Original Article

Chemiluminescence analysis of the prooxidant and antioxidant effects of epigallocatechin-3-gallate

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The aim of this study was to investigate the mechanism of antioxidant and prooxidant effects of epigallocatechin-3-gallate (EGCG) using chemiluminescence analysis. Results showed that EGCG scavenged superoxide radical and H_2O_2 in a dose dependent manner. EGCG scavenged 50% of superoxide radical at 0.31 mM and scavenged 50% of H_2O_2 at 0.09 mM, demonstrating that EGCG has a stronger reactive oxygen species (ROS) scavenging activity than ascorbic acid. Effects of EGCG on free radical-induced DNA oxidative damage were investigated. EGCG had protective effect on DNA at low concentrations (2-30 mM), but it enhanced the DNA oxidative damage at higher concentrations (>60 mM), exhibiting a prooxidant effect on DNA. EGCG showed a greater reducing power on iron ions, reducing Fe^{3+} to Fe^{2+}, which accelerates the generation of hydroxyl radical from the Fenton reaction. At low concentrations, ROS scavenging activity of EGCG might predominate over its reducing power and lead to its protective effect on DNA. However, relatively higher reducing power of EGCG at higher concentrations may gradually predominate over its ROS scavenging activity and result in the prooxidant effect of EGCG on DNA.

Key Words: epigallocatechin-3-gallate, chemiluminescence analysis, prooxidant effect, reactive oxygen species scavenging activity, reducing power

Introduction

Tea is a popular beverage in Asia–Pacific region. It has been reported to have multiple healthy effects,[1] including anticancer and antimicrobial effects. It can lower blood lipid and glucose. Epidemiology shows that it can decrease the risk of tuberculosis and cardiovascular diseases.[2] These healthy effects were mostly attributed to its antioxidant components - polyphenols including epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), and epigallocatechin-3-gallate (EGCG). EGCG accounts for more than 40% of the total polyphenols and has been identified as the most potent antioxidant component in tea.[3] EGCG had remarkable protective effects against lipid peroxidation in synaptosomes.[4] It was found to inhibit the paraquat-induced microsomal malondialdehyde (MDA) productions in rat liver.[5] However, there are conflicting reports on the effects of EGCG. Recently, negative results of EGCG on resistance of lipid peroxidation in humans have been reported.[6] EGCG at high doses showed a cellular toxicity on experimental animals.[7] This active component seems to have different effects at different treatment doses and conditions. It was reported that EGCG had different effects in different cellular compartments of PC12 cells, and high concentrations of EGCG treatment resulted in an increased DNA breakdown and activation of apoptotic markers in the cells.[8] However, the mechanism of the concentration-dependent effects of EGCG on biological macromolecules such as DNA has not been clearly elucidated.

In the present paper, the antioxidant and prooxidant effects of EGCG were investigated by using chemiluminescence analysis. EGCG has dual activities: reactive oxygen species (ROS) scavenging activity and reducing power on metal ions. These activities were measured and the possible mechanism of its prooxidant effect on DNA was discussed.

Materials and methods

Materials

EGCG and ascorbic acid were purchased from Sigma Chemical Company (St. Louis, MO, USA). The structures of these two compounds are shown in Figure 1. The samples were dissolved in deionized water or dimethyl sulfoxide (DMSO) according to different assays. Calf thymus DNA (sodium salt) and luminol were purchased from Sigma Chemical Company. All other chemicals were of analytical grade.

Measurement of scavenging activity of EGCG on Superoxide radical (O_2^-)

O_2^- was generated from a pyrogallol autoxidation system.[9] The reaction mixture contained 50 μL of pyrogallol (0.3125 mM), 10 μL of tested sample or DMSO (in control experiment).

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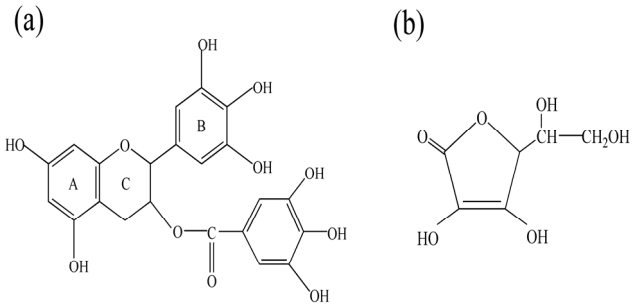

Figure 1 Structures of EGCG (a) and ascorbic acid (b).

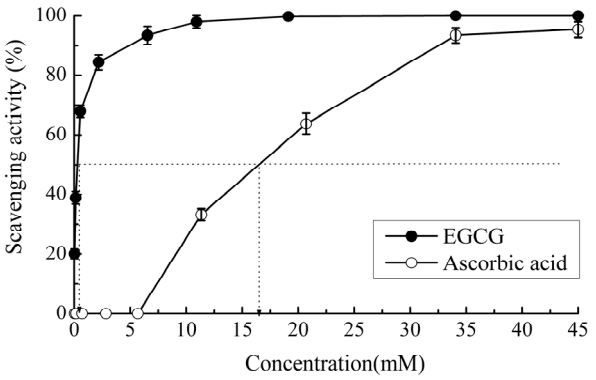

Figure 2. Scavenging effects of EGCG and ascorbic acid on O_2^-. The O_2^- was generated in a pyrogallol autoxidation system, to which 10 µl tested sample at various concentrations and 400 µl luminol (1mM) were added. Dotted line shows the value of EC_{50} (50% effective concentration). Average values of triplicate experiments are shown.

Luminescence was counted every 6 s (expressed as 'Counts/6s') on a BPCL Model Ultra Weak Chemiluminescence Analyzer (Institute of Biophysics, Academia Sinica, Beijing, China) at 30 ˚C. The amount of luminosity (total counts) was integrated.

Measurement of scavenging activity of EGCG on H_2O_2

H_2O_2 scavenging activity was assayed according to the method as described by Olinescu.[10] The reaction mixture was made of 50 µL of luminol (1 mM), 700 µL of 50 mM carbonic acid buffered saline solution (pH 10.2, containing 0.1 mM EDTA), and 10 µL of tested sample or DMSO (in control experiment). 10 µL of 50 mM H_2O_2 was added to trigger the chemiluminescence reaction. Luminescence was counted every second (expressed as 'Counts/s') on BPCL Model Ultra Weak Chemiluminescence Analyzer at 37 ˚C. The amount of luminosity (total counts) was integrated. Scavenging activities (%) were calculated by the equation as described.[9]

Effect of EGCG on hydroxyl radical induced DNA damage

The effects of EGCG on DNA damage induced by hydroxyl radical were assayed using the method as described in our previous paper with some modifications.[9] Reactants were added in turn and mixed as following: 50 µL of 12 mM $FeSO_4$, 50 µL of 1 mM luminol, 840 µL of 50 mM phosphate buffer (pH 7.4), 20 µL of 50 µg/mL DNA and 10 µL of tested sample at various concentrations. After recording the luminosity of background (CL_0), the reaction was started by the addition of 50 µL of 0.8 M H_2O_2. Luminescence was counted once every 5 s at 37 ˚C. The amount of luminosity was integrated, and the antioxidant or prooxidant effect of EGCG was represented by its luminosity percentage of the control. Chemiluminsence peak lag time was expressed as the difference value between the peak time of tested sample and the control.

Measurements of reducing power of EGCG on iron ions

Reducing power on iron ion was measured according to the method as described by Yen et al.[11] 0.5 mL of sample was added into 0.5 mL of 1% potassium ferricyanide [$K_3Fe(CN)_6$] and the mixture was incubated at 50 °C for 20 min. 0.5 mL of 10% trichloroacetic acid was added, and then the mixture was centrifuged at 3000 × g for 10 min. The upper layer of the solution (1.0 mL) was mixed with 1 mL of distilled water and 0.2 mL of 0.1 % ferric chloride ($FeCl_3$), and the absorbance was measured at 700 nm. Higher absorbance indicated greater reducing power.

Statistical analysis

Data analyses were processed using Microcal Origin 6.0 software (Microcal Software, Inc., Northampton, MA, USA). All the experiments were performed three times and the values were represented as mean ± SD. Results were assessed by Student's t test, and $p<0.05$ was considered as significant.

Results

Figure 2 shows the scavenging activities of EGCG on O_2^-. O_2^- generated in a pyrogallol autoxidation system induced the chemiluminescence reaction. The chemiluminescence intensity (luminosity) is dependent on the quantity of O_2^- generated from the reaction system. Therefore, the chemiluminescence reaction system can be used to evaluate the scavenging activity of tested samples on O_2^-. The scavenging activities of EGCG on O_2^- dose-dependently increased from 0 to 5 mM and kept at higher than 90% with the increase of concentration. Ascorbic acid showed little scavenging ability on O_2^- at low concentrations (0-5 mM), but it exhibited increasing scavenging activity with the increasing concentration. EGCG had higher scavenging activities on O_2^- with a 50% effective concentration (EC_{50}, the concentration of tested sample scavenging 50% of free radicals) of 0.31 mM, compared to that of ascorbic acid (EC_{50}=16.53 mM). The stronger scavenging effect of EGCG on O_2^- than ascorbic acid may be due to the different structure between these two compounds as shown in Figure 1. EGCG is a polyphenol compound with multiple phenolic hydroxyl group substitutions that were considered to be a characteristic of strong free radical scavengers.[4]

As presented in Figure 3, both EGCG and ascorbic acid acted as strong scavengers of H_2O_2 in a concentration-dependent manner. EGCG showed higher scavenging activity on H_2O_2 than ascorbic acid. At concentrations of 0.24 mM, 0.96 mM and 1.4 mM, EGCG scavenged H_2O_2 by about 83%, 97% and 99% respectively (EC_{50}=0.09 mM). However, at concentrations of 0.35 mM, 0.96 mM and 1.4 mM, ascorbic acid scavenged H_2O_2 by about 57%,77% and 85% respectively (EC_{50}=0.23 mM). Com-

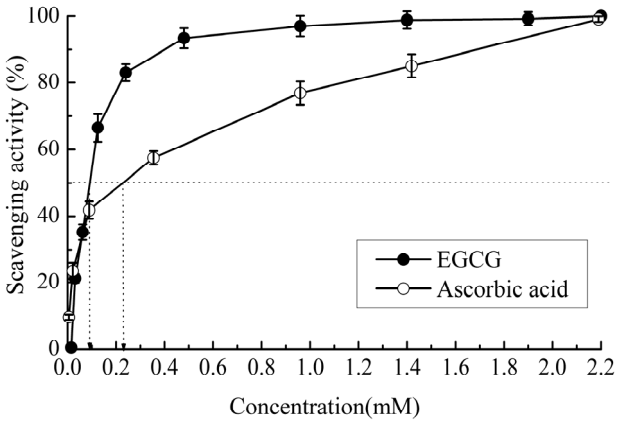

Figure 3. Scavenging effects of EGCG and ascorbic acid on H_2O_2. Chemiluminescence was induced by H_2O_2 reacting with luminol and scavenging activity of tested samples at various concentrations was measured. Dotted line shows the value of EC_{50} (50% effective concentration). Average values of triplicate experiments are shown.

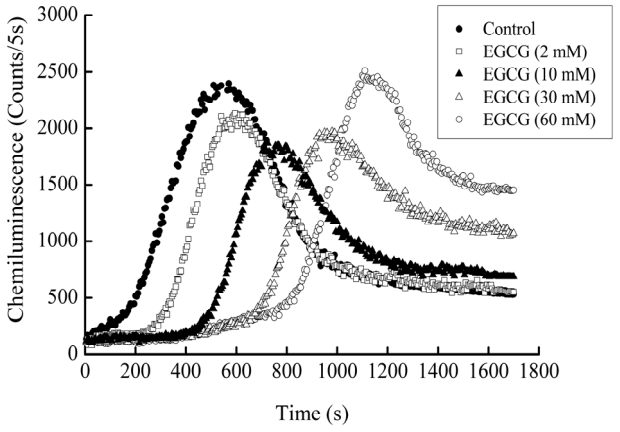

Figure 4. Effects of EGCG on hydroxyl radical induced DNA damage chemiluminescence. Hydroxyl radical was generated from a Fenton reaction system, to which tested samples at various concentrations were added.

paring with their scavenging effects on O_2^-, EGCG and ascorbic acid showed higher H_2O_2 scavenging abilities even at low concentrations.

Oxidized bases formed during DNA damage induced by hydroxyl radical (·OH) have characteristic chemiluminescence that reflects the extent of DNA damage as shown in Figure 4. EGCG at different concentrations showed different effects on ·OH induced DNA damage. At low concentrations from 2 – 30 mM, EGCG remarkably reduced the DNA damage chemiluminescence. At the concentrations of 2 mM and 10 mM, EGCG suppressed the chemiluminescence of ·OH induced DNA damage by about 13% and 19% as listed in Table 1. However, EGCG at concentration higher than 60 mM significantly enhanced the DNA damage, indicating a prooxidant effect of EGCG on DNA. Moreover, the chemiluminescence peak shifted backward in the presence of EGCG as shown in Figure 4, suggesting that the ·OH induced chemiluminescence reaction was delayed by EGCG (Table 1). These results indicated that the presence of EGCG had different effects on DNA at different concentrations and changed the progress of ·OH induced DNA damage reaction.

In a Fenton reaction, Fe^{2+} reacts with H_2O_2 and results

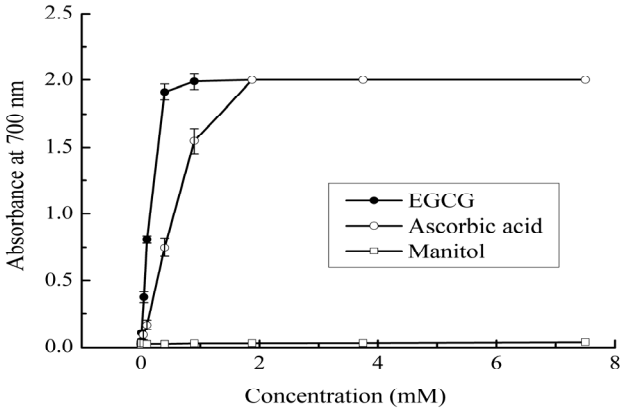

Figure 5. Reducing power of EGCG, ascorbic acid and manitol. Values are means of three experiments ± standard deviation (mean ± SD). Higher absorbance at 700 nm indicated greater reducing power.

Table 1. Chemiluminescence assay of antioxidant and prooxidant effects of EGCG on DNA.

Concentration (mM)	Luminosity (%)	Peak lag time (s)
2	87.4 ±1.94 *	25 ± 2.5
10	81.5 ± 2.16 *	155 ± 4.5
30	90.9 ± 2.07 *	445 ± 5.0
60	104.9 ± 1.84 *	540 ± 6.5

Luminosity (%) is the luminosity percentage of tested sample (EGCG) of the control (in the absence of EGCG). *Significantly different from the control, as calculated by Student's *t* test (p<0.05). Chemiluminsence peak lag time was expressed as difference value between the peak time of tested sample and the control. All data was obtained from triplicate experiments and represented as means ± SD.

in the production of hydroxyl radical, which was considered to be the most harmful radical to biomolecules.[12] Fe^{2+} is oxidized to Fe^{3+} in Fenton reaction. By many reductants, the oxidized form of iron ions can be reduced to its reduced form (Fe^{2+}), which can enhance the generation of hydroxyl radicals and resulted in the increased DNA damage. To elucidate the role of reducing power in the prooxidant effect of EGCG, the ability of EGCG on reduction of Fe^{3+} to Fe^{2+} was measured as shown in Figure 5. EGCG exhibited greater reducing power than that of ascorbic acid and manitol. The absorbance at 700 nm of EGCG was higher than 1.9 when its concentration was at 0.9 mM and above. The reducing power of EGCG increased as its concentration increasing. Manitol exhibited little reducing power at all tested concentrations, indicating that it has a weak reducing power on iron ions.

Discussion

ROS including superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH) are generated during normal aerobic metabolism and can be increased under the oxidative stress.[13] ROS are considered to cause oxidative damage to DNA, lipids, proteins and other biological macromolecules, which can lead to cancer and other diseases.[14-15] Drinking tea has been proved to be beneficial to human health. Tea polyphenols including

EGCG have been suggested as cancer chemopreventatives, due to its proxyl radicals scavenging and lipid peroxidation inhibiting effects.[16] Dietary EGCG decreased total plasma cholesterol and non-HDL cholesterol concentrations in rat.[17] It was also reported that EGCG alleviated the oxidation stress and normalized renal function of rats.[18] These suggest that EGCG can work as an important antioxidant in living systems.

However, there are conflicting reports on the effects of EGCG. Excessive doses use of EGCG was found to have harmful effect (prooxidant effect) on experimental animals.[8] It has been reported that tea polyphenols in cell culture systems produce free radicals, which may be responsible for its cellular toxicity.[7] The active component seems to have different effects at different treatment doses and conditions. However, it has not been clearly understood on the concentration-dependent effects of EGCG on biomolecules. In this paper, the ROS scavenging effects of EGCG were investigated at a wide range of concentration, and the prooxidant effect of EGCG on ·OH induced DNA damage was also evaluated using a chemiluminescence method. Our results demonstrated that EGCG is a strong scavenger on ROS including O_2^- and H_2O_2, compared with ascorbic acid. EGCG was also reported to have strong scavenging effect on hydroxyl radical.[4] Its antioxidant activity may correlate with the number of phenolic hydroxyl group on its structure, which can donate electrons to react with radicals. Moreover, the stability of the formed semiquinone radicals from the reaction of EGCG with ROS may also contribute a lot to its scavenging effect on ROS.

Our results showed that EGCG at low concentrations had protective effect on DNA in the ·OH induced DNA damage reaction system. At higher concentrations, EGCG showed a prooxidant effect on DNA. In the Fenton reaction system, Fe^{2+} reacts with H_2O_2, resulting in the production of hydroxyl radical, which leads to the DNA damage. Fe^{2+} is oxidized to Fe^{3+} in the Fenton reaction. The oxidized form of iron ions can be reduced by reductants to its reduced form (Fe^{2+}), which can enhance the generation of hydroxyl radicals. EGCG exhibited a great reducing power on iron ions, especially at high concentrations. The differential effects (prooxidant or antioxidant effect) of EGCG on DNA might be due to the balance of its dual activities: ROS scavenging activity, and reducing power on iron ions which may drive the Fenton reaction via reduction of iron ions as indicated in Figure 6. The relatively stronger ROS scavenging ability of EGCG at low concentrations protects DNA against oxidative damage. With the increase of concentration, the reducing power of EGCG on iron ion, which accelerates the generation of hydroxyl radical from the Fenton reaction, may gradually predominate over its ROS scavenging ability and result in its prooxidant effect on DNA. The toxicity of EGCG on cells was decreased in the presence of deferoxamine, a chelator of iron ion.[19] This also supported that the prooxidant effect of EGCG is due to its reduction on iron ions. It was reported that in vitro Cu^{2+}-induced- human low density lipoprotein oxidation can be accelerated in the presence of EGCG,[20] which converts Cu^{2+} into Cu^+. The prooxidant effect of EGCG was similar to that obtained by ascorbic acid which acts as a prooxidant in the presence of free transition metal ions by accelerating the Fenton reaction and lipid peroxidation.[5] Some phenolic compounds including ascorbic acid at high concentrations were reported having the ability of reducing iron ions and generating the cytotoxic hydroxyl radical from the Fenton reaction in culture media.[21] It was found that the concentration of EGCG in healthy human plasma was in the range of 0.3 – 4 mM.[22] These facts indicate that catechins can act as antioxidants at a lower concentration than expected. Moreover, we observed that the DNA damage chemiluminscence peak was delayed in the presence of EGCG, and the peak lag time increased with the increasing concentration of EGCG, indicating that the reaction progress was retarded with the effect of EGCG. The delayed reaction may be due to the scavenging ability of EGCG on H_2O_2, which is one of the reactants in Fenton system, however, the detail mechanism need to be further investigated.

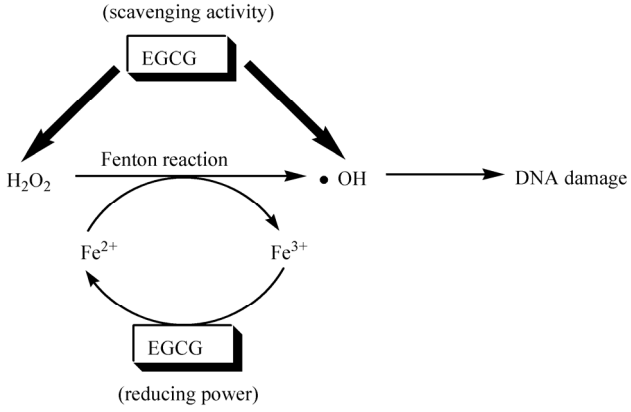

Figure 6. Scheme of effects of EGCG on hydroxyl radical-induced DNA damage.

In this paper, EGCG was investigated for its antioxidant and prooxidant effects on DNA using a chemiluminescence method. EGCG showed a concentration-dependent antioxidant or prooxidant effect on DNA. The effects of EGCG on DNA may be due to the balance of two activities including ROS scavenging activity and reducing power on iron ions. The predomination of reducing power on iron ions over ROS scavenging activity of EGCG at high concentrations might result in the prooxidant effect of EGCG on DNA. Although further research is necessary to investigate the antioxidant and prooxidant mechanisms of EGCG at physiological levels, the present results suggest that dose selection may be very important in the application of EGCG.

Acknowledgements

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References

1. Zhao WH, Chen JS. Candidate foods in the Asia–Pacific region for cardiovascular protection: Oriental tea. Asia Pacific J Clin Nutr 2001; 10: 138 –142.
2. Anand PK, Kaul D, Sharma M. Green tea polyphenol inhibits *Mycobacterium tuberculosis* survival within human macrophages. Int J Biochem Cell Biol 2006; 38: 600-609.
3. Orzechowski A, Ostaszewski P, Jank M, Berwid SJ. Bioactive substances of plant origin in food-impact on genomics. Reprod Nutr Dev 2002; 42: 461-477.

4. Guo Q, Zhao BL, Li MF, Shen SR, Xin WJ. Studies on protective mechanism of four components of green tea polyphenols against lipid peroxidation in synaptosomes. Biochim Biophys Acta 1996; 1304: 210-222.
5. Higuchi A, Yonemitsu K, Koreeda A, Tsunenari S. Inhibitory activity of epigallocatechin gallate (EGCg) in paraquat-induced microsomal lipid peroxidation - a mechanism of protective effects of EGCg against paraquat toxicity. Toxicology 2003; 183: 143 –149.
6. Van het Hof KH, de Boer HSM, Wiseman SA, Lien N, Weststrate JA, Tijburg LBM. Consumption of green or black tea does not increase resistancs of low-density lipoprotein to oxidation in humans. Am J Clin Nutr 1997; 66:1125-1132.
7. Chai PC, Long LH, Halliwell B. Contribution of hydrogen peroxide to the cytotoxicity of green tea and red wines. Biochem Biophys Res Commun 2003; 304: 650-654.
8. Raza H, John A. Green tea polyphenol epigallocatechin-3-gallate differentially modulates oxidative stress in PC12 cell compartments. Toxicol Appl Pharmacol 2005; 207: 212 - 220.
9. Tian B, Wu Y, Sheng D, Zheng Z, Gao G, Hua Y. Chemiluminescence assay for reactive oxygen species scavenging activities and inhibition on oxidative damage of DNA in *Deinococcus radiodurans.* Luminescence 2004; 19: 78-84.
10. Olinescu RM, Kummerow FA. Fibrinogen is an efficient antioxidant. J Nutr Biochem 2001; 12: 162-169.
11. Yen GC, Duh PD, Chuang DY. Antioxidant activity of anthraquinones and anthrone. Food Chem 2000; 70: 437 - 441.
12. Meneghini R. Iron homeostasis, oxidative stress, and DNA damage. Free Rad Biol Med 1997; 23: 783-792.
13. Fridovich I. The biology of oxygen radicals. Science 1978; 201: 875 -880.
14. Floyd RA, West MS, Eneff KL, Schneider JE. Methylene blue plus light mediates 8-hydroxyguanine formation in DNA. Arch Biochem Biophys 1989; 273: 106 -111.
15. Pansarasa O, Bertorelli L, Vecchiet J, Felzani G, Marzatico F. Age-dependent changes of antioxidant activities and markers of free radical damage in human skeletal muscle. Free Rad Biol Med 1999; 27: 617- 622.
16. Saffari Y, Sadrzadeh SM. Green tea metabolite EGCG protects membranes against oxidative damage in vitro. Life Sci. 2004; 74: 1513 - 1518.
17. Raederstorff DG, Schlachter MF, Elste V, Weber P. Effect of EGCG on lipid absorption and plasma lipid levels in rats. J Nutr Biochem 2003; 14: 326 - 332.
18. Rhee SJ, Kim MJ, Kwag OG. Effects of green tea catechin on prostaglandin synthesis of renal glomerular and renal dysfunction in streptozotocin-induced diabetic rats. Asia Pacific J Clin Nutr 2002; 11: 232 - 236.
19. Weisburg JH, Weissman DB, Sedaghat T, Babich H. In vitro cytotoxicity of epigallocatechin gallate and tea extracts to cancerous and normal cells from the human oral cavity. Basic Clin Pharmacol Toxicol 2004; 95:191-200.
20. Hu C, Kitts DD. Evaluation of antioxidant activity of epigallocatechin gallate in biphasic model systems in vitro. Mol Cell Biochem 2001;218:147-155.
21. Harel S, Kanner J. Haemoglobin and myoglobin as inhibitors of hydroxyl radical generation in a model system of “iron redox” cycle. Free Radical Res Commun 1989; 6: 1-10.
22. Ueno T, Kondo K, Itakura H, Takeo T. Analysis of (-)-epigallocatechin gallate in human serum obtained after ingesting green tea. Biosci Biotech Biochem 1996; 60:1558–1559.