Fitoterapia 81 (2010) 205–209

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Reactive oxygen species scavenging activities and inhibition on DNA oxidative damage of dimeric compounds from the oxidation of (−)-epigallocatechin-3-O-gallate

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ARTICLE INFO

Article history:
Received 12 February 2009
Accepted in revised form 3 September 2009
Available online 12 September 2009

Keywords:
Tea
Dehydrotheasinensin A
Theacitrin C
Antioxidant activity
Chemiluminescence

ABSTRACT

The dimeric catechins dehydrotheasinensin A (2) and theacitrin C (3) were prepared from the oxidation of (−)-epigallocatechin-3-O-gallate (EGCG, 1), and their antioxidant activity was investigated using a chemiluminescence (CL) method *in vitro*. Both compounds showed significant inhibitory effects on reactive oxygen species ($O_2^{\cdot-}$, H_2O_2 and •OH) and DNA oxidative damage, with 2 being more potent than 3 and EGCG itself.

1. Introduction

(−)-Epigallocatechin-3-O-gallate (EGCG,1) is the major polyphenol of green tea leaves, accounting for ca. 5.0%–10% of its dry biomass, and ca. 40%–55% of the total catechins contents[1,2]. Green tea has been shown to possess antixoidant [3,4], hepatoprotective [5], chemopreventive [6] and anticarcinogenicactivity[7,8], making it an attractive topic for biomedical research. EGCG is the most active polyphenol of green tea, and is primarily responsible for its beneficial properties [9]. EGCG displays potent antioxidant properties [10] related to the presence of two galloyl (adjacent triphenolic motif) moieties, whose presence is critical for bioactivity [11,12].On the other hand, over the past few years there has been growing evidence that also the oxidized products of tea catechins show bioactivity [13–15]. To give a contributed to this debated issue, we have compared the antioxidative properties of EGCG with those of two of its oxidative dimers in assay of reactive oxygen species (ROS) scavenging activities and inhibition of DNA damage.

2. Experimental

2.1. General

UV spectra were obtained on a Shimadzu UV 1700 spectrophotometer. ^1H spectra and ^{13}C NMR spectra were recorded, respectively with a Bruker DMX-500 NMR spectrometer (Bruker, German) in methanol-d_4 (Sigma Aldrich Co.), operating at 500 MHz for ^1H and 300 MHz for ^{13}C NMR spectroscopy. And tetramethylsilane was used as internal standard. High-performance liquid chromotography–diode array detector–electrospray–ionization mass spectroscopy (HPLC–DAD–ESI/MS) analyses were performed on an Agilent 1100 series HPLC system (Agilent Technologies, Ville St-Laurent, Canada), equipped with an auto-sampler, binary pump, degasser, and a DAD connected directly to the mass detector (Agilent G2440A SD-Trap-XCT ion trap mass spectrometer) with an ESI source. Analytical HPLC was performed on a Luna C_{18} column

Abbreviations: EGCG, (−)-epigallocatechin-3-O-gallate; CL, chemiluminescence; ROS, reactive oxygen species; $O_2^{\cdot-}$, superoxide anion radical; •OH, hydroxyl radical; H_2O_2, hydrogen peroxide; HPLC–DAD–ESI/MS, high-performance liquid chromotography–diode array detector–electro spray-ionization mass spectroscopy; CBSS, carbonic acid-buffered saline solution; PBS, phosphate-buffered saline; ESR, electron spin resonance.

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doi:10.1016/j.fitote.2009.09.004

(5 μm, 250×4.6 mm i.d., Phenomenex, Torrance, CA, USA) at column temperature 40 °C with gradient elution from 8% to 31% (50 min) and 31% to 8% (3 min) of acetonitrile in 2.0% acetic acid at a flow rate of 0.8 ml/min, and UV–vis absorption spectra was recorded online at 280 nm. The MS parameters were as follows: nebulizer pressure, 30.0 psi (N_2); dry gas, N_2 (8.0 l/min); dry gas temperature, 300 °C; spray capillary voltage, 4000 V; and scan range, m/z 100–2000.Ultrapure He was used as the collision gas. The antioxidant activities of test samples were assessed using a BPCL Model Ultra Weak Chemiluminescence Analyzer (Institute of Biophysics, Academia Sinica, Beijing, China) at 30 °C, Hi-V 800, Kv-1.

2.2. Chemicals

(−)-EGCG (purity>99%), luminol, calf thymus DNA, pyrogallic and 1, 10-phenanthroline were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of analytical grade.

2.3. Oxidation of EGCG and isolation of dimeric derivatives

The oxidation of EGCG was based on X. C. Wan and R.G.Bailey et al method [16,17] with some modification. 200 mg EGCG was reacted with 300 mg potassium hexacyanoferrate (III) and 100 mg sodium hydrogen carbonate in 10 ml of distilled water at 25 °C, pH 7 for 15 min. The reaction mixture was extracted immediately with ethyl acetate (3×10 ml) at pH 7. After evaporation of the solvent of ethyl acetate phase in vacuum, the residue was dissolved in water (10 ml) and re-extracted with ethyl acetate (3×10 ml) at pH 2 to obtain the aqueous phase (Fraction A) and ethyl acetate phase (Fraction B).

The Fraction A was adsorbed onto column (20 cm×1.0 cm i.d.) of D_{101} macro-porous absorption resin (Naikai University Chemical Co, Najing, China) and then eluted with distilled water until the water-soluble impurity was eluted clear. The adsorbed oxidized products of EGCG was then eluted with 100 ml ethanol (80%) from the resin. The ethanol elution was concentrated under reduced pressure at low temperature (<40 °C) and was re-extracted with ethyl acetate to give compound 2 (4.48 mg).

The Fraction B was evaporated by a Rota-vapor (Buchi) in vacuum at 40 °C to remove the solvent, and the residue, which was suspended in water and was then subjected to a Sephadex LH-20 gel filtration (25–100 μm, Pharmacia Fine Chemical Co. Ltd., Uppsala, Sweden) column chromatography (40 cm×1.5 cm i.d.) eluting with 70% methanol at a flow rate of 1 ml/min. Fractions were combined based on their UV absorptions at 280 nm to yield three fractions F1–F3. According to the HPLC analysis, F2 was concentrated and purified repeatedly to yield compound 3 (9.58 mg).

Compounds 2 and 3 were identified on the basis of their spectral data. Compound 2, a yellow amorphous powder, was assigned the molecular formula of $C_{44}H_{34}O_{23}$ determined by negative-ion LC/ESI/MS ($[M–H]^-$ at m/z 929, t_R = 18.56 min), as well as from its NMR data, which displayed a signal pattern similar to that of dehydrotheasinensin A(2) (Fig. 1) reported in the literatures [18,19]. Compound 3 was isolated as an orange amorphous powder. The negative-ion LC/ESI/MS of 3 displayed a molecular ion peak at m/z $[M–H]^-$ 911 (t_R = 24.28 min), supporting a molecular formula of $C_{44}H_{32}O_{22}$. The NMR and UV spectra of 3 corresponded to theacitrin C (3) (Fig. 1) reported by Davis et al among the oxidation products from black tea [20].

2.4. Determination of antioxidant activity

2.4.1. Determination of superoxide anion scavenging activity

O_2^- was generated from a pyrogallol autoxidation system [21]. The reaction mixture contained 50 μl pyrogallol (50 mM), 200 μl carbonic acid-buffered saline solution (CBSS, pH 10.2) containing 0.1 mM EDTA, 10 μl test samples (CBSS replaced the sample in the control), and 400 μl luminol (1 mM). The emission light intensity was recorded every 3 s, and the total integral of the light intensity of 300 s was determined.

2.4.2. Determination of hydroxyl radical scavenging activity

•OH was generated by $CuSO_4$-Phenanthroline-Vc-H_2O_2 system [22]. The reaction mixture containing 50 μl test samples (borate–borax buffer was used in control), 50 μl $CuSO_4$ (1 mM), 50 μl phenanthroline (1 mM), 700 μl borate–borax buffer (50 mM). After incubation, 100 μl Vc (1 mM) and 50 μl of H_2O_2 (1.5 mg/ml) were added into the mixture. The luminescence intensity was recorded every 3 s.

2.4.3. Determination of hydrogen peroxide scavenging activity

H_2O_2 was generated by Luminol-H_2O_2 system [23]. 600 μl of 50 mM phosphate-buffered saline (PBS, pH 8.0) with 200 μl test samples and 200 μl of 10 mg/ml H_2O_2 were incubated for 20 min at 37 °C. In the control experiment, the test samples were replaced with PBS, 150 μl of 1 mM luminol was added into the mixture to start the chemiluminescence reaction. The luminescence intensity was recorded every 2 s.

2.4.4. Determination of preventing DNA damage effect

Preventing the DNA damage effect of test samples was determined by $CuSO_4$–Phenanthroline–Vc–H_2O_2–DNA chemiluminescence system [24]. The reactants were added in turn and mixed as follows: 100 μl $CuSO_4$ (20 mM), 70 μl Vc (2 mM), 70 μl phenanthroline (2 mM), 440 μl of 0.1 M acetate buffer (pH 5.5, NaAc–HAc buffer), 20 μl DNA (50 μg/ml) and 100 μl test samples or acetate buffer (in control experiment). The reaction was started by the addition of 200 μl H_2O_2 (0.8 M). The luminescence intensity was recorded every 10 s.

2.4.5. Calculation of antioxidant activity

Scavenging rate (%) was calculated by the following equation: $scavenging\,rate\,(\%) = \frac{(CLcontrol-CLo)-(CLsample-CLo)}{CLcontrol-CLo} \times 100\%$, where $CL_{control}$ is the luminosity of the control, CL_0 is the luminosity of the background and CL_{sample} is the luminosity of the test samples. All analyses were run in triplicate and averaged.

2.5. Statistical analysis

Data were presented as means ± standard deviation (SD) of three determinations. Statistical analyses were performed using a one-way analysis of variance. The IC_{50} value was determined to be the effective concentration at which chemiluminescence intensity was inhibited by 50%. The IC_{50} values were calculated by linear-regression analysis. Results

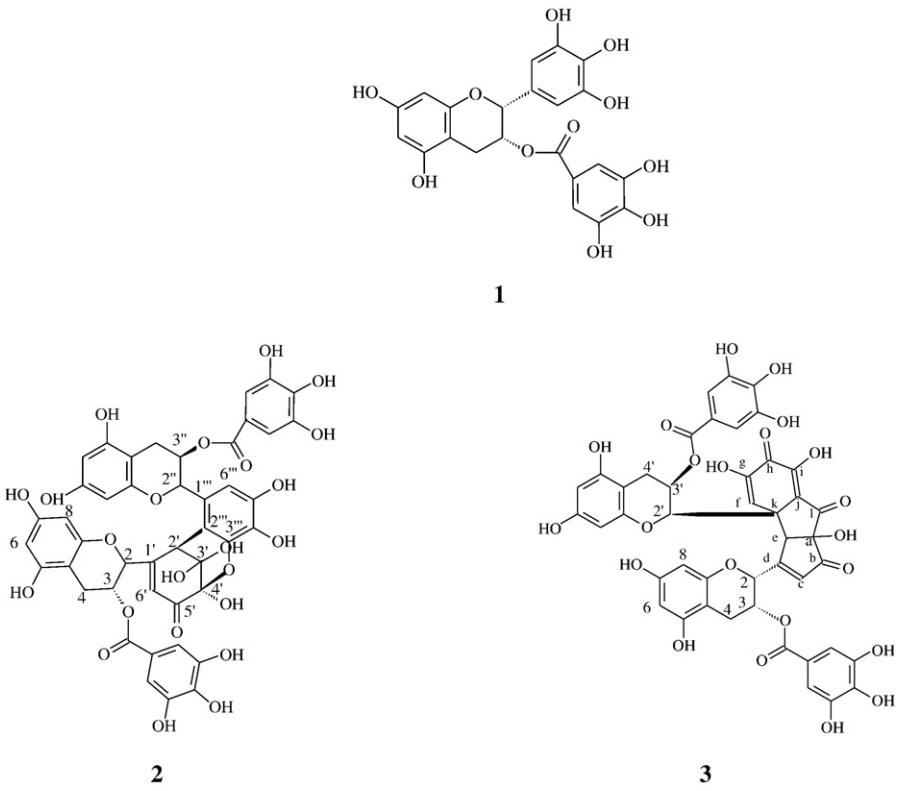

Fig. 1. Structures of compounds 1–3.

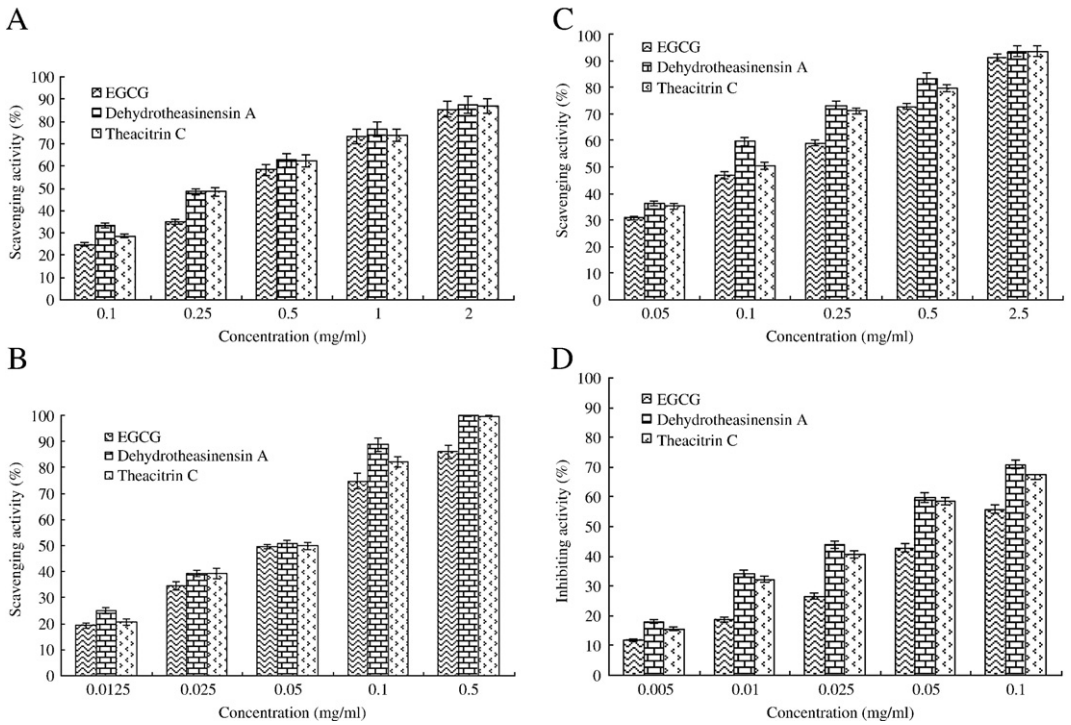

Fig. 2. Inhibiting activities of compounds 1–3 on superoxide anion (A), hydroxyl radical (B), hydrogen peroxide (C) and •OH-induced DNA oxidative damage (D). Values are expressed as mean ± SD ($n = 3$).

were calculated by employing the statistical software (SPSS 13.0, SPSS Inc., USA).

3. Results and discussions

Nowadays, many methods have been developed to detect ROS and evaluate antioxidants, such as radiolysis technology, CL, fluorescence assay, and electron spin resonance (ESR). Among them, CL has been widely used as a sensitive assay for monitoring free radicals and reactive metabolites from enzyme, cell, or organ systems [25,26]. Because of its high sensitivity and rapidity, the chemiluminescence method has been widely used in antioxidation effect evaluation [27].

The scavenging effects and dose-dependent relation of EGCG, compounds 2 and 3 on ROS (O_2^-, H_2O_2 and •OH) were presented in Fig. 2(A, B, C). The test samples were all exhibited significant inhibitory effects on ROS. Comparison of IC_{50} values measured in different assay systems (Table 1), Compound 2 showed a highest scavenging effect on O_2^- ($IC_{50}=0.254$ mg/ml), •OH ($IC_{50}=0.037$ mg/ml) and H_2O_2 ($IC_{50}=0.069$ mg/ml), followed by compound 3, which scavenged O_2^-, •OH and H_2O_2 with IC_{50} of 0.285 mg/ml, 0.041 mg/ml, and 0.094 mg/ml, respectively, while EGCG exhibited the lowest inhibition capacity. Furthermore, gradual increases in inhibiting effect on •OH-induced DNA damage with an increase in the concentration of EGCG, compounds 2 and 3 were also observed in Fig. 2 (D). The scavenging rate of EGCG was less than 56% at concentration of 0.10 mg/ml, while compounds 2 and 3 at the same concentration were 70.89% and 67.36%, respectively. Inhibiting effects of compounds 2 ($IC_{50}=0.029$ mg/ml) and 3 ($IC_{50}=0.035$ mg/ml) were 2.86 times and 2.37 times that of EGCG ($IC_{50}=0.083$ mg/ml) (Table 1). It is suggesting that the dimeric derivatives oxidized of EGCG possess higher activities on inhibiting of DNA oxidative damage induced by •OH radical. In addition, their inhibition capacities of DNA oxidative damage were correlated well with the scavenging effects on ROS.

The purification, identification and formation mechanism of oligomeric catechin derivatives including compounds 2 and 3, theaflavins and thearubigins in the simulation systems and during the fermentation stage of black tea manufacture had been reported in some literatures [16–20,28,29]. However, there is only little information about bioactivities of some oxidized products apart from theaflavines and tea pigment from black tea [13,15,21]. Our study, for the first time, compared the antioxidative activities of EGCG with its oxidative dimers, and indicated that all of them had remarkable inhibiting effects on ROS and DNA oxidative damage, with the dimeric EGCG derivatives, compounds 2 and 3 exhibiting more potent than EGCG.

Previous the structure–activity relationship studies indicated that flavonoids with an o-dihydroxy or trihydroxy B ring are the most effective antioxidants. Moreover, a gallate ester moiety at the 3-position of catechins (such as EGCG and ECG) has the highest antioxidant activity [30]. The excellent antioxidant effects of compounds 2 and 3 maybe related to their structures. The scavenging activities of these two compounds on free radical increased significantly with the increase of number of hydrogen groups or catechol moieties in the molecule. Compared to EGCG, they could afford more ortho-hydroxyl in B-ring structures, which are crucial for the enhanced antioxidant activity because of the easy formation of ortho-quinone [31,32]. In addition, compounds 2 and 3 possess typical hydrated quinone structure and a three fused ring system joined a flavan-3-ol and a flavan-3-O-gallate together separately, that is formed from co-oxidation between two pyrogallol B-rings of EGCG [20]. These structural features may provide more interaction sites with radicals, strengthen intra- molecular hydrogen bonds, and improve the complexation capacity of metal ions. Therefore, dimeric derivatives oxidized of EGCG had greater antioxidant properties in different assay systems than their precursor EGCG.

Biochemical studies suggested that oxidative damage to cell membrane lipids induced by ROS may be important in a number of pathological conditions such as cancer and aging [33,34]. The higher antioxidant potentials of dimeric EGCG derivatives proved in this study may expand the utility of tea polyphenols as a resource for antioxidant development and broaden their therapeutic applications towards the prevention of degenerative and neoplastic diseases of various organs.

Table 1
Comparative antioxidant capacities of compounds 1–3 measured in different assay systems.

Compound	Regression equation	R^2	IC_{50} (mg/ml)
1	$y_{O2-}=21.496\mathrm{Ln}(x)+71.15$	0.9775	0.374
	$y_{OH}=18.805\mathrm{Ln}(x)+105.73$	0.9288	0.052
	$y_{H2O2}=15.176\mathrm{Ln}(x)+79.68$	0.9833	0.141
	$y_{DNA}=14.663\mathrm{Ln}(x)+86.56$	0.9592	0.083
2	$y_{O2-}=18.537\mathrm{Ln}(x)+75.37$	0.9978	0.254
	$y_{OH}=21.652\mathrm{Ln}(x)+121.64$	0.9085	0.037
	$y_{H2O2}=13.923\mathrm{Ln}(x)+87.14$	0.9057	0.069
	$y_{DNA}=17.189\mathrm{Ln}(x)+110.29$	0.9883	0.029
3	$y_{O2-}=19.296\mathrm{Ln}(x)+74.22$	0.9972	0.285
	$y_{OH}=22.087\mathrm{Ln}(x)+120.42$	0.9486	0.041
	$y_{H2O2}=14.881\mathrm{Ln}(x)+85.23$	0.9426	0.094
	$y_{DNA}=16.968\mathrm{Ln}(x)+107.02$	0.9807	0.035

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