monitored at 280 nm and the chromatogram was recorded. Each peak fraction (Fig. 1) was manually collected according to the obtained chromatogram and evaporated under vacuum. The residues were further purified by a preparative HPLC system [Elite, Dalian; Equipped with a Sinochrom ODS-BP column (20 × 250 mm; Id: 5 μm; Elite, Dalian); a Model UV23 II monitor and a Model P270 high-pressure constant-flow pump] and recrystallization. Ultimately, four compounds were isolated and identified by means of ESI-MS and NMR spectrometry. HPLC was used for the identification, quantification of the main compounds in the fractions and as purity criteria for the isolated compounds before NMR measurements and antioxidant assays. The separation scheme of the compounds is summarized in Fig. 2.

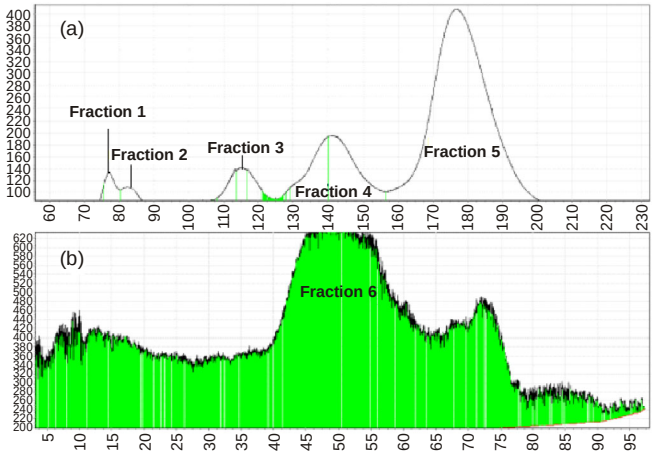

Fig. 1. HSCCC of sub-fraction FC. (a): the HSCCC apparatus was run in forward rotation model; (b): the forward rotation model was converted to the reverse rotation mode 230 min after the sample injection

Determination of total phenolic contents: The total phenolic contents of the *C. citratus* ethanol extract and its fractions were determined spectrophotometrically using Folin-Ciocalteu reagent by the method described by Wang *et al.*[28]. Briefly, 0.5 mL of sample solution (through appropriate dilution to obtain absorbance within the range of the prepared calibration curve) was mixed with 1 mL of Folin-Ciocalteu reagent (diluted 10 times before use). After an interval of 5 min at 30 °C in dark, 2 mL of saturated Na_2CO_3 solution was added and the mixture was allowed to stand for 1 h before the absorbance at 747 nm was measured with an evolution 220 UV-visible spectrophotometer (Thermo Scientific, USA). A calibration curve based on gallic acid was used for conversion of the absorbance to phenol concentration in gallic acid equivalent.

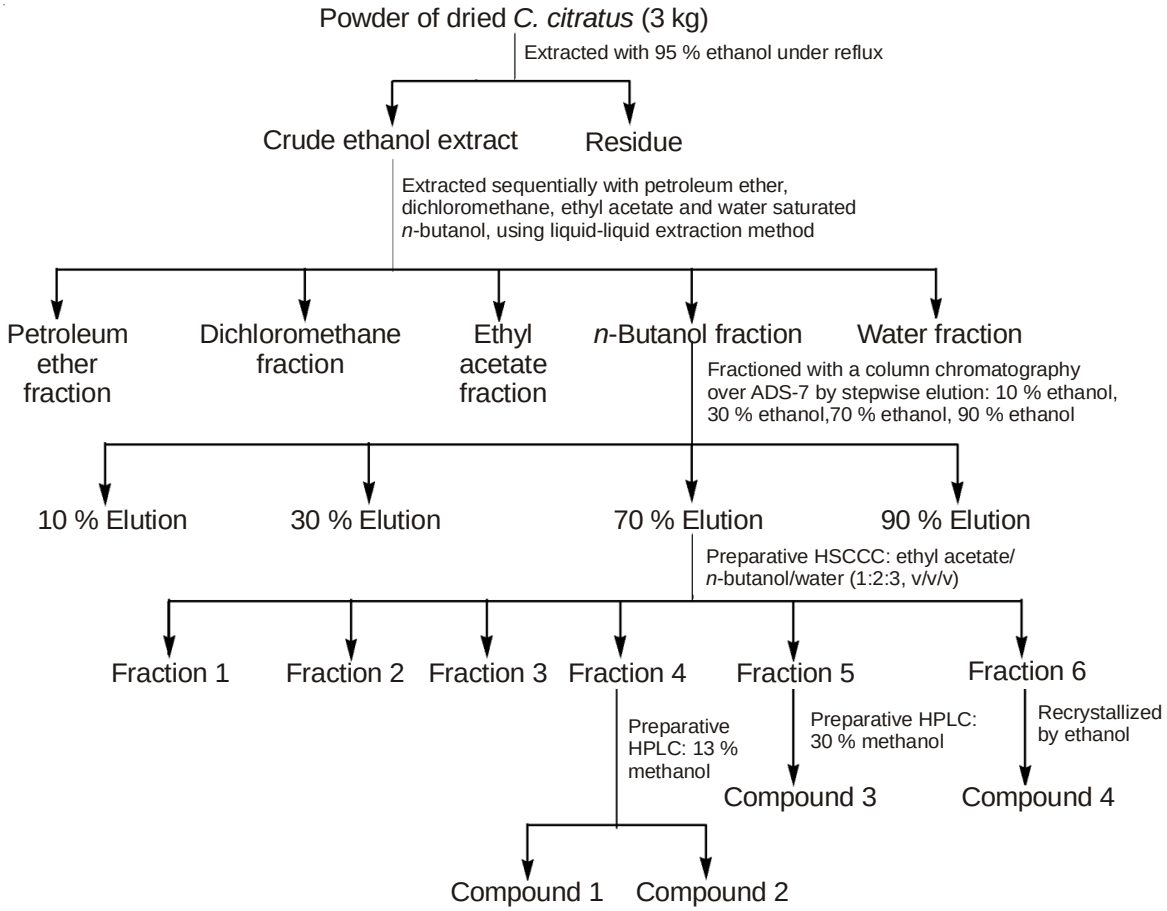

Fig. 2. Isolation scheme of the compounds from *C. citratus*

DPPH radical-scavenging assay: This assay was carried out as previously reported by Romano *et al.*[7] with some modifications. Briefly, 200 µL of DPPH• ethanol solution (120 µM) and 20 µL of sample solution (with different concentrations) were mixed in the well of a 96-well microplate. The plate was then covered and allowed to stand at room temperature for 0.5 h in dark. The absorbance at 492 nm was measured on an enzyme-linked immunosorbent assay (ELISA) Reader (Thermo Scientific Multiskan MK3, USA). Standard curves for DPPH• at 492 and 515 nm were developed in order to convert the values at 492 nm to the corresponding ones at 515 nm. The radical-scavenging activity was calculated as a percentage of DPPH• scavenging or DPPH• remnant according to the following equations:

$$\text{DPPH}^{\bullet}\ \text{Scavenging}\ (\%) = [(A_{control}-A_{sample})/A_{control}] \times 100$$

$$\text{DPPH}^{\bullet}\ \text{Remnant}\ (\%) = [(A_{sample}-A_{control}) \times 100$$

where A_{sample} is the absorbance of 120 µM DPPH• with sample at different concentrations and $A_{control}$ is the absorbance of 120 µM DPPH• without sample added. IC_{50} values, the concentrations required to quench 50 % of the initial DPPH•, were calculated using the SPSS software package to further evaluate the antioxidant activity. All tests and analyses were carried out in triplicate.

Determination of antioxidants by HPLC: The concentrations of antioxidants in the reaction solutions were determined by HPLC, which was performed with an Agilent 1260 HPLC system equipped with a ZORBAX Eclipse XDB-C_{18} column (250 mm × 4.6 mm; Id: 5 µm; Column temperature: 30 °C) by using the following mobile phases at a flow rate of 1 mL/min and the peaks were detected at the following wavelengths. An aliquot of 20 µL of reaction solution was injected. The mobile phases, detection wavelengths and the retention times were as fellows. For α-tocopherol: methanol, 285 nm, 12 min; For caffeic acid: methanol/water (35:65, v/v), 243 nm, 12 min; For chlorogenic acid: methanol/water (25:75, v/v), 330 nm, 6 min; For isoorientin 2"-*O*-rhamnoside: methanol/water (30:70, v/v), 285 nm, 6 min; For isoorientin: methanol/water (40:60, v/v), 285 nm, 5 min. The amount of each antioxidant in the sample solutions was determined by comparing the peak area to those obtained from the standard solutions.

Combination system: Combination index (CI) analysis, a numerical value calculated as described in the following equation, provides a quantitative measure of the extent of drug interaction.

$$CI = C_{A,x}/C_{x,A} + C_{B,x}/C_{x,B}$$

where CI < 1, = 1 and > 1 indicate synergism, addition and antagonism, respectively. $C_{A,x}$ and $C_{B,x}$ are the concentrations of drugs A and B used in combination to achieve an inhibition effect of x%. $C_{x,A}$ and $C_{x,B}$ are the concentrations for single agents to achieve the same effect.

Isobolographic analysis also evaluates the nature of interaction of two drugs, *i.e.*, drug A and drug B, as follows. First, the concentrations of drugs A and B required to produce a defined single-agent effect (*e.g.*, IC_{50}), when used as single agents, are placed on the x and y axes in a two-coordinate

plot, corresponding to (C_A, 0) and (0, C_B), respectively. The line connecting these two points is the line of equivalent addition. Second, the concentrations of the two drugs used in combination to achieve the same effect, denoted as (c_A, c_B), are placed in the same plot. Synergism, addition and antagonism are indicated when (c_A, c_B) is located below, on and above the line of equivalent addition, respectively.

In the present study, both combination index and isobolographic analyses at IC_{50} were employed to show the interaction types between the respective *C. citratus* polyphenols and α-tocopherol according to the method described by Zhao *et al.*[29]. And a fix-fraction design was used with 13 different mole ratios (100:1, 50:1, 20:1, 8:1, 4:1, 2:1, 1:1, 1:2, 1:4, 1:8, 1:20, 1:50 and 1:100) of the respective *C. citratus* phenolic compounds to α-tocopherol.

Statistical analysis: All experimental data were analyzed statistically using the SPSS software package. The results were given as the mean ± standard deviation (SD). The significance of differences between groups was evaluated with the analysis of variance, followed by Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Bioassay-guided isolation of polyphenols from *C. citratus*: *C. citratus* extracts obtained with 95 % ethanol were rich in the content of phenolic compounds. Table-1 shows the yields, total phenolic contents and DPPH radical-scavenging activities of various solvent-extracted fractions from the ethanol extract. Among these fractions, water layer fraction had the highest yield, while ethyl acetate fraction had the lowest. The total phenolic contents of the extracts decreased in the order of BF > DF > EF > WF > PF and the highest radical-scavenging effect was also obtained in BF with the lowest IC_{50} value in the DPPH radical-scavenging assay. Accordingly, BF was chosen as research object. To discover the constituents of the phenolic compounds of BF, this fraction was fractionated into four sub-fractions (FA, FB, FC and FD) by ADS-7 macroporous adsorbent resin column with ethanol/water gradient. The total phenolic contents and DPPH radical-scavenging effects of the sub-fractions both decreased in the order of FC > FD > FB > FA. Further purification of FC, which exhibited the highest total phenolic content and the strongest antioxidant activity with the lowest IC_{50} value in the DPPH radical-scavenging assay among the four sub-fractions (Table-1), was conducted sequentially with HSCCC, preparative-HPLC and recrystallization. From ESI-MS, ^1H and ^{13}C NMR data, four purified compounds were identified as caffeic acid (**1**), chlorogenic acid (**2**), isoorientin 2''-*O*-rhamnoside (**3**) and isoorientin (**4**), respectively. The spectral data of these compounds, which agreed with the earlier published data[30-33], are given below:

Caffeic acid (1): Yellow crystalline powder; ESI-MS *m/z* 181 $[M + H]^+$; ^1H NMR (400 MHz, CD_3OD, ppm) δ: 7.49 (1H, d, *J* = 16.00 Hz, 8-H), 7.03 (1H,d, *J* = 1.96 Hz, 2-H), 7.01 (1H, d, *J* = 2.00 Hz, 6-H), 6.84 (1H, d, *J* = 8.24 Hz, 5-H); 6.25 (1H, d, *J* = 15.96 Hz, 7-H). ^{13}C NMR (100 MHz, CD_3OD, ppm) δ: 171.04 (C-9), 149.44 (C-4), 147.05 (C-3), 143.83 (C-7), 127.80 (C-1), 122.89 (C-5), 116.49 (C-2), 115.53 (C-6), 115.08 (C-8).

Chlorogenic acid (2): White crystalline powder; ESI-MS *m/z* 355 $[M + H]^+$; ^1H NMR (400 MHz, CD_3OD, ppm) δ: 7.41 (1H, d, *J* = 16.00 Hz, 7'-H), 6.99 (1H, brs, 2'-H), 6.93 (1H, brs, 6'-H), 6.78 (1H, d, *J* = 8.00 Hz, 5'-H), 6.15 (1H, d, *J* = 16.00 Hz, 8'-H), 5.15 (1H, m, 3-H), 4.12 (1H, m, 5-H), 3.75 (1H, m, 4-H), 2.03 (4H, m, 2-H, 6-H). ^{13}C NMR (100 MHz, CD_3OD, ppm) δ: 177.08 (C-7), 168.59 (C-9'), 147.04 (C-4'), 146.15 (C-7'), 144.16 (C-3'), 126.84 (C-1'), 122.68 (C-6'), 116.08 (C-5'), 115.04 (C-2'), 114.25 (C-8'), 74.81 (C-1), 71.30 (C-3), 70.57 (C-4), 69.08 (C-5), 36.44 (C-6), 36.36 (C-2).

Isoorientin 2''-*O*-rhamnoside (3): Yellow powder; ESI-MS *m/z* 595 $[M + H]^+$; ^1H NMR (400 MHz, DMSO-d_6, ppm) δ: 13.56 (1H,s, OH), 10.60 (1H, s, OH), 9.93 (1H, s, OH), 9.42 (1H, s, OH), 6.88 (1H, d, *J* = 8.00 Hz, 5'-H), 6.67 (1H, s, 3-H), 6.46 (1H, s, 8-H); 3.15 (3H, s, OCH_3). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 163.44 (C-2), 102.60, 102.80 (C-3), 181.62, 181.98 (C-4), 159.86, 161.21 (C-5), 108.56, 108.93 (C-6), 162.52, 163.44 (C-7), 92.78, 94.09 (C-8), 156.20, 156.34 (C-9), 103.13, 103.70 (C-10), 121.33 (C-1'), 113.15 (C-2'), 145.72 (C-3'), 149.67 (C-4'), 116.04 (C-5'), 118.86 (C-6'), 71.51, 71.28 [C-1 (Glc)], 75.70, 74.57 [C-2 (Glc)], 79.96, 79.57 [C-3 (Glc)], 70.30, 70.56 [C-4 (Glc)], 81.42 [C-5 (Glc)], 61.68, 61.18 [C-6 (Glc)], 100.64, 100.33 [C-1 (Rha)], 70.30, 70.56 [C-2 (Rha)], 70.90, 71.28 [C-3 (Rha)], 70.30, 70.56 [C-4 (Rha)], 68.25 [C-5 (Rha)], 17.50, 17.74 [C-4 (Rha)].

Isoorientin (4): Yellow amorphous powder; ESI-MS *m/z* 449 $[M + H]^+$; ^1H NMR (400 MHz, DMSO-d_6, ppm) δ:13.58

TABLE-1
YIELDS, TOTAL PHENOLIC CONTENTS AND DPPH RADICAL-SCAVENGING ACTIVITIES OF FRACTIONS FROM THE ETHANOL EXTRACT OF *C. citratus*

Fraction	Yield[a] (mg/g)	TPC[b] (mg/g)	IC_{50}/DPPH$^•$ (μg/mL)
Petroleum ether fraction	30.15 ± 3.89	13.76 ± 1.08	173.38 ± 3.05
Dichloromethane fraction	16.80 ± 2.22	25.24 ± 1.46	320.75 ± 4.70
Ethyl acetate fraction	7.87 ± 0.93	21.31 ± 1.23	104.03 ± 6.25
n-Butanol fraction	39.18 ± 3.46	37.25 ± 2.19	93.84 ± 3.63
Water layer fraction	134.22 ± 4.10	16.23 ± 1.34	782.21 ± 8.82
Fraction-A (FA)	7.26 ± 0.67	22.72 ± 1.72	112.16 ± 6.42
Fraction-B (FB)	11.55 ± 1.28	33.74 ± 2.29	89.15 ± 4.94
Fraction-C (FC)	14.61 ±1.34	46.21 ± 2.52	45.37 ± 2.48
Fraction-D (FD)	5.80 ± 0.54	36.45 ± 2.17	67.41 ± 3.16
V_E[c]	NA[d]	NA[d]	130.03 ± 3.89

Results are presented as means ± standard deviations (*n* = 3); [a]Yield: calculated as the weight (mg) of each fraction/the weight (g) of dried *C. citratus*; [b]TPC: total phenolic content, expressed as gallic acid equivalents; [c]V_E: α-tocopherol; [d]NA: not analyzed

(1H, s, 5-OH),10.61 (1H, s, 7-OH), 9.95 (1H, s, 4'-OH), 9.44 (1H, s, 3'-OH), 7.43 (2H, m, 6'-H), 6.89 (1H, d, J = 8.00 Hz, 5'-H), 6.69 (1H, s, 3-H), 6.48 (1H, s, 8-H); ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 181.84 (C-4), 163.58 (C-2), 163.19 (C-7), 160.66 (C-5), 156.13 (C-9), 149.64 (C-4'), 145.69 (C-3'), 121.37 (C-1'), 118.94 (C-6'), 115.99 (C-5'), 113.25 (C-2'), 108.83 (C-6), 103.35 (C-10), 102.76 (C-3), 93.40 (C-8), 81.58 (C-5'), 78.90 (C-3'), 72.97 (C-1'), 70.58 (C-2'), 70.09 (C-4'), 61.46 (C-6'). The structures of these compounds are shown in Fig. 3.

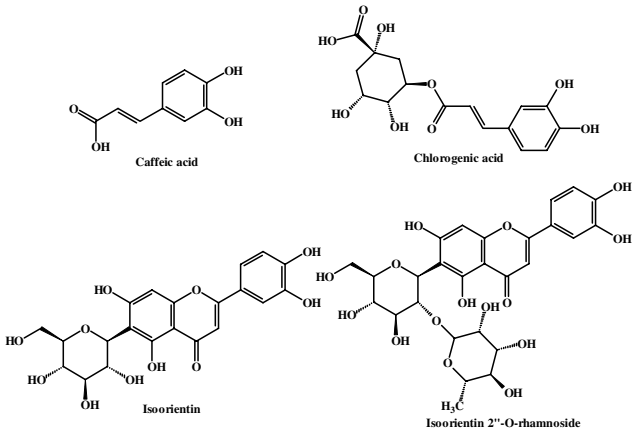

Fig. 3. Structures of the compounds isolated from *C. citratus*

Antioxidant capacities of phenolic compounds: The respective antioxidant activities of the isolated phenolics were determined by the DPPH radical-scavenging assay. The experimental results indicated that all tested compounds produced dose-dependent scavenging effects against DPPH•. The highest DPPH• scavenging effect was obtained in isoorientin with the lowest IC_{50} value of 0.166 mM, followed by isoorientin 2''-*O*-rhamnoside (IC_{50} = 0.225 mM), caffeic acid (IC_{50} = 0.272 mM), α-tocopherol (the positive control, IC_{50} = 0.302 mM) and chlorogenic acid (IC_{50} = 0.365 mM). Therefore, these four phenolic compounds included in *C. citratus* are potent free radical scavengers and may be considered an excellent source of antioxidants.

Antioxidant capacities of polyphenols coupled with α-tocopherol: The antioxidant capacities of the respective polyphenols combined with α-tocopherol at different mole ratios, including 100:1, 50:1, 20:1, 8:1, 4:1, 2:1, 1:1, 1:2, 1:4, 1:8, 1:20, 1:50 and 1:100 (each phenolic compound to α-tocopherol), were also analyzed based on the DPPH radical-scavenging assay, in order to assess how their interactions contribute to the total antioxidant capacities in the binary mixtures. combination index values and isobol graphs were therefore generated (Fig. 4).

As shown in Table-2 and Fig. 4, the interactions of isoorientin and α-tocopherol for all tested ratios were statistically

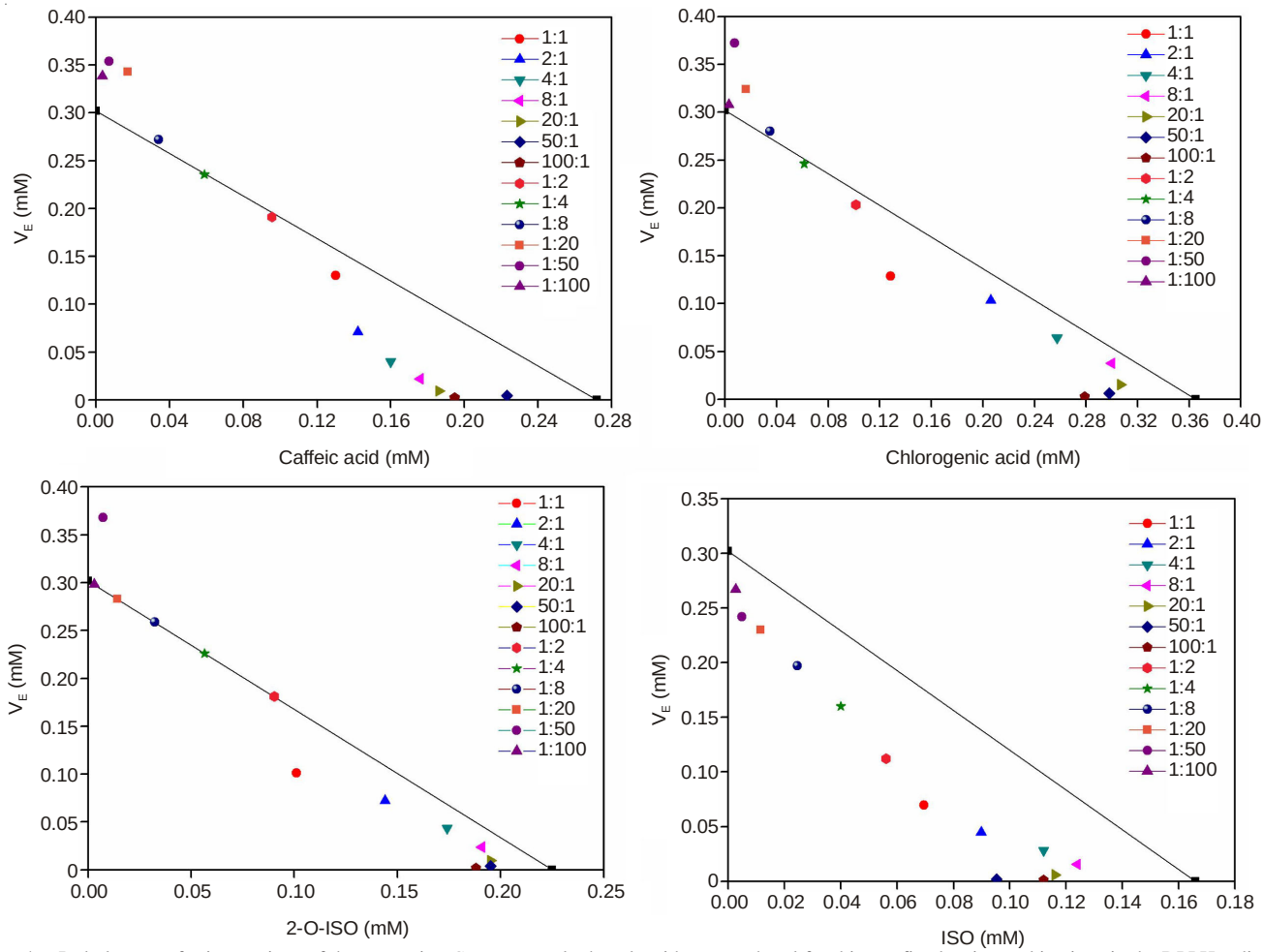

Fig. 4. Isobolograms for interactions of the respective *C. citratus* polyphenols with α-tocopherol for thirteen fixed-ratio combinations in the DPPH radical-scavenging assay. V_E: α-tocopherol; 2-O-ISO: isoorientin 2''-*O*-rhamnoside; ISO: isoorientin

TABLE-2
CI ANALYSES OF THE INTERACTIONS OF CAFFEIC ACID, CHLOROGENIC ACID, ISOORIENTIN 2"-*O*-RHAMNOSIDE AND ISOORIENTIN IN BINARY MIXTURES WITH α-TOCOPHEROL DURING THE DPPH RADICAL-SCAVENGING ASSAY

Interacting compounds	Dose ratio	IC_{50} (mM)		CI	Interaction
		Polyphenol	V_E[a]		
Caffeic acid: V_E[a]	1:0	0.272 ± 0.019	–	–	–
Caffeic acid: V_E[a]	100:1	0.195 ± 0.016	0.00195 ± 0.00015	0.72	Synergism
Caffeic acid: V_E[a]	50:1	0.223 ± 0.019	0.00446 ± 0.00037	0.83	Synergism
Caffeic acid: V_E[a]	20:1	0.186 ± 0.012	0.00930 ± 0.00077	0.71	Synergism
Caffeic acid: V_E[a]	8:1	0.176 ± 0.009	0.0220 ± 0.0016	0.72	Synergism
Caffeic acid: V_E[a]	4:1	0.160 ± 0.013	0.0400 ± 0.0033	0.72	Synergism
Caffeic acid: V_E[a]	2:1	0.142 ± 0.012	0.0711 ± 0.0052	0.76	Synergism
Caffeic acid: V_E[a]	1:1	0.130 ± 0.008	0.130 ± 0.006	0.90	Synergism
Caffeic acid: V_E[a]	1:2	0.0956 ± 0.0062	0.191 ± 0.011	1.00	Addition
Caffeic acid: V_E[a]	1:4	0.0589 ± 0.0031	0.236 ± 0.016	1.00	Addition
Caffeic acid: V_E[a]	1:8	0.0340 ± 0.0029	0.272 ± 0.015	1.00	Addition
Caffeic acid: V_E[a]	1:20	0.0172 ± 0.0014	0.343 ± 0.022	1.20	Antagonism
Caffeic acid: V_E[a]	1:50	0.00708 ± 0.00043	0.354 ± 0.029	1.19	Antagonism
Caffeic acid: V_E[a]	1:100	0.00338 ± 0.00029	0.338 ± 0.031	1.13	Antagonism
Caffeic acid: V_E[a]	0:1	–	0.302 ± 0.022	–	–
Chlorogenic acid: V_E[a]	1:0	0.365 ± 0.021	–	–	–
Chlorogenic acid: V_E[a]	100:1	0.279 ± 0.019	0.00279 ± 0.00018	0.77	Synergism
Chlorogenic acid: V_E[a]	50:1	0.298 ± 0.013	0.00597 ± 0.00043	0.84	Synergism
Chlorogenic acid: V_E[a]	20:1	0.307 ± 0.012	0.0153 ± 0.00092	0.89	Synergism
Chlorogenic acid: V_E[a]	8:1	0.300 ± 0.011	0.0375 ± 0.0022	0.95	Synergism
Chlorogenic acid: V_E[a]	4:1	0.257 ± 0.012	0.0644 ± 0.0036	0.92	Synergism
Chlorogenic acid: V_E[a]	2:1	0.206 ± 0.009	0.103 ± 0.0073	0.91	Synergism
Chlorogenic acid: V_E[a]	1:1	0.129 ± 0.007	0.129 ± 0.008	0.83	Synergism
Chlorogenic acid: V_E[a]	1:2	0.102 ± 0.008	0.204 ± 0.014	0.95	Synergism
Chlorogenic acid: V_E[a]	1:4	0.0601 ± 0.0032	0.241 ± 0.019	1.00	Addition
Chlorogenic acid: V_E[a]	1:8	0.0350 ± 0.0021	0.280 ± 0.013	1.00	Addition
Chlorogenic acid: V_E[a]	1:20	0.0162 ± 0.0011	0.324 ± 0.013	1.12	Antagonism
Chlorogenic acid: V_E[a]	1:50	0.00745 ± 0.00049	0.372 ± 0.020	1.25	Antagonism
Chlorogenic acid: V_E[a]	1:100	0.00308 ± 0.00040	0.308 ± 0.014	1.00	Addition
Chlorogenic acid: V_E[a]	0:1	–	0.302 ± 0.022	–	–
2-O-ISO[b]: V_E[a]	1:0	0.225 ± 0.017	–	–	–
2-O-ISO[b]: V_E[a]	100:1	0.188 ± 0.013	0.00188 ± 0.00016	0.84	Synergism
2-O-ISO[b]: V_E[a]	50:1	0.195 ± 0.009	0.00390 ± 0.00042	0.88	Synergism
2-O-ISO[b]: V_E[a]	20:1	0.195 ± 0.011	0.00976 ± 0.00063	0.90	Synergism
2-O-ISO[b]: V_E[a]	8:1	0.191 ± 0.011	0.0239 ± 0.0014	0.93	Synergism
2-O-ISO[b]: V_E[a]	4:1	0.174 ± 0.009	0.0436 ± 0.0025	0.92	Synergism
2-O-ISO[b]: V_E[a]	2:1	0.144 ± 0.006	0.0721 ± 0.0048	0.88	Synergism
2-O-ISO[b]: V_E[a]	1:1	0.101 ± 0.006	0.101 ± 0.008	0.78	Synergism
2-O-ISO[b]: V_E[a]	1:2	0.0904 ± 0.0044	0.181 ± 0.008	1.00	Addition
2-O-ISO[b]: V_E[a]	1:4	0.0565 ± 0.0042	0.226 ± 0.013	1.00	Addition
2-O-ISO[b]: V_E[a]	1:8	0.0323 ± 0.0034	0.259 ± 0.019	1.00	Addition
2-O-ISO[b]: V_E[a]	1:20	0.0142 ± 0.0012	0.283 ± 0.018	1.00	Addition
2-O-ISO[b]: V_E[a]	1:50	0.00735 ± 0.00039	0.368 ± 0.017	1.25	Antagonism
2-O-ISO[b]: V_E[a]	1:100	0.00298 ± 0.00037	0.298 ± 0.020	1.00	Addition
2-O-ISO[b]: V_E[a]	0:1	–	0.302 ± 0.022	–	–
ISO[c]: V_E[a]	1:0	0.166 ± 0.010	–	–	–
ISO[c]: V_E[a]	100:1	0.112 ± 0.008	0.00112 ± 0.00012	0.68	Synergism
ISO[c]: V_E[a]	50:1	0.0952 ± 0.007	0.00191 ± 0.00020	0.58	Synergism
ISO[c]: V_E[a]	20:1	0.116 ± 0.010	0.00582 ± 0.00062	0.72	Synergism
ISO[c]: V_E[a]	8:1	0.124 ± 0.008	0.0155 ± 0.0016	0.80	Synergism
ISO[c]: V_E[a]	4:1	0.112 ± 0.008	0.0281 ± 0.0029	0.77	Synergism
ISO[c]: V_E[a]	2:1	0.0898 ± 0.010	0.0449 ± 0.0062	0.69	Synergism
ISO[c]: V_E[a]	1:1	0.0696 ± 0.0060	0.0696 ± 0.0073	0.65	Synergism
ISO[c]: V_E[a]	1:2	0.0561 ± 0.0058	0.112 ± 0.012	0.71	Synergism
ISO[c]: V_E[a]	1:4	0.0400 ± 0.0066	0.160 ± 0.018	0.77	Synergism
ISO[c]: V_E[a]	1:8	0.0246 ± 0.0024	0.197 ± 0.021	0.80	Synergism
ISO[c]: V_E[a]	1:20	0.0115 ± 0.0012	0.230 ± 0.022	0.83	Synergism
ISO[c]: V_E[a]	1:50	0.00484 ± 0.00065	0.242 ± 0.025	0.80	Synergism
ISO[c]: V_E[a]	1:100	0.00267 ± 0.00027	0.267 ± 0.025	0.90	Synergism
ISO[c]: V_E[a]	0:1	–	0.302 ± 0.029	–	–

Results are presented as means ± standard deviations (n = 3); [a]V_E: α-tocopherol; [b]2-O-ISO: isoorientin 2"-*O*-rhamnoside; [c]ISO: isoorientin

synergistic, while synergism seems to require the presence of caffeic acid, chlorogenic acid and isoorientin 2''-*O*-rhamnoside with a concentration considerably higher than that of α-tocopherol. Remarkably, when the proportions of the three polyphenols to α-tocopherol were 100:1, 50:1, 20:1, 8:1, 4:1, 2:1 and 1:1, much lower combination index values were obtained and the experimental $IC_{50, mix}$ values fell far lower the line of equivalent addition. Whereas visible antagonistic interactions were mainly observed between caffeic acid, chlorogenic acid, or isoorientin 2''-*O*-rhamnoside and α-tocopherol at the fixed ratios of 1:20, 1:50 and 1:100, since the combination index values were larger than 1 and the experimental $IC_{50, mix}$ values were significantly above the line of equivalent addition in the isobolograms. And the remaining combinations between the three respective phenolic compounds and α-tocopherol at the fixed ratios of 1:2, 1:4 and 1:8 primarily showed pure additivity tendencies, since the combination index values were equal to 1 and the experimental $IC_{50, mix}$ values were just on the line of equivalent addition in the isobolograms.

According to above analysis, caffeic acid, chlorogenic acid, isoorientin 2''-*O*-rhamnoside and isoorientin could act synergisticly with α-tocopherol in the DPPH radical-scavenging assay and these synergisms were dependent on the antioxidants involved and the ratios at which they are mixed. Table-3 shows the proportions of hydroxyl in the four polyphenols. Among these compounds, isoorientin, which has the highest proportion of hydroxyl, showed the strongest synergistic effect with α-tocopherol, since their interactions for all tested ratios were statistically synergistic and the lowest combination index value 0.58 was observed for their combinations. At the molar ratio of 1:1, the synergistic effects of the four polyphenols combined with α-tocopherol decreased in the order of isoorientin > isoorientin 2''-*O*-rhamnoside > chlorogenic acid > caffeic acid, which was in accordance with the decreased order of hydroxyl proportions included in the four polyphenols. These results indicated that the differences in antioxidant synergisms between the four *C. citratus* polyphenols and α-tocopherol could be ascribed to the proportions of hydroxyl contained in the polyphenols.

In order to obtain additional information about the synergistic interactions in the binary mixtures, the decay kinetics of DPPH• that follow the addition of the respective phenolic compounds combined with α-tocopherol at the fixed molar ratio of 1:1 were studied. Thus, to the DPPH• ethanol solution (120 μM), the polyphenols and α-tocopherol alone, or combined mixtures of the respective polyphenols and α-tocopherol were added. After the reaction mixtures were kept in dark for 0.5 min, time courses of the DPPH• consumptions were determined by the decreases of absorbance at 510 nm. As shown in Fig. 5, the observed DPPH• remnant percentages in the presence

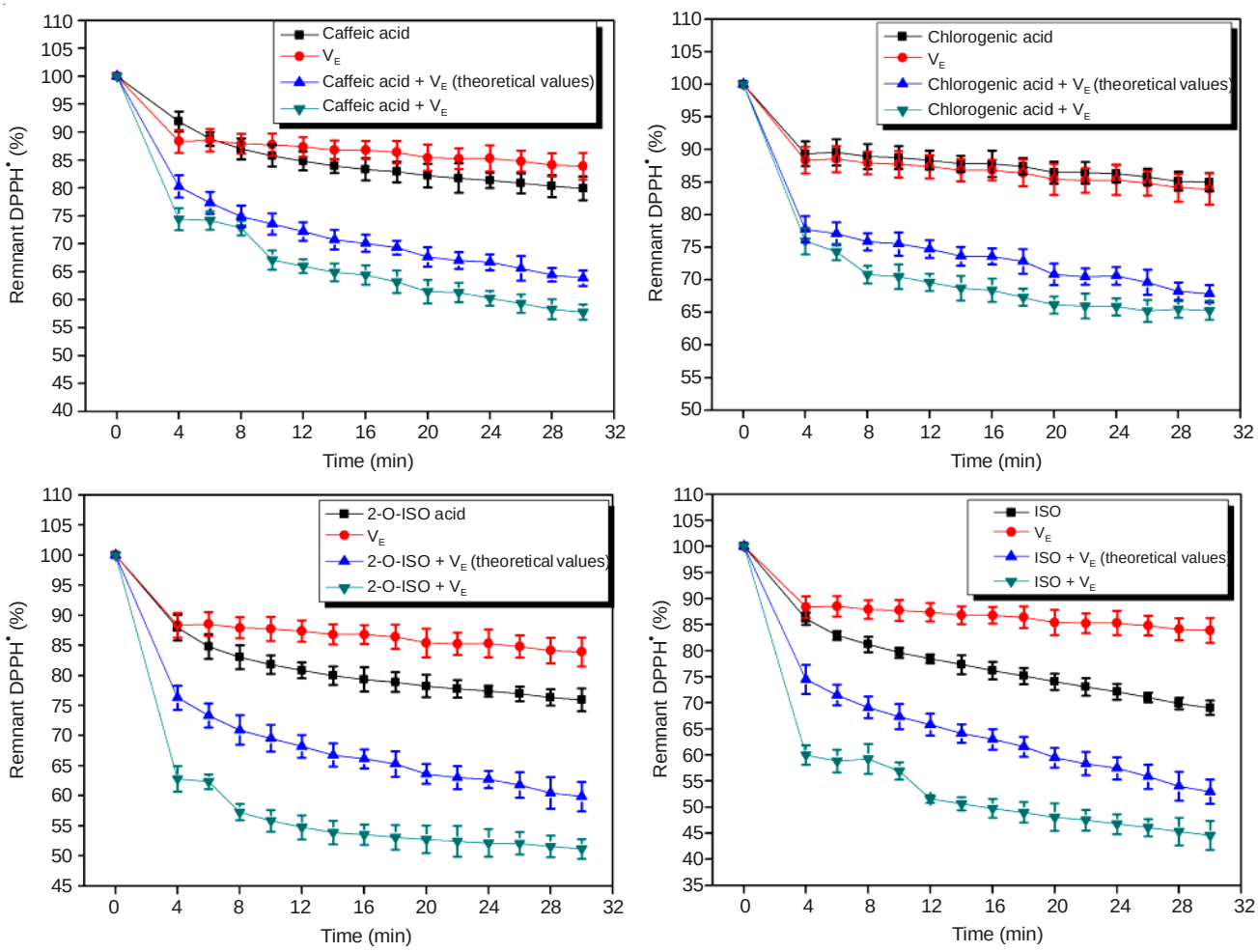

Fig. 5. Time courses of DPPH• depletion in the presence of the *C. citratus* polyphenols combined with α-tocopherol. VE: α-tocopherol; 2-*O*-ISO: isoorientin 2''-*O*-rhamnoside; ISO: isoorientin

TABLE-4
TIME COURSES OF α-TOCOPHEROL CONSUMPTION IN THE PRESENCE OF THE *C. citratus* POLYPHENOLS DURING THE DPPH RADICAL-SCAVENGING ASSAY

Samples	V_E[a] concentration (mM)					
	0 min	2 min	7 min	15 min	25 min	32 min
V_E[a]	0.0084	0.00159	0.000336	0.00028	0.00014	0
ISO[b] + V_E[a]	0.0084	0.00191	0.00087	0.00065	0.00038	0
Increase percentage (%)	0	20.1	158.9	132.1	171.4	0
V_E[a]	0.0089	0.00121	0.00036	0.00025	0.00016	0
2-O-ISO[c] + V_E[a]	0.0089	0.00142	0.00078	0.00055	0.00037	0
Increase percentage (%)	0	17.4	116.7	120.0	131.3	0
V_E[a]	0.013	0.00179	0.00052	0.00047	0.00026	0
Chlorogenic acid + V_E[a]	0.013	0.00182	0.00092	0.00099	0.00059	0
Increase percentage (%)	0	1.7	76.9	110.6	126.9	0
V_E[a]	0.013	0.00179	0.00052	0.00047	0.00026	0
Caffeic acid + V_E[a]	0.013	0.000194	0.000723	0.000681	0.00038	0
Increase percentage (%)	0	8.4	39.0	44.9	46.2	0

Results are presented as means (n = 3); [a]V_E: α-tocopherol; [b]ISO: isoorientin; [c]2-*O*-ISO: isoorientin 2''-*O*-rhamnoside

TABLE-3
PROPORTION OF HYDROXYL IN THE *C. citratus* POLYPHENOLS

C. citratus polyphenol	Hydroxyl number	Hydroxyl proportion
ISO[a]	8	0.304
2-O-ISO[b]	10	0.286
Chlorogenic acid	5	0.24
Caffeic acid	2	0.189

[a]ISO: isoorientin; [b]2-*O*-ISO: isoorientin 2''-*O*-rhamnoside

of the respective phenolic compounds combined with α-tocopherol were obviously lower than those theoretical values (predicted sum of the DPPH• remnant percentages of the individual polyphenols and α-tocopherol), which also indicated the evident synergistic effects between these compounds and α-tocopherol. Notablely, the individual phenolic compounds or α-tocopherol reacted rapidly with DPPH• before it reached a steady state at about 4 min, whereas the tested combinations showed first a fast reduction of DPPH• with a short plateau that was immediately followed by another slow DPPH• reduction starting after 8 min. These results further indicated that the antioxidant efficiency of the combinations involved the combinatory effect of the two individual component constituents[7].

Preventive effects of the *C. citratus* polyphenols on the decomposition of α-tocopherol: Previous studies have shown the sparing effects of plant polyphenols on α-tocopherol, which have been ascribed to the regeneration of α-tocopherol by the polyphenols, in effect leading to antioxidant synergisms[8]. In current study, the interactions between the four *C. citratus* polyphenols and α-tocopherol were further studied by observing the preventive effects of the *C. citratus* polyphenols on the decomposition of α-tocopherol during the DPPH radical-scavenging assay. As a result, α-tocopherol was decreased rapidly in the initial stage and completely disappeared after 7 min in the reaction solution of α-tocopherol without added *C. citratus* polyphenols. When combined with caffeic acid, chlorogenic acid, isoorientin 2''-*O*-rhamnoside, or isoorientin, α-tocopherol was also decomposed rapidly from the start, but its decomposition was moderately prevented between 2-32 min, during which the concentrations of α-tocopherol were obviously increased compared with when α-tocopherol was added alone. At 25 min, the increase percentages of α-tocopherol concentration by isoorientin, isoorientin 2''-*O*-rhamnoside, chlorogenic acid and caffeic acid reached maximum, which were 171.4, 131.3, 126.9 and 46.2 %, respectively (Table-4). The decreased order was also in accordance with the decreased order of hydroxyl proporptions contained in the polyphenols. Results also showed that the concentrations of the four *C. citratus* polyphenols were basically invariant in the experiments with or without α-tocopherol added. The antioxidant activities of phenolics are ascribed to the existence of substituted hydroxyls[34]. All these results indicated that α-tocopherol was to some extent protected from decomposition by the four respective polyphenols, which was correlated with the hydroxyl proportions contained in the polyphenols. As the previously reported polyphenols, the synergistic effects between the four *C. citratus* polyphenols and α-tocopherol can be explained by the regeneration of α-tocopherol by the polyphenols[12,13]

Conclusion

In conclusion, this work demonstrated that the types of interactions (synergism, addition and antagonism) exhibited by the phenolic antioxidants from *C. citratus*, including caffeic acid, chlorogenic acid, isoorientin 2''-*O*-rhamnoside and isoorientin, with α-tocopherol in DPPH radical-scavenging assay were dependent on not only the antioxidants involved, but also their ratios in the mixtures. The most important finding here was that several combinations of the respective polyphenols and α-tocopherol with certain ratios showed evident synergistic effects. The enhanced antioxidant efficacies were associated with the sparing effects of *C. citratus* polyphenols on α-tocopherol, which have been ascribed to the regeneration of α-tocopherol by the polyphenols.

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