Epigallocatechin-3-gallate and Epicatechin-3-gallate from Green Tea Decrease Plasma Non-Transferrin Bound Iron and Erythrocyte Oxidative Stress

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Abstract: β-Thalassemia patients suffer from secondary iron overload caused by increased iron absorption and multiple blood transfusions. Excessive iron catalyzes free-radical formation, causing oxidative tissue damage. Non-transferrin bound iron (NTBI) detected in thalassemic plasma is highly toxic and chelatable. Desferrioxamine and deferiprone are used to treat the iron overload, but many side effects are found. Epigallocatechin gallate (EGCG) and epicatechin gallate (ECG) in green tea (GT) show strong antioxidant properties. We separated the EGCG and ECG from GT extract using an HPLC, and examined their iron-binding and free-radical scavenging activities. They bound Fe^{3+} rapidly to form a complex with a predominant absorption at 560 nm. EGCG and ECG bound chemical Fe^{3+} and chelated the NTBI in a time- and dose dependent manner. They also decreased oxidative stress in iron-treated erythrocytes. In conclusion, EGCG and ECG could be natural iron chelators that efficiently decrease the levels of NTBI and free radicals in iron overload.

Key Words: Epigallocatechin-3-gallate, epicatechin-3-gallate, green tea, iron overload, non-transferrin bound iron, oxidative stress.

INTRODUCTION

Iron is a transition element essential for all living organism and functions for oxygen transport and many redox reactions. Loss of iron homeostasis can result in iron deficiency or iron overload. Iron overload in β-thalassemia patients is caused by repeated blood transfusions and elevated dietary iron absorption [1]. Plasma iron and transferrin saturation in these patients is often excessive and can lead to the formation of non-transferrin-bound iron (NTBI) [2]. The uptake of plasma NTBI into tissues can lead to excess iron accumulation and contribute to increased intracellular labile iron pools (ICLIP) [3,4]. The two toxic forms of iron potentially generate reactive oxygen species, leading to oxidative tissue damage and vital organ dysfunction [5]. Iron overload is the main cause of death in β-thalassemia patients [6]. Iron chelation therapy aims ultimately to decrease levels of accumulated iron by removing plasma NTBI or ICLIP and can reverse iron-mediated disease in the short term [7]. Parenteral desferrioxamine (DFO) reduces tissue iron stores, prevents iron-induced organ damage, and reduces morbidity and mortality, with little serious toxicity. However, due to prolonged continuous infusions, high cost and noncompliance, many patients eliminate the treatment. Deferiprone (DFP), an oral iron chelator, less effective and more toxic than DFO and may not adequately control iron overload; however, compliance and quality of life are improved. A novel oral iron chelator, deferasirox (ICL670), is assessed in a comprehensive series of multi-center clinical trials [8]. Green tea polyphenols are natural antioxidant that have strong scavenging effects on reactive oxygen radicals [9-11] and decrease the utilization of dietary iron [12,13]. At least five catechins like epigallocatechin (EGC), epicatechin (EC), catechin (C), epigallocatechin gallate (EGCG) and epicatechin gallate (ECG) have been found in all the tea infusions examined (Fig. **1**), ranging in concentration from 1-13 mg/dl and of them EGCG is the most abundant [14,15].

Green tea catechins having 3', 4'-dihydroxy and/or galloyl groups in their structures are the likely iron-binding compounds accounting for the antioxidant effects [16,17]. Current evidence has reported that green tea catechins have brain-permeable, transitional metal (iron and copper)-chelatable/radical scavenger properties [18,19], and recently, Srichairatanakool *et al*. [15] demonstrated that green tea extract decreased levels of plasma NTBI and erythrocyte oxidative stress. Here we investigate the efficacy of EGCG and ECG from green tea in chelating plasma NTBI and scavenging erythrocyte free-radicals *in vitro*.

MATERIALS AND METHODS

Chemicals

All catechins (EGC, C, EC, EGCG and ECG), aluminium chloride hexahydrate, 3-[N-morpholino]propanesulfonic acid (MOPS) and hydrogen peroxide were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). HPLC-grade acetronitrile and methanol were purchased from

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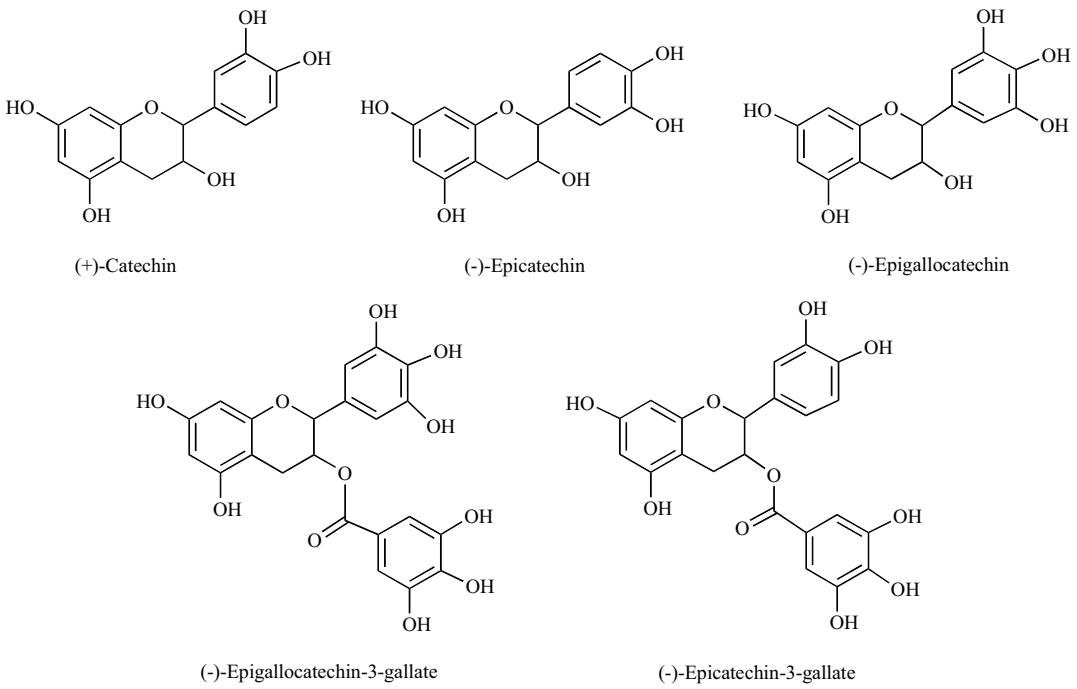

Fig. (1). Chemical structures of catechin.

Merck Company. All other chemicals and reagents used were of AnalaR grade. CP22 (1-methyl-2-propyl-3-hydroxy-pyridin-4-one) was kindly donated by Dr. Chada Phisalaphong, Thailand Government Pharmaceutical Organization, Bangkok. A stock of ferric nitrate (1 ppm or 18.16 mM iron in 1% nitric acid) was used as the iron source for other preparations. Stock ferric citrate and ferric nitrilotriacetate solutions were prepared by consecutive mixing of ferric nitrate with the chelators citric acid and nitrilotriacetic acid (at a 1:5 molar ratio of Fe^{3+} to chelator). Various iron concentrations were prepared in 10 mM MOPS buffer, pH 7.0, just before use.

Tea Samples

Tea (*Camellia sinensis*) shoots were harvested from the tea fields of the Royal Project Foundation in Chiang Mai and were immediately dried in a microwave oven according to the method recently reported by Srichairatanakool and colleagues [15].

Green Tea Extract

Green tea (GT) leaves (2.0 g in dry weight) were extracted in 100 ml of hot water (80°C) for 10 minutes. This crude GT extract was initially passed through Whatman No.1 filter paper (Whatman International Ltd, Maidstone, England) and subsequently through a cellulose acetate filter membrane (0.45 µm pore size). The extract was freshly prepared and used on the same day because of its instability in aqueous solution.

Analysis of Green Tea Catechins

Catechin derivatives in the extract were quantified using a high performance liquid chromatography (HPLC) system [20] consisting of a ternary delivery dual-pump (Model 3500; LDC Milton-Roy Analytical Inc., Rochester, NY, USA) equipped with a flow cell detector (SpecMonitor 3200, LDC Milton-Roy Analytical Inc.). Briefly, 50 µl of crude extract (2.0 g%) was injected manually and fractionated on the analytical column (Spherisorb ODS2, 250 x 4.7 mm, 5 µm) capped with a guard column (Spherisorb ODS2, 10 mm x 4.7 mm, 5 µm). Individual catechins were eluted isocratically with a solvent mixture of 0.05% H_2SO_4:acetonitrile: ethyl acetate (86:12:2, v/v/v) at a flow-rate of 1.0 ml/min. and monitored at 280 nm. Authentic EGC, EC, C, EGCG, and ECG standards at the concentrations of 0-10 mM were used to calibrate the column and to build standard curves. Identification and determination of each catechin concentration were performed by a comparison of their retention times with those of the standards.

Isolation of Green Tea Catechins

Individual GT catechin isomers were isolated using semi-preparative HPLC [21]. Green tea extract (5.0 g%) (0.5 ml) was injected manually into the HPLC system consisting of a main column (Luna C18(2), 250X10 mm, 5 µm, Phenomenex®, Torrance, California, USA) connected to a guard column (Luna C18(2), 50x10 mm, 5 µm, Phenomenex®, Torrance, California, USA). Catechins species were eluted isocratically with a mobile-phase (methanol:H_2O = 29:71, *v*/*v*) at a flow rate of 1.0 ml/min and collected (1 ml/fraction) using a BioRad® Fraction Collector. The fractions were measured at absorption of 280 nm using a UV-VIS Spectrophotometer (Shimadzu, Japan), and the individual catechin isomers were pooled. Each individual catechins fraction was checked for the purity using the analytical HPLC as described above. Methanol constituted in the pooled fractions

was removed under vacuum using a freeze-dry technique. Lyophilized catechins powder was stored in the dark at -20°C until use.

Blood Samples

Venous blood was collected from β-thalassemia patients attending the Thalassemia Clinic, Maharaj Nakorn Chiang Mai, Faculty of Medicine, Chiang Mai University, Thailand. Normal healthy volunteers were also included as a control group. The Faculty of Medicine ethics Committee for Human Research approved the protocol for blood collections (Reference number 0605(8)/217). Blood was collected in heparinized tubes and centrifuged at 3,000 rpm, 4°C for 15 min. Plasma was removed and kept frozen at -80°C until analysis. Red blood cells (RBC) were washed three times with phosphate-buffered saline (PBS), pH 7.0 solution and used in oxidative stress experiments on the same day.

Measurement of Chemical Iron Binding

This procedure was adopted from the method of Srichairatanakool *et al.* method [15]. First, solutions of catechin fractions (200 μM) (1 ml) previously dissolved in 5 mM MOPS buffer, pH 7.0 were mixed with the Fe^{3+}-NTA solution (100 μM) in polypropylene tubes to obtain the indicated concentrations. After 10 minutes, the absorption was monitored between 400-800 nm against a reagent blank (each catechin solution only) using a double-beam UV-VIS scanning spectrophotometer (Shimadzu Corporation, Analytical & Measuring Instruments Division, Kyoto, Japan). Second, various concentrations of catechin fractions (0-200 μM) were added to the Fe^{3+}-NTA solution (100 μM) and incubated at room temperature (25°C) for 15 minutes. Third, the Fe^{3+}-NTA solution (10-400 μM) was incubated with EGCG and ECG solutions (200 μM) at room temperature for 0-30 minute. Fourth, iron-binding affinity was examined by incubating the solutions of ferrous ammonium sulfate and Fe^{3+}-NTA with the EGCG and ECG fractions at room temperature for 15 minutes. The absorbance of iron-catechin complexes was measured at 560 nm.

NTA Chelation/HPLC-based Measurement of Plasma NTBI

Plasma NTBI measurement was undertaken essentially as described by Singh and colleagues [22]. Plasma (450 μl) from β-thalassemia patients was incubated with 800 mM nitrilotriacetic acid (NTA), pH 7.0 solution (50 μl) at room temperature to transform NTBI into Fe-NTA. Afterwards, plasma proteins were removed by centrifugation of the treated plasma using an ultracentrifugation filtration device (NanoSep®, 30-kDa cut off, polysulfone type; Pall Life Sciences, Ann Arbor, MI USA) at 12,000 rpm (10620*g*, Hettich Centrifugation, Germany), at 15°C for 45 minutes. The ultrafiltrate was analyzed using a non-metallic HPLC system. The HPLC conditions were as follows: a dual-piston high pressure pump (ConstaMetric®3500 LDC Analytical, Inc., Florida, USA), a glass analytical column (ChromSep ODS1, 100x10 mm, 5 μm; Chrompack International, Middelburg, the Netherlands), mobile-phase solvent containing 3 mM CP22 (1-methyl-2-propyl-3-hydroxypyridin-4-one) in 19% acetonitrile (HPLC grade, E. Merck, Darmstadt, Germany) buffered with 5 mM MOPS, pH 7.0 at a flow rate of 1.0 ml/min. Column effluents were monitored at 450 nm using a flow-cell detector (SpecMonitor2300; LDC Milton-Roy Inc., Florida, USA) and conducted with the BDS software (BarSpec Ltd., Rehovot, Israel). The NTBI peak was calculated from a calibration curve of standard iron solutions (0-16 μM Fe-NTA in 80 mM NTA, pH 7.0). The NTA solution (800 mM) was prepared by adding N,N-bis[carboxymethyl]glycine disodium (purity minimum 99%, Sigma-Aldrich Co., St. Louis, MO, USA) solution (800 mM) to N,N-bis[carboxymethyl]glycine trisodium (purity 98%, Sigma-Aldrich Chemie GmbH, Steinheim, Switzerland) solution (800 mM) to reach a final pH of 7.0.

The NTBI measurement was also slightly modified by including an aluminum blocking step to saturate free iron-binding sites on iron chelator molecule and preventing iron shuttling by NTA when excess unbound iron chelator remained in chelator-treated plasma [15,23]. Aluminium chloride solution (200 μM) was added to chelator-treated plasma, and the mixture was further incubated for 1 hour. The plasma was then quantified for NTBI as described above.

Measurement of Erythrocyte Oxidative Stress

Erythrocyte oxidative stress was assayed basically as described by Amer *et al*. [24]. Briefly, RBC suspension (40% hematocrit) was prepared in the PBS solution. The RBC suspension (2 μl) was diluted with 9 ml of PBS. The RBC suspension (2 ml) was incubated with a 2',7'-dichlorofluorescein diacetate (DCFH-DA) solution (10 mg/ml in methanol) at 37°C under 5% CO_2 atmosphere for 15 minutes. The RBC was then washed three times with the PBS solution and subsequently exposed to 3% H_2O_2 for 15 minutes before being analysed for green fluorescence on a FACscan Becton Dickinson (Mountain View, CA, U.S.A.) flow cytometer. Arithmetic mean fluorescence channel (MFC) was derived by CellQuest® software. Increased green fluorescence intensity indicates increased intracellular oxidative stress.

Statistical Analysis

The data were expressed as mean ± standard error of measurements. The statistical difference of analyzed data was determined by using the Student's *t*-test. Comparison between groups was done using the non-parametric Mann-Whitney U test.

RESULTS AND DISCUSSION

HPLC Assay of Green Tea Catechins

Consistent with a recent study [15], six catechin species; GA (3.5 min), EGC (4.7 min), C (6.2 min), EC (7.7 min), EGCG (10.9 min) and ECG (23.2 min) were detected in the microwave-dried GT, and EGCG was the major component (Fig. **2**). The semi-preparative HPLC method separated the GT crude extract into four fractions; F1 (GA and EGC), F2 (EGCG), F3 (C), and F4 (ECG) respectively (Fig. **3**).

When each fraction was rechromatographed with the analytical HPLC, at least 80% peak purity was obtained. When compared to organic solvent extraction this adsorption chromatography is easy and efficient in preparation of individual GT catechins. The microwave drying method is more rapid and convenient than the conventional method for the

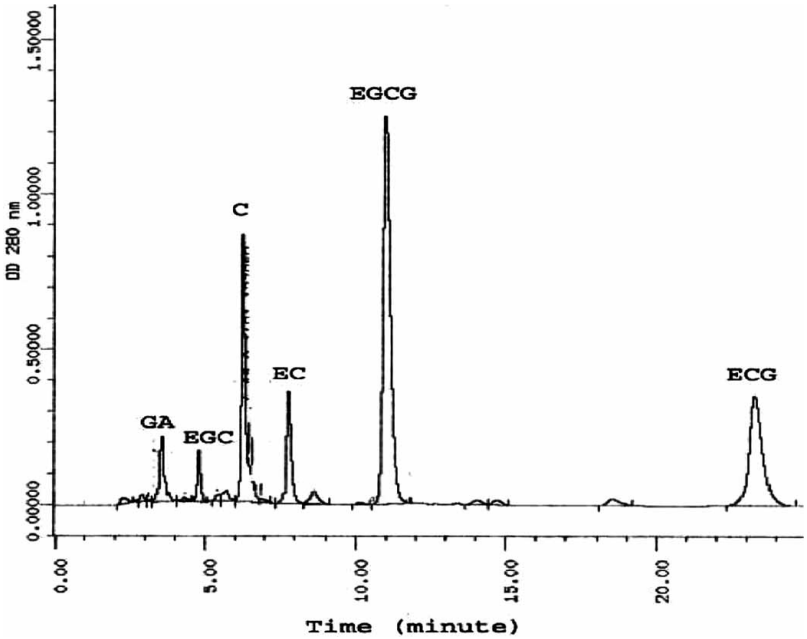

Fig. (2). HPLC analysis of catechin species from the microwave-processed green tea. GA = gallic acid, EGC = epigallocatechin, C = catechin, EC = epicatechin, EGCG = epigallocatechin 3-gallate, and ECG = epicatechin 3-gallate.

production of green tea. It can inhibit polyphenol oxidase (PPO)-catalyzed conversion of catechins to other products such as theaflavins and thearubigins and therefore high yield of catechins (particularly EGCG and ECG) are obtained.

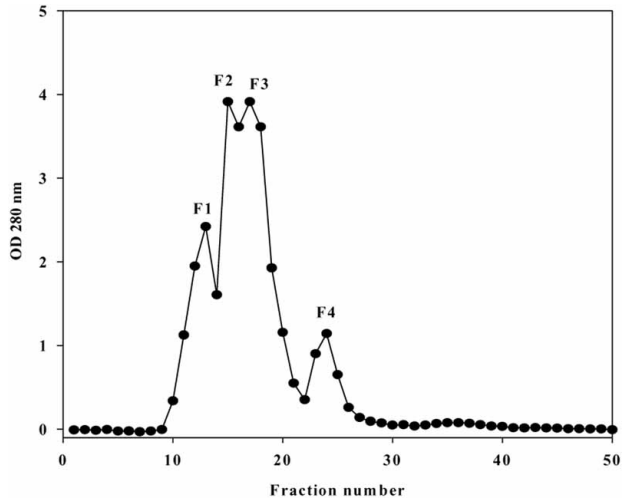

Fig. (3). Fractionation of four catechins from green tea extract. F1 (GA and EGC), F2 (EGCG), F3 (C) and F4 (ECG) were separated by a semi-preparative HPLC. Eluents were detected at a wavelength of 280 nm.

Measurement of Chemical Iron Binding

Having added the catechin fractions (200 μM) to the Fe-NTA solution (100 μM), Fe^{3+}-catechin complexes were formed and measured between 400 and 800 nm. A predominant absorption peak was observed at 560 nm for the ECG, EGCG and EGC fractions, and also at 620 nm for the C fraction (Fig. **4**).

The formation of such Fe^{3+}-catechin complexes was dose dependent and the EGCG exhibited the highest absorption and ECG was the second (Fig. **5**). Clearly, the binding of EGCG and ECG seemed to be saturated at the iron concentration above 200 μM and gradually decreased between 200-400 μM (Fig. **6**).

As shown in Fig. **7**, the formation of Fe^{3+}-EGCG and Fe^{3+}-ECG complexes was also dependent upon the incubation time when using lower iron concentrations (10-40 μM). The iron binding with EGCG and ECG was accelerated when their concentrations were increased. Their binding with iron (at 100 μM and 200 μM) was complete within 10 minutes and unchanged until 30 minutes.

Our recent spectrophotometric study correlates with the result of Grinberg *et al*. [17] and show that the GT extract bound Fe^{3+} in dose- and time-dependent manners [15]. Chromatographic and electrophoretic experiments show the interaction iron with different polyphenol fractions of green tea [25]. Interestingly, EGCG and ECG can complex with the iron *in vitro* and the galloyl group in the molecules may contribute to this activity [26].

Kinetic Removal of Plasma Non-Transferrin Bound iron

For a dose-response study, thalassemic plasma (0.45 ml) was incubated with catechin fractions (0-10 μM) (10 μl) at 37°C for 1 hour and quantified for NTBI concentration with the NTA chelation/HPLC method as mentioned above. It was found that all catechin fractions were able to decreased plasma NTBI level in the order EGCG>ECG>C>EGC, GA (Fig. **8**). Removal of NTBI by these catechin fractions was dose-dependent in the range of 0-1.25 μM, and almost unchanged even using higher concentrations (2.0-10 μM).

Results in Fig. **9** showed that the efficiency of EGCG and ECG in chelation of NTBI when assayed with the original

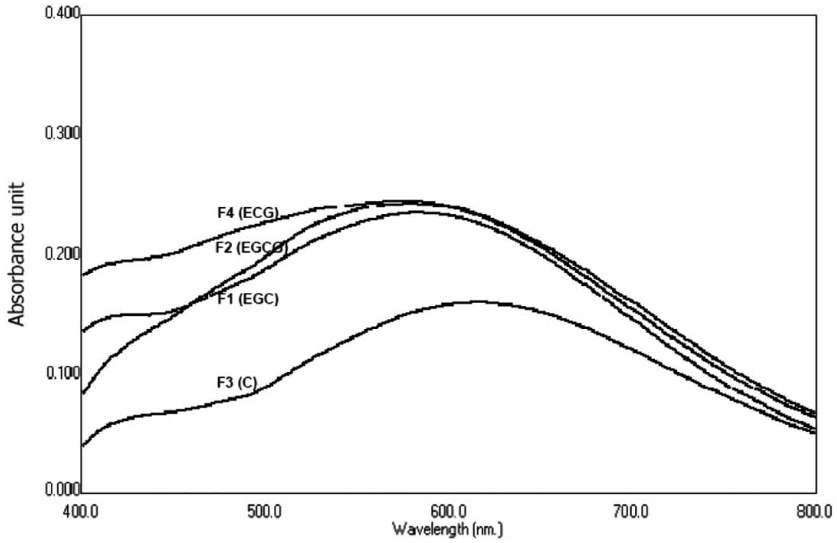

Fig. (4). Spectra of Fe^{3+}-catechin complexes.

method [22] and the modified method [15,23]. The shuttling of NTBI by excess unbound EGCG and ECG to a weak iron chelator NTA was seen, and this led to overestimated NTBI chelation.

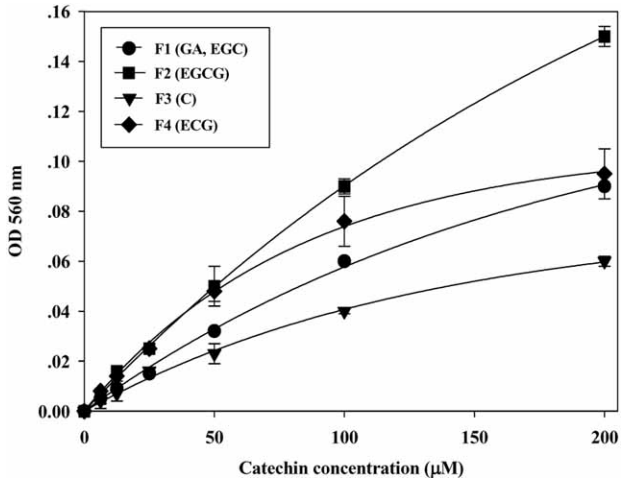

Fig. (5). Chemical binding of iron to four catechin fractions. Results obtained from three separate experiments were expressed as mean±SEM.

For a time-course study, thalassemic plasma (0.45 ml) was incubated with catechin fractions (2.5 μM) (10 μl) at 37°C for 0-8 hours and quantified for NTBI concentration with the NTA chelation/HPLC method. As shown in Fig. **10**, EGCG and ECG (2.5 μM) seemed to decrease NTBI concentrations (assayed without aluminium blocking) more rapidly than the ones assayed with the blocking step. When the iron shuttling was prevented with aluminium, they removed NTBI very slowly in time-dependent manner between 0 and 2 hours. This means that the NTBI chelation by EGCG and ECG can be divided into two phases, a rapid phase during 0-2 hours and a slow phase during 2-8 hours. Apparently, one pool of NTBI is easily chelated with a low concentration of EGCG and ECG (2.5 μM) by 2 hours, and another pool of NTBI is difficult to be chelated even at the concentrations up to 10 μM and longer incubation time up to 8 hours. When the thalassemic plasma was treated with 10 μM iron chelator for 1 hour, the EGCG, ECG, DFO and DFP removed 2.12 μM, 1.92 μM, 2.38 μM and 0.79 μM NTBI respectively. This indicates the efficacy of EGCG and ECG compared to standard iron chelators, DFO and DFP, in decreasing the levels of NTBI in thalassemic plasma.

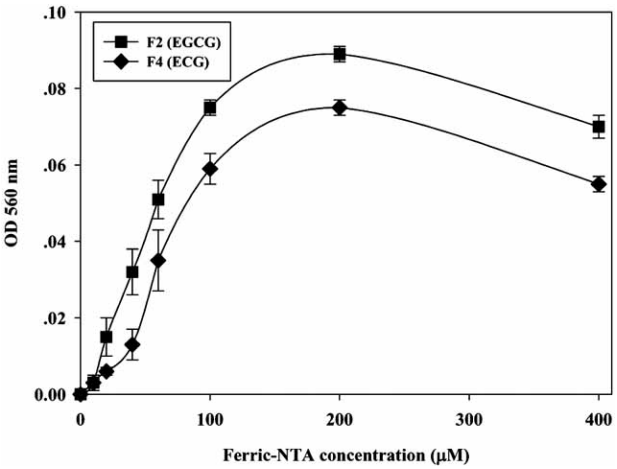

Fig. (6). Iron binding affinity of green tea EGCG and ECG. Results obtained from three independent experiments were shown as mean±SEM.

It is proposed that there may be two possible pools of NTBI, high-molecular-weight NTBI (HMW-NTBI) and low-molecular-weight NTBI (LMW-NTBI), existing in the thalassemic plasma. HMW-NTBI could be the iron loosely bound to proteins (such as albumin and ceruloplasmin) or polymerized forms (e.g. ferric oxyhydroxide polymer), and LMW-NTBI would be the iron bound to small ligands (e.g. citrate, phosphate and glutamate). The LMW-NTBI is easily chelatable, and the HMW-NTBI is inaccessible by the chelators, probably due to steric hindrance and rigid structure.

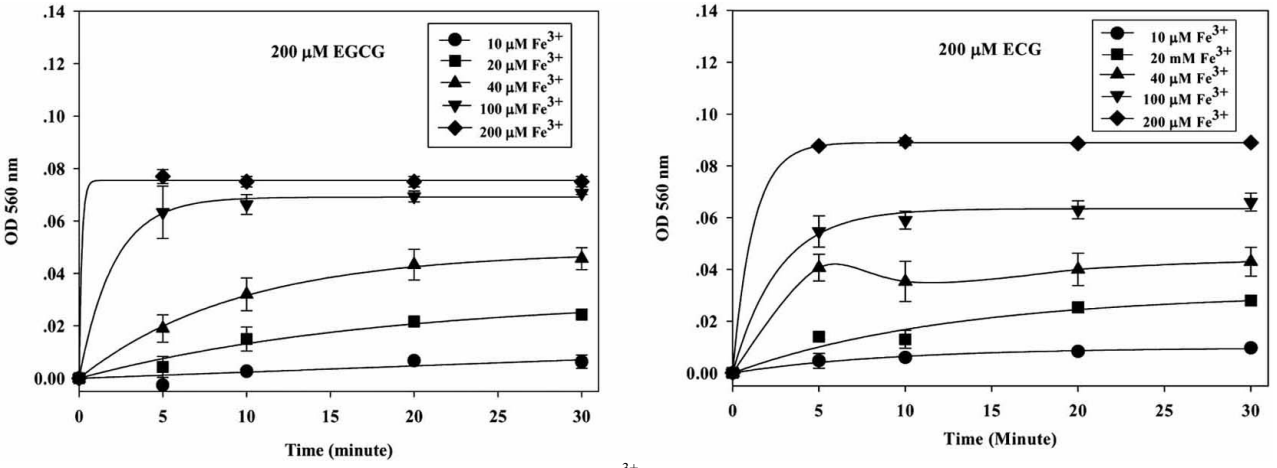

Fig. (7). Time-course binding of green tea EGCG and ECG with Fe^{3+}. Results obtained from three separate experiments were expressed as mean±SEM.

Recent studies [27,28] have suggested that labile plasma iron (LPI) (synonymous to the NTBI) is chelatable and redox-active, sustaining the LIP level with iron chelation therapy would compromise vital organ functions and the survival of the thalassemia patients.

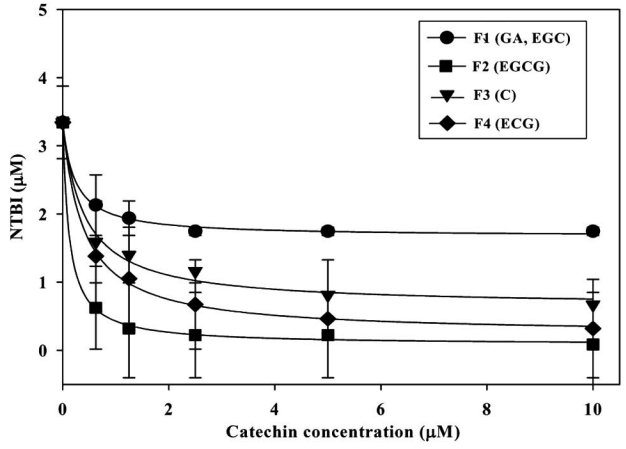

Fig. (8). NTBI chelation in pooled thalassemic serum by fractionated green tea catechins. NTBI concentration was measured using the NTA chelation/HPLC without an aluminium blocking step. Results obtained from five independent experiments were expressed as mean±SEM.

Inhibitory Effect of Iron-Induced Erythrocyte Oxidative Stress

Oxidants used in this study were chemical iron (100 μM Fe^{2+}-EDTA and 100 μM Fe^{3+}-NTA), and biological iron as thalassemic serum (TS) containing 3.2 μM NTBI. Briefly, human red blood cells were treated with EGCG and ECG (10 μM) for 1 hour before or concomitant with the oxidant treatment and the mixtures were further incubated at 37°C for the indicated time. Fluorescence intensity (FI) in the treated cells was measured using a DCF labeling/flow cytometry. As shown in Fig. **11**, oxidative stress levels were increased in the cells treated with Fe^{2+}-EDTA and Fe^{3+}-NTA, but not with TS. EGCG and ECG decreased the oxidative

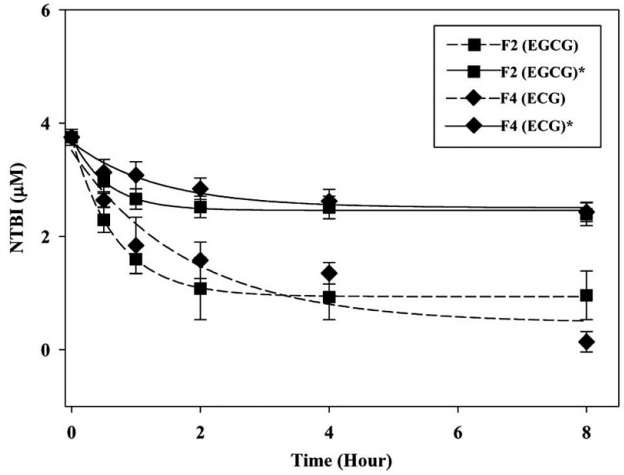

Fig. (9). Removal of plasma NTBI by F2 (EGCG) and F4 (ECG) fractions (2.5 μM) for 0-8 hours. NTBI concentration was measured using the NTA chelation/HPLC without and with the aluminium blocking step (*). Data were obtained from ten thalassemic plasmas and shown as mean±SD.

stress levels in treated cells after incubation for 1 and 8 hours. They also reduced persisting oxidative stress in the red blood cells being incubated for 8 hours. The results suggest that EGCG and ECG, both pretreatment and immediate treatment could decrease the levels of erythrocyte oxidative stress effectively by means of free-radicals scavenging or/and iron-chelating mechanisms.

Considering the flow cytometric results of erythrocyte oxidative stress, the beneficial effects may possibly be due to one of hydroxyl groups or galloyl groups in the EGCG and ECG molecules [29]. This evidence relates to a previous study done by Grinberg *et al.* [17] that one or more major GT catechins like EGCG and ECG would be potential to bind iron and account for the antioxidant effects on red blood cells. Investigation of GT catechins using diphenyl picryhydrazine (DPPH) method indicated the order of scavenging activity as ECG ≥ EGCG ≥ EGC ≥ EC [30]. However, EGCG has also been reported as a pro-oxidant being the

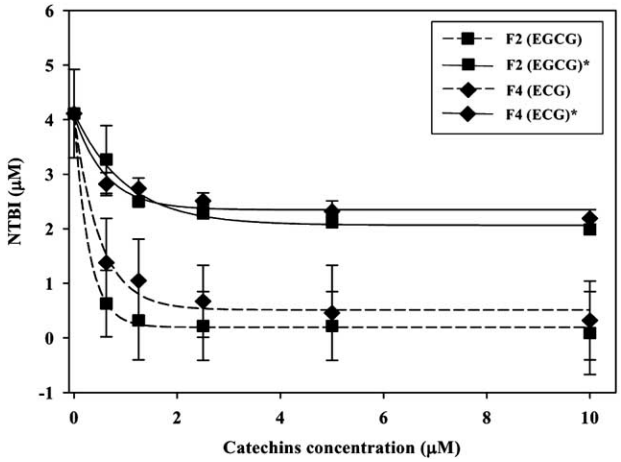

Fig. (10). Removal of plasma NTBI by F2 (EGCG) and F4 (ECG) fractions (0-10 μM) for 1 hour. NTBI concentration was measured using the NTA chelation/HPLC without and with the aluminium blocking step (*). Data were obtained from ten thalassemic plasmas and shown as mean±SD.

most effective apoptotic agent [31]. Therefore, it is realized that EGCG is a potent antioxidant at suitable concentrations and a pro-oxidant at a high dose.

These findings demonstrate that EGCG and ECG in green tea products exhibits bifunctional iron-chelating and antioxidant activities *in vitro*, possibly due to the presence of galloyl and catechol groups in the molecules. It suggests that they could be useful as phytochemical therapeutic agents in some pathological conditions, particularly iron overload in the transfusion-dependent patients with β-thalassemia who suffer from iron-induced oxidative tissue damage. It needs to further investigate the iron-chelating and antioxidative properties of GT crude extract and catechin derivatives in both animals and humans.

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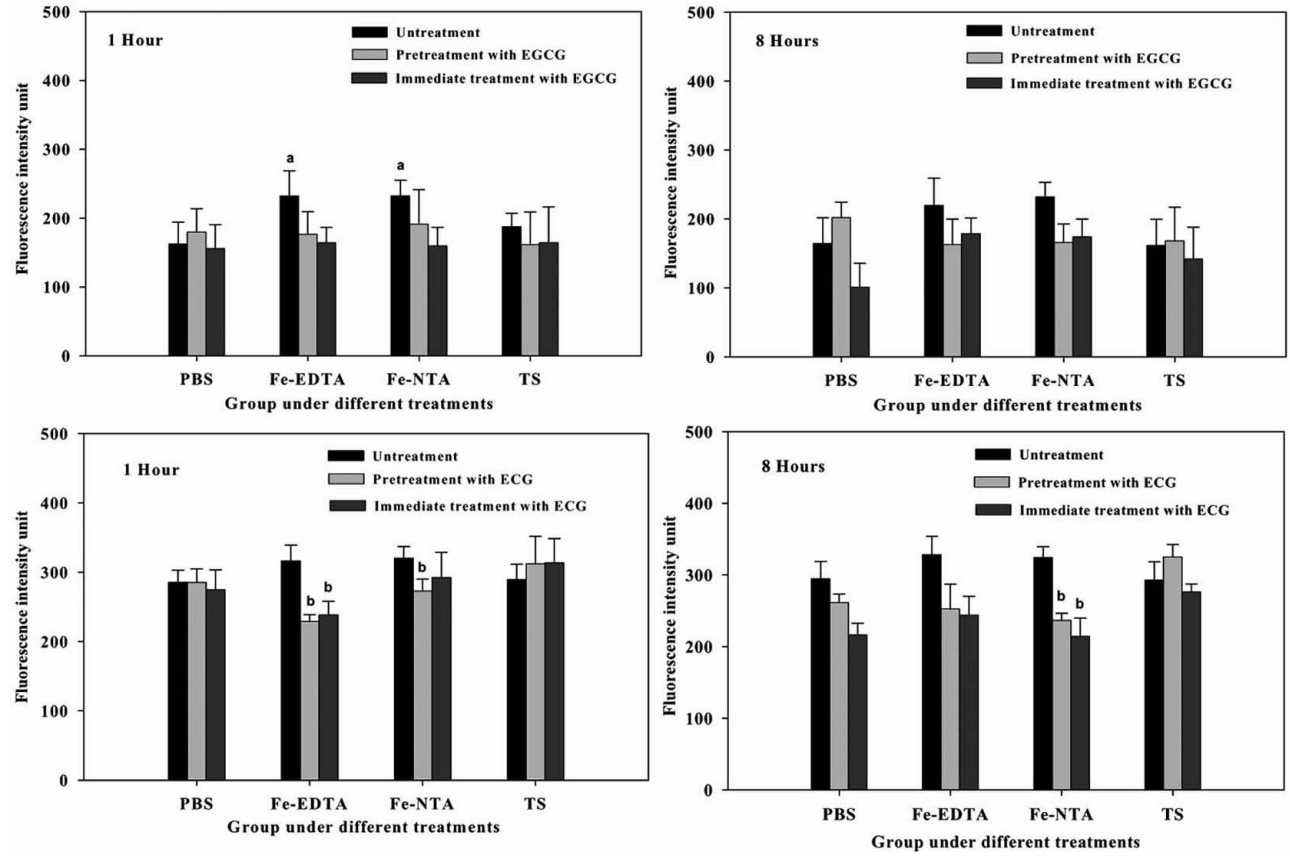

Fig. (11). Effect of EGCG and ECG treatment on oxidative stress in iron-induced human erythrocytes. Data obtained from five separate experiments and are shown as mean ± SEM. $^{a}p<0.05$ compared to PBS, $^{b}p<0.05$ compared to untreatment.

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