J. Life Science. (2002), 12(2), 75~79

JOURNAL OF
Life Science

Scavenging Effects of Tea Catechins on Superoxide and Hydroxy Radical

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Received: December 20, 2002

Abstract Tea catechins, the most important compounds in tea polyphenols, can efficiently scavenge superoxide anion free-radical ($O_2^-\cdot$), hydroxyl radical. ($\cdot OH$) The mechanism of scavenging active oxygen free radicals was investigated by ESR spin trapping technique and Chemiluminescence. Results showed that various tea catechins constitute an antioxidant cycle in accordance with the decreasing order of the first reductive potential, and produce the effect of cooperative strength each other. Esterificated catechins could scavenge active oxygen free radicals more effectively than the non-esterificated ones. When $\cdot OH$ and $O_2^-\cdot$- were scavenged by (-)-epigallocatechin gallate [(-)- EGCG], the stoichiometric factors were 6, and the rate constants of scavenging reaction reached 7.71×10^6 and 3.52×10^{11} L mol^{-1} s^{-1}, respectively. In the mean time, tea catechins could scavenge superoxide anion fiee radical ($O_2^-\cdot$) and hydroxyl radical ($\cdot OH$) in a dose dependent manner. But at higher concentration or pH value, tea catechins can induce the prooxidant.

Key words: polyphenols, superoxide, free radical

Introduction

Tea, including black tea, green tea and oolong tea, is one of the most widely consumed beverages in the world. Although consumption of tea has been primarily associated with countries in Asia and Europe, the heightened popularity of this beverage throughout the world in recent years may be due in part to the evidence of a relationship between tea consumption and the prevention of certain human diseases[3,5,6]. These days, the roles of tea consumption and tea polyphenols in the prevention of cancer and cardiovascular disease have received a great deal of attention [,8,14].The chemopreventive effects of tea against these human diseases have been attributed to the biochemical and pharmacological activities of polyphenolic compounds (especially catechins) in tea[2,3,7,16]. The chemical structures of the four most abundant naturally occurring tea catechins, are illustrated (-)-epicatechin ((-)-EC), (-)-epigallocatechin ((-)-EGC), (-)-epicatechin gallate ((-)-ECG) and (-)-epigallocatechin gallate ((-)-EGCG).

Tea catechins, belonging to the flavanol, a subclass of the flavonoid family, have received considerable attention in recent years due to their numerous potentially beneficial medicinal properties including inhibition of carcinogenesis, tumorigenesis and mutagenesis, as well as the inhibition of tumor growth and metastasis[2,9,14]. A common discussed mechanism of their biological and pharmacological effects is related to the antioxidative activities of these compounds[10,11,12]. In this paper, we evaluated the scavenging effects of four catechins both on superoxide anions ($O_2^-\cdot$) and hydroxyl free radical ($\cdot OH$). In addition, their synergic effects, structures activity relationships as well as the stoichiometric factors and rate constants for the reactions of $\cdot OH$ and $O_2^-\cdot$ with EGCG were discussed.

Materials and Methods

Reagents

EGCG, EGC, ECG, EC, and (+)-C were purified by HPLC. DMPO (5,5-dimethyl-1-pyrroline-1-oxide) purchased from Sigma was purified with active charcoal before used. All the other chemicals made in China were at the analytical grade.

$O_2^-\cdot$ and $\cdot OH$ measurement

Superoxide anion and hydroxyl radical were generated by the xanthine and xanthine oxidase system and fenton reaction[4], respectively. The corresponding radicals were trapped by DMPO, and the ESR spectra of DMPO/$O_2^-\cdot$ or $\cdot OH$ radical adducts were observed. All ESR measurement was made using a varian-109 ESR spectrometer. Reactants were mixed in test tubes. And the reaction mixture was then transferred to a flat cell for ESR measurement. The

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conditions of ESR measurement were as fallows: microwave power 20mw, modulation frequency 100kHz, modulation amplitude 0.1mT, scan width 200G, time constant 0.1s, temperature 298k, and center field 324.5mT.

Chemiluminescence was measured under the same conditions reported by Shen S.R.[5].

Statistical analysis

Data were analyzed by Student's t-test and presented as mean±standard deviation. P<0.05 was considered significant.

Results

Scaveging activities of tea catechins on $O_2^-\cdot$

The xanthine and xanthine oxidise system was used to generate $O_2^-\cdot$ radicals. As shown in Fig. 1a, a mixture of xanthine and xanthine oxidase in the presence of DMPO formed a spin adduct spectrum. Addition of tea catechins and EGCG significantly decreased the spectral intensity of DMP0/$O_2^-\cdot$ (Fig. 1c, Fig. 1d). The control, which tea catechins were replaced by equivalent volume PBS (pH 7.4, 0.05 mol/L), did not alter the spectral intensity (Fig. 1b), and this indicated that both tea catechins and EGCG appear to have $O_2^-\cdot$ scavenging activity[3].

The chemiluminescence was used to measure the scavenging of $O_2^-\cdot$ by different concentration of tea catechins[12]. The results in Fig. 2 showed that the scavenging percentage were concentration-dependent. The maximal scavenging percentage was observed at 6×10^{-3}mg/mL, reached 97%, but when their concentrations exceeded 6×10^{-3}mg/mL, the scavenging percentage decreased significantly.

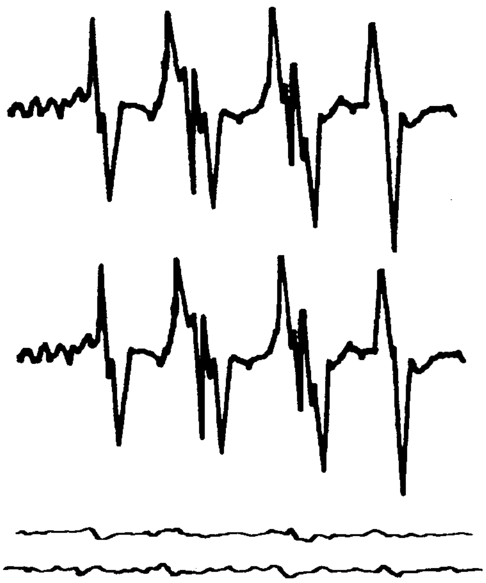

Fig. 1. Electron spin resonance(ESR)spectra of DMPO/$O_2^-\cdot$ adducts tained in the absence of catechins in X/XO system.
a. ESR spectrum of DMPO/$O_2^-\cdot$ b. Control
c. Tea catechin(0.0043mg/mL) d. EGCG(0.0043mg/mL)

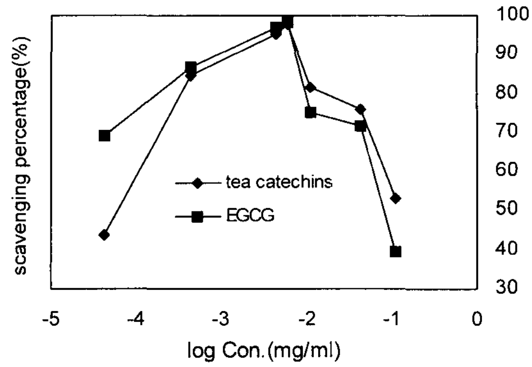

Fig. 2. Effect of concentrations on scavenging rate($O_2^-\cdot$)

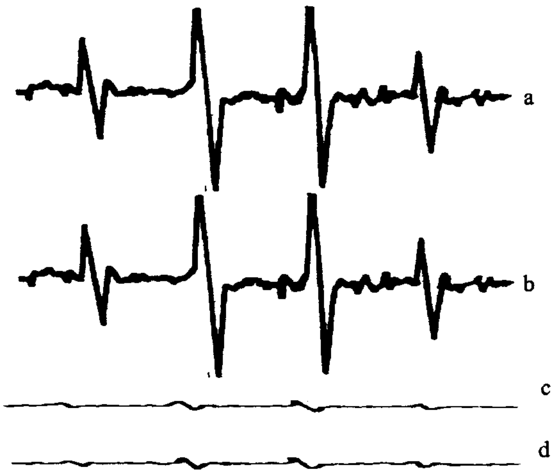

Fig. 3. Electron spin resonance(ESR) spectra of DMPO/·OH adducts obtained in the abscence and presence of catechins in Fenton's reaction system.
a. ESR spectrum of DMPO/·OH b. Control
c. Tea catechin(0.1mg/mL) d. EGCG(0.1mg/mL)

Scavenging activities of tea catechines on ·OH

The Fenton reaction was used as a source of ·OH radicals. As shown in Fig. 3a, an aqueous solution containing Fe(ll), H_2O_2 and a spin trapper(DMPO) in a phosphate-buffered solution(pH 7.4), generated a DMPO/·OH spin adduct spectrum. Addition of 0.1mg/mL tea catechins or EGCG can effectively reduced the intensity of the DMPO/·OH spin adduct signal (Fig. 3c, Fig. 3d). The control, which tea catechins were replaced by equivalent volume PBS (pH 7.4, 0.05mol/L), did not alter the spectral intensity (Fig. 3b), demonstrating that both tea catechin and EGCG are ·OH radical scavengers[9].

The Chemiluminescence is used to measure the scavenging of ·OH by different concentrations of tea catechins. The results in Fig. 4 show that the scavenging percentage eached the maximum (99.97%) when tea catechins or EGCG was at the concentration of 0.1mg/mL, but when their concentration exceeded 0.1mg/mL, the scavenging

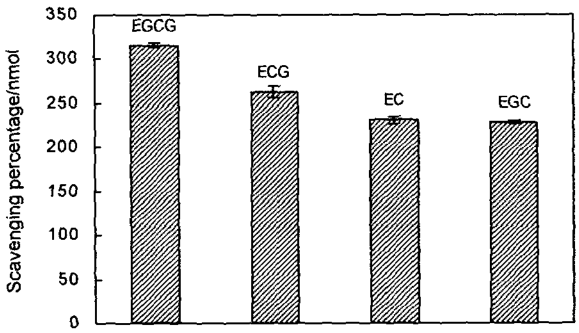

Fig. 4. Scavenging persentage of per nmol catechin.

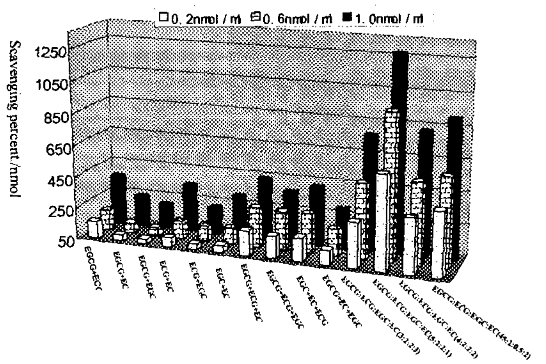

Fig. 5. Synergic effects of 2-catechin, 3-catechin and 4-catechin combination on scavenging $O_2^{-}\cdot$.

percentage decreased sharply, showing remarkable prooxidant. This result suggested that the concentration range for effectively scavenging ·OH of tea catechins is very limited, and tea catechins can show preferable scavenging activity only at the concentrations between 0.043mg/mL and 0.1mg/mL.

Scavenging effect of four catechins on $O_2^{-}\cdot$

To determine the optimum concentration of tea catechins for scavenging superoxide anion free radical ($O_2^{-}\cdot$), we investigated the scavenging percentage per nmol catechin by Chemiluminescence (CL) in the xanthine-xanthine oxidase system. Results showed that the scavenging percentage per nmol catechin keeps constant at the catechin concentration less than 0.5 umol/mL (data not shown). As shown in Fig. 5, the superoxide scavenging activities of four catechins increased in the order of (-)-EC < (-)-ECC < (-)-ECG < (-)-ECCG. The scavenging effects of ECG and EGCC with a gallate group at the C-3 position were stronger than the nongalloylated ones (EC and EGC).

Scavenging effects of 2-catechin, 3-catechin and 4-catechin on $O_2^{-}\cdot$

With the total molar concentrations remained uniform, the experiment was done at the ratio of 1 : 1 for the combinations of two catechins, and 1:1:1 for the combinations of three-catechins. Results in Fig. 6 showed that ECCG+ECG was the most effective, 131lowe6 by ECG+EC, among the 2-catechin combinations and they were stronger than the application of only one catechin. Scavenging effects of 3-catechin combination decreased in the order of ECCG+ECG+EC > EGC+EC+ECG > EGCG+ECG+EGC > ECCC+EC+ECG, and they were stronger than the 2-catechin combinations. The 4-catechin combination at the ratio of 5:2:2:1 (EGCG:ECC:ECC:EC) in the natural tea leaves possessed the highest antioxidative effect[15]. The synergic effect was positively correlated to the molecular concentrations of catechifs[13]. The higher the concentration, the stronger the scavenging effect. Measured by the V-A method, the first reducing potentials of catechins (data not shown) were in the order of EGCC > ECG > ECC > EC, and this could be the foundation of the synergic offsets of catechins.

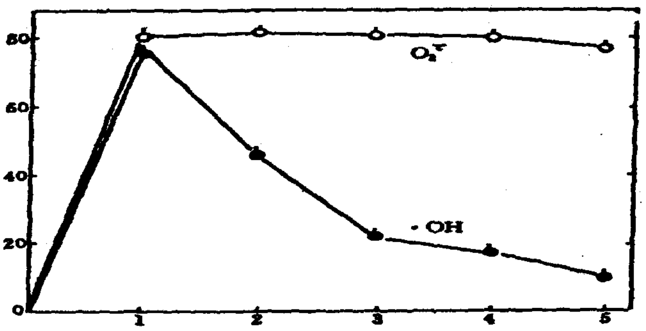

Fig. 6. Scavenging effect of (-)-EGCG 20umol·L^{-1}on $O_2^{-}\cdot$(O) and 300umol·L^{-1}·OH(●).

Effect of tea catechin concentrations and scavenging time on prooxidant

As mentioned previously scavenging percentages of tea catechins and EGCG declined significantly at higher concentrations (Fig. 2 and Fig. 4) and far ·OH, the prooxidant

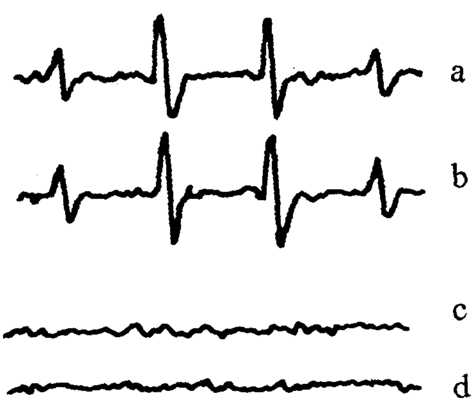

Fig. 7. ESR spectrum of DMPO/·OH formed by Tea catechins (a) and (-)-EGCG(1.0mg/ml) in an alkaline solution of pH10.1 (b)tea catechins+SOD (c)(-)-EGCG+SOD(d).

was induced remarkably. The relationship between scavenging effect and time is also an important parameter for assessing scavenging abilities. Fig. 7 shows the scavenging effects of (-)-ECCG on ·OH and O_2^{-}· measured at different time. Results indicated the scavenging effects on O_2^{-}· and ·OH increased linearly with time and reached the maximum at 1 minute, and then the farmer keeps constant, but the latter declined rapidly, which is likely correlated with the formation of proxidant reaction.

Effect of pH on prooxidant

As shown in Fig. 8 and Fig. 9, both superoxide anion and semiquinone anion radicals were engaged by tea catechins and (-)-ECCG in alkaline solution *in vitro*. The inducing capability of (-)-EGCC stronger than that of tea catechins. Different free radicals could be generated according to the different conditions. At pH 10.1, they could produce O_2^{-}· and at pH 12.0, semiquinone anion free radicals were formed. Different free radicals have different inducing capabilities of prooxidant for tea catechins and EGCG.The more ·OH active has stronger ability than the less active O_2^{-}·.

Relationship between catechin structures and their antioxidative activities

To examine the relationship between the free radical scavenging activities and the chemical structures of four catechins, we investigated their scavenging effects on ·OH and O_2^{-}· The results showed that the scavenging effects of galloylated catechins (EGCG and ECG) were better than

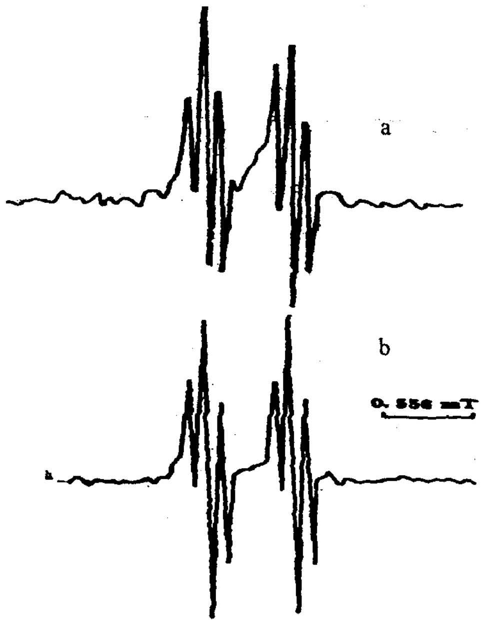

Fig. 8. ESR spetra of (-)-EGCG radical in an alkaline solution. Autoxidation condition: 1.0mg/ml sample in pH12.0 buffer at 25±1℃.
a. Pyrogallol(0.1mg/mL) b. EGCG(0.0043mg/mL)

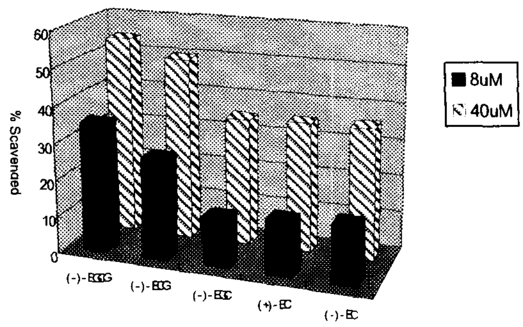

Fig. 9. Scavenging effect of tea catechins on O_2^{-}·.

the of nongalloylated ones (EGC, EC, (+)-C), and the scavenging effect of EGC was stronger than those of EC and (+)-C. Thus, it is suggested that the presence of the gallate group at 3 position plays a very important role in their free radical-scavenging abilities and an additional insertion of the hydroxyl group at the C-5'position of B ring also contributes to their scavenging activities.

Calculation of stoichiometric factors and reaction rate constant of EGCC with·OH and O_2^{-}·

To determine the reaction rate constant of EGCG with O_2^{-}· and ·OH radicals, kinetics studies were carried out according to methods reported by Burton et al(6) and Suzuki et at(f. The reactions of (-)-EGCG with ·OH or O_2^{-}· may be expressed as:

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