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AN *IN VIVO* AND *IN VITRO* STUDIES ON THE ANTIOXIDANT PROPERTY OF EPIGALLOCATECHIN GALLATE ON SODIUM FLUORIDE INDUCED TOXICITY IN RATS

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ABSTRACT

In the present study was designed to explore the protective effect of EGCG against NaF induced changes in plasma lipid peroxidation and antioxidant status such as Vitamin C , E, and reduced glutathione and *In vitro* free radical scavenging assays of EGCG. Administration of NaF (25 mg/kg bw) orally for 4 weeks significantly ($p<0.05$) elevated the levels of lipid peroxidation and decreased the levels of non-enzymatic antioxidants like GSH, Vit.C and E in the plasma of rats. Administration of EGCG revealed a significant ($p<0.05$) decrease in lipid peroxidation with a significant ($p<0.05$) increase in the antioxidant status in the plasma of fluoride treated rats. For *in vitro* scavenging effect of EGCG, five different concentrations (10, 20, 30, 40, and 50 µM) were used. Free radical scavenging activity was evaluated by following invitro assays such as 1,1-diphenyl-2- picrylhydrazyl (DPPH•), 2,2-azinobis-(3-ethylbenzothiazoline- 6-sulfonic acid) (ABTS), superoxide anion (O•), hydroxyl (OH•) radicals, reducing power assay, total antioxidant assay, metal chelating assay and nitric oxide (NO) assay. In addition to that, ascorbic acid and butylated hydroxyl toluene was used as the standard antioxidants for free radical scavenging assays. The results confirmed that, the free radical scavenging and antioxidant activity of EGCG has increasing with increasing concentrations. Interestingly, among the different concentrations tested, 40 µM of EGCG showed the highest antioxidant and free radical scavenging activities in almost all the *in vitro* assays. Hence the present study proved that the EGCG has potent *In vitro* and *in vivo* antioxidant activity which augmenting its therapeutic potential against fluoride induced toxicity.

Key words: EGCG, Antioxidant, *in vivo*, *in vitro*, NaF, Rat.

INTRODUCTION

Fluoride is ubiquitous in the environment; therefore, sources of drinking water are likely to contain small amount of fluoride. However, in areas of the world in which endemic fluorosis of the skeleton or teeth has been well documented, the level of fluoride in drinking water supplies range from 3 to more than 20 mg/l. In areas in which drinking water is fluoridated (i.e., fluoride is intentionally added for the prevention of dental caries), the concentration of fluoride in drinking water generally ranges from 0.7 to 1.2 mg/l. Low intakes of fluoride are associated with an increased incidence of dental caries and addition of fluoride, at 1 mg/kg to water supplies reduces this. The body burden of fluoride is regulated by renal excretion. Fluorosis is excessive deposition of fluoride particularly in the bones and teeth. It occurs when the daily intake exceeds 20 mg of fluoride (WHO, 2002). F^- is a potent inhibitor of many enzymes and was used as an important tool to define certain steps in the glycolytic pathway (Whitford 1996). Recent studies revealed that NaF induces excessive production of oxygen free radicals, and might cause the depletion in biological activities of some antioxidant enzymes (Chlubek 2003). Free radicals have been implicated in the causation of several problems like asthma, cancer, cardiovascular disease, cataract, diabetes, gastrointestinal inflammatory disease, liver disease, muscular degeneration and other

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inflammatory process (Sen *et al*., 2008). Reactive oxygen species (ROS) are continuously produced during cell metabolism and under normal circumstances they are scavenged and renewed to nonreactive species by diverse intracellular enzymatic and non-enzymatic antioxidant system (Shao *et al*., 2008). Over production of ROS may induce oxidative stress and cause damage to all types of biomolecules such as proteins, lipids and nucleic acids (Droge 2002). Antioxidants may act as free radical scavengers, reducing agents, chelating agents for transition metals, quenchers of singlet oxygen molecules and or activators of antioxidative defense enzyme system to suppress the radical damages in biological systems (Murphy *et al*., 2011; Venkatesh *et al*., 2009). Antioxidants thus play an important role in the protection of human body against damage by reactive oxygen species (Peng *et al*., 2011; Ling *et al*., 2011). Therefore, inhibition of free radical-induced oxidative damage by supplementation of antioxidants has become an attractive therapeutic strategy for reducing the risk of these diseases. In recent years, it has been investigated that many plant species are serving as source of antioxidants and received therapeutic significance (Pari and Amutha, 2011). Antioxidants thus play an important role in the protection of human body against damage by reactive oxygen species (Thambiraj *et al*., 2012). Therefore, inhibition of free radical-induced oxidative damage by supplementation of antioxidant has become an attractive therapeutic strategy for reducing the risk of these diseases. In recent year epigallocatechin gallate (EGCG), known to be most abundant in green tea, is an effective scavenger of ROS *in vitro* and may also function indirectly as antioxidant through its effects on transcription factors and enzyme activities (Na and Surh, 2008; Sriram *et al*., 2009). EGCG is also a useful agent to protect against protein oxidation- and glycation-associated diseases (Sriram *et al*., 2009). EGCG is known to block many targets in signal transduction pathways, including NFkB and Nrf2 (Na and Surh, 2008; Sriram *et al*., 2009). Therefore the objective of the present investigation was intended to explore the ameliorative potency of epigallocatechin gallate via its *In-vitro* and *In-vivo* models.

MATERIALS AND METHODS

Drug and Chemicals

EGCG was purchased from Sigma Aldrich, USA. Sodium fluoride, reduced glutathione, butylated hydroxytoluene (BHT), 2,2′- dipyridyl, xylenol orange, 4-dinitrophenylhydrazine (DNPH), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), DPPH, ABTS and butylated hydroxytoulene , ascorbic acid were obtained from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals and solvents were of certified analytical grade and purchased from S.D. Fine Chemicals, Mumbai or Hi media Laboratories Pvt. Ltd., Mumbai, India. Reagent kits were obtained from span Diagnostics, Mumbai, India.

Animals

Male albino rats of Wistar strain with a body weight ranging from 160 to 180 g, were procured from Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College and Hospital, Annamalai University, and were maintained in an air conditioned room (25±1 °C) with a 12 h light/12 h dark cycle. Feed and water were provided ad libitum to all the animals. The study was approved by the Institutional Animal Ethics Committee of Rajah Muthiah Medical College and Hospital (Registration Number: 160/1999/CPCSEA, Proposal number: 952/2012), Annamalai University, Annamalainagar.

Experimental design

The animals were randomly divided into four groups of six rats in each group.

Group 1: Control rats treated with normal saline and corn oil for 28 days.

Group 2: Normal rats received EGCG (40mg/kg body weight) (Thangapandiyan and Miltonprabu 2013) dissolved in corn oil and administered orally using an intragastric tube for 28 days.

Group 3: Rats received fluoride as sodium fluoride (25 mg/kg body weight) (Chinoy 1991) in normal saline for 28 days.

Group 4: Rats received NaF (25mg/kg body weight) with oral pre-administration of EGCG (40 mg/kg body weight) for 28 days

At the end of the experimental period, animals in different groups were sacrificed by cervical decapitation. Blood samples were collected in heparinised tubes, for plasma. Plasma separated by centrifugation was used for various biochemical estimations.

BIOCHEMICAL ASSAYS

In vivo assays

Estimation of lipid peroxidation

Lipid peroxidation in plasma was estimated spectrophotometrically by measuring thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides (LOOH) by the method of Niehiaus and Samuelsson (1968) and Jiang (1992), respectively. In brief, plasma (0.1 ml) was treated with 2ml of TBA–trichloroacetic acid (TCA)–HCl reagent (0.37% TBA, 0.25N HCl and 15% TCA, 1:1:1 ratio) placed in a water bath for 15 min and cooled and centrifuged at room temperature, clear supernatant was measured at 535 nm against a reagent blank. A 0.1 ml aliquot of plasma was treated with 0.9 ml of Fox reagent (88 mg of butylated hydroxytoluene, 7.6 mg of xylenol orange and 0.8 mg of ammonium iron sulfate were added to 90 ml methanol and 10 ml of 250

mM sulfuric acid) and incubated at 37°C for 30 min. The colour that developed was read at 560 nm.

Determination of non-enzymatic antioxidants

Ascorbic acid (vitamin C) concentration was measured by Omaye (1979). To 0.5 mL of plasma, 1.5 mL of 6% TCA was added and centrifuged (3500 ×g, 20 min). To 0.5 mL of supernatant, 0.5 mL of DNPH reagent (2% DNPH and 4% thiourea in 9N sulfuric acid) was added and incubated for 3 h at room temperature. After incubation, 2.5 mL of 85% sulfuric acid was added and colour developed was read at 530 nm after 30 min. Vitamin E was estimated by the method of Desai (1984). Vitamin E was extracted from plasma by addition of 1.6 mL ethanol and 2.0 mL petroleum ether to 0.5 mL plasma and centrifuged. The supernatant was separated and evaporated on air. To the residue, 0.2 mL of 0.2% 2, 2-dipyridyl, 0.2 mL of 0.5% ferric chloride was added and kept in dark for 5 min. An intense red coloured layer obtained on addition of 4 mL butanol was read at 520 nm. Reduced glutathione (GSH) was determined by the method of Ellman (1959). 1 mL of supernatant was treated with 0.5 mL of Ellman's reagent (19.8 mg of 5, 5-dithiobisnitro benzoic acid in 100 mL of 0.1% sodium citrate) and 3.0 mL of phosphate buffer (0.2 M, pH 8.0) was added and the absorbance was read at 412 nm in spectrophotometer.

DETERMINATION OF *IN-VITRO* ACTIVITY

Superoxide anion scavenging activity

Superoxide anion scavenging activity of EGCG was determined by the method of Nishmiki *et al*.,(1972) with modification. One ml of NBT (100 µMoles of NBT in 100 mM phosphate buffer, pH 7.4), 1 ml of NADH solution (14.68 µMoles of NADH in 100 mmoles phosphate buffer, pH 7.4) and varying concentrations of EGCG (10, 20, 30, 40, and 50 µMoles) were mixed well. The reaction was started by the addition of 100 µMoles of PMS (60 µMoles) 100 mmoles of phosphate buffer, pH 7.4). The reaction mixture was incubated at 30ºC for 15 min. The absorbance was measured at 560 nm in a spectrophotometer (Elico- Sl177). Incubation without EGCG was used as blank. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging.

Hydroxyl Radical Scavenging Assay

The hydroxyl radical scavenging activity was determined by the method of Halliwell *et al*.,(1989). The following reagents were added in the order stated below. The incubation mixture in a total volume of 1 ml contained 0.1 ml of 100 mmoles of potassium dihydrogen phosphate- KOH buffer, varying concentrations of EGCG (10, 20, 30, 40, and 50 µMoles), 0.2 ml of 500 mmoles of ferric chloride, 0.1 ml of 1 mmoles of ascorbic acid, 0.1 ml of 10 mmoles of H2O2 and 0.2 ml of 2-deoxy ribose. The contents were mixed thoroughly and incubated at room temperature for 60 min. Then added, 1 ml of 1% TBA (1 gm in 100 ml of 0.05 N sodium hydroxide) and 1 ml of 28% TCA. All the tubes were kept in a boiling water bath for 30 min. The absorbance was read in a spectrophotometer at 532 nm (Elico- Sl177) with reagent blank containing distilled water in a place of EGCG. The percentage scavenging activity was determined. Decreased absorbance of the reaction mixture indicated increased hydroxyl radical scavenging activity.

Free Radical Scavenging Activity

The ability to scavenging the stable free radical, DPPH was measured as a decrease in absorbance at 517 nm by the method of Mensor *et al*., (2001) to a methanolic solution of DPPH (90.25 mmoles), an equal volume of EGCG (10, 20, 30, 40,and 50 µMoles) dissolved in distilled water was added and made up to 1.0 ml with methanolic DPPH. An equal amount of methanol was added to the control. After 20 min, the absorbance was recorded at 517 nm in a Spectrophotometer (Elico-Sl177).

Formula for calculations:

$$\text{RSA activity (\%)} = \frac{\text{Control OD-Sample OD}}{\text{Control OD}} \times 100$$

Total Antioxidant Activity Assay

Total antioxidant potential of EGCG was determined by the ABTS assay, as described by Miller *et al*., (1996). The reaction mixture contained ABTS (0.002 M), varying concentrations of EGCG (10, 20, 30, 40 and 50 µMoles) and buffer in a total volume of 3.5 ml. The absorbance was measured at 734 nm in a Spectrophotometer (Elico- Sl177).

Formula for calculations:

$$\text{TAA activity (\%)} = \frac{\text{Control OD-Sample OD}}{\text{Control OD}} \times 100$$

Metal chelating activity

The chelating of ferrous ions by EGCG was estimated by the method described by Dinis *et al*.,(1994) Various concentrations of the EGCG (10, 20, 30, 40 and 50 µMoles) were added with 1 mL of 2mM FeCl2 separately. The reaction was initiated by the addition of 5 mM ferrozine (1 mL). Absorbance was measured at 562nm after 10min.

Formula for calculations:

$$\text{Chelating activity (\%)} = \frac{\text{Control OD-Sample OD}}{\text{Control OD}} \times 100$$

Reducing Power

The reducing power was determined according to the Oyaizu, (1986) method. Different concentrations

EGCG (10, 20, 30, 40, and 50 µM) were prepared in methanol mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [K3Fe (CN) 6] (2.5 mL, 1%). The mixture was incubated at 50oC for 20 min and 2.5 mL of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and FeCl3 (0.5 mL, 0.1%). The absorbance was measured at 700 nm in a spectrophotometer (Elico-Sl177). Increased absorbance of the reaction mixture indicated increased reducing power.

Determination of nitric oxide (NO) radical scavenging activity

The nitric oxide level was determined by Griess Illosvoy reaction (Sakat *et al*., 2010). Various concentrations (1.9-500 mg/mL) of EGCG (10, 20, 30, 40, and 50 µM) (3 mL) and ascorbic acid were dissolved in methanol and incubated at 25_C for 150 minutes. The prepared sample was reacted with reagents (1% (w/v) sulphanilamide, 2% O-phosphoric acid and 0.1% N-(1-napthyl)ethylenediamine hydrochloride). The absorbance at 546 nm of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylene diamine was measured. The EC_{50} values were determined as the concentration of the test mixture that gave 50% reduction in the absorbance compared to the control blank.

Formula for calculations:

$$\text{NO Scavenging activity (\%)} = \frac{\text{Control OD - Sample OD}}{\text{Control OD}} \times 100$$

Statistical Analysis

The values are given as mean ± S.D. for five rats in each group. The data for various biochemical parameters were analysed using analysis of variance (ANOVA) and the group means were compared by Duncan's Multiple Range Test (DMRT). Values were considered statistically significant when $p<0.05$ (Duncan, 1957).

RESULTS

EGCG activity on plasma lipid peroxidation

Table 1 shows the changes in plasma lipid peroxidative markers (TBARS and LOOH) in control and experimental rats. In rats treated with fluoride, the levels of plasma TBARS and LOOH were significantly increased ($p<0.05$). Administrations of EGCG significantly decrease fluoride induced LPO compared with fluoride alone treated rats. EGCG alone treated rats did not show any changes in the LPO markers compared with control group.

EGCG activity on antioxidant levels

The levels of non-enzymatic antioxidants (vitamin C, vitamin E and GSH) in plasma of normal and experimental rats are shown in Table 2. In fluoride treated rats, the non-enzymatic antioxidants were found to be significantly ($P < 0.05$) decreased when compared to control rats. Administration with EGCG to fluoride treated rats showed a significant ($P < 0.05$) increase in non-enzymatic antioxidants levels compared with fluoride alone treated rats. EGCG alone administrated rats exhibits no changes in their antioxidant levels.

EGCG activity on *In-vitro* superoxide anion scavenge

Figure 3 shows the superoxide radical scavenging ability of EGCG on Invitro. The superoxide radical scavenging activities of EGCG were exhibit dose dependent manner (10, 20, 30, 40, and 50 µM). The percentage scavenging activity of EGCG on superoxide radical was increases with increasing concentrations. The maximum free radical scavenging ability of EGCG were observed in the fifty percent of effective concentration (EC_{50}%) at the value of 54.5% at the concentration of 40 µM compared with ascorbic acid was observed.

EGCG activity on *In-vitro* Hydroxyl radical scavenge

Figure 4 shows the hydroxyl radicals scavenging effect of EGCG on Invitro. EGCG scavenges hydroxyl radicals scavenging activities in a dose dependent manner (10, 20, 30, 40 and 50 µM). The percentage scavenging activity of EGCG on hydroxyl radicals scavenging activities increases with increasing concentration. The maximum activities observed in the highest concentration and the fifty percent of effective concentration (EC_{50}%) value of EGCG is 52.3, at the concentration of 40 µM compared with ascorbic acid was observed.

EGCG activity on *In-vitro* Free radical scavenge

Figure 5 shows the percentage of free radical scavenging effect of EGCG Invitro by using 1,1-diphenyl-2-picryl-hydrazyl (DPPH•) assay. The hydrogen atom or electron donation abilities of the compounds were measured from the bleaching of the purple-colored methanol solution of 2,2′-diphenyl-1-picrylhydrazyl (DPPH). EGCG scavenges DPPH radical in a dose dependent manner (10, 20, 30, 40 and 50 µM). The percentage scavenging activity of EGCG on DPPH radical increases with increasing concentration. The maximum activities observed in the highest concentration and the fifty percent of effective concentration (EC_{50}%) value of EGCG is 40.2, at the concentration of 40 µM compared with ascorbic acid was observed.

EGCG activity on *In-vitro* total antioxidant

Figure 6 shows the total antioxidant scavenging effect of EGCG Invitro by using 2, 2′-azinobis- (3-ethyl-benzothiazoline-6-sulfonic acid) radical assay. EGCG

scavenges 2, 2'-azinobis-(3-ethyl-benzothiazoline- 6-sulfonic acid) radical in a dose dependent manner (10, 20, 30, 40 and 50 μM). The percentage scavenging activity of EGCG on 2, 2′-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) radical increases with increasing concentration. The maximum activities observed in the highest concentration and the fifty percent of effective concentration (EC_{50}%) value of EGCG is 58.3, at the concentration of 40 μM compared with ascorbic acid was observed.

EGCG activity on *In-vitro* Metal chelation

Figure 7 shows the metal chelating activities of EGCG in Invitro. The EGCG metal chelation was increased in a dose dependent manner (10, 20, 30, 40 and 50 μM). The percentage scavenging activity of EGCG on metal chelation increases with increasing concentration. The maximum activities observed in the highest concentration and the fifty percent of effective concentration (EC_{50}%) value of EGCG is 45.5 at the concentration of 40 μM compared with ascorbic acid was observed.

EGCG activity on *In-vitro* Reducing power

Figure 8 shows the reducing power of EGCG in Invitro. The EGCG reducing power was increased in a dose dependent manner (10, 20, 30, 40, and 50 μM). The percentage of EGCG on reducing power activities increases with increasing concentrations. The percentage scavenging activity of EGCG on reducing power increases with increasing concentration. The maximum activities observed in the highest concentration and the fifty percent of effective concentration (EC_{50}%) value of EGCG is 0.06 at the concentration of 40 μM compared with ascorbic acid was observed.

EGCG activity on *In-vitro* Nitric oxide scavenge

Figure 9 shows the nitric oxide (NO) scavenging activities of EGCG in Invitro. The EGCG nitric oxide (NO) scavenging activities were increased in a dose dependent manner (10, 20, 30, 40 and 50 μM). The percentage scavenging activity of EGCG on nitric oxide (NO) increases with increasing concentration. The maximum activities observed in the highest concentration and the fifty percent of effective concentration (EC_{50}%) value of EGCG is 64.3 at the concentration of 40 μM compared with ascorbic acid was observed.

Table 1. Changes in the levels of plasma TBARS and LOOH of control and experimental rats

Group	Parameters in plasma	
	TBARS (mM/dl)	LOOH (×10-5 mM/dl
Control	0.14 ± 0.02^{a}	12.68 ± 0.85^{a}
EGCG (40 mg/kg bw)	0.11 ± 0.01^{b}	10.07 ± 0.78^{b}
Fl (25 mg/kg bw)	0.25 ± 0.03^{c}	16.70 ± 0.82^{c}
Fl + EGCG (25 mg/kg bw)+ EGCG (40 mg/kg bw)	0.18 ± 0.02^{d}	13.42 ± 1.05^{d}

Values are given as mean ± S.D. from six rats in each group; Values not sharing a common letter (a-d) differ significantly at $p<0.05$ (DMRT).

Table 2. Changes in the levels of vitamin - C, vitamin – E and reduced glutathione (GSH) in plasma of control and experimental rats

Group	Parameters in Plasma		
	GSH (mg/dl)	Vit.C (mg/dl)	Vit.E (mg/dl)
Control	19.16 ± 1.34^{a}	1.63 ± 0.19^{a}	1.20 ± 0.08^{a}
EGCG (40 mg/kg bw)	21.18 ± 1.54^{b}	1.76 ± 0.16^{a}	1.40 ± 0.09^{a}
Fl (25 mg/kg bw)	13.65 ± 1.15^{c}	1.18 ± 0.11^{b}	0.70 ± 0.03^{b}
Fl + EGCG (25 mg/kg bw)+ EGCG (40 mg/kg bw)	18.45 ± 1.08^{d}	1.50 ± 0.06^{c}	1.13 ± 0.18^{c}

Values are given as mean ± S.D. from six rats in each group; Values not sharing a common letter (a-d) differ significantly at $p<0.05$ (DMRT)

Fig 1.The structure of EGCG

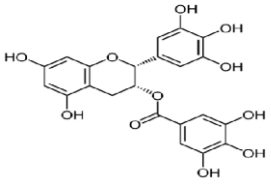


Fig 2. The structure of Ascorbic acid

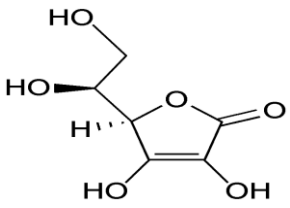


Fig 3. Superoxide radical scavenging assay

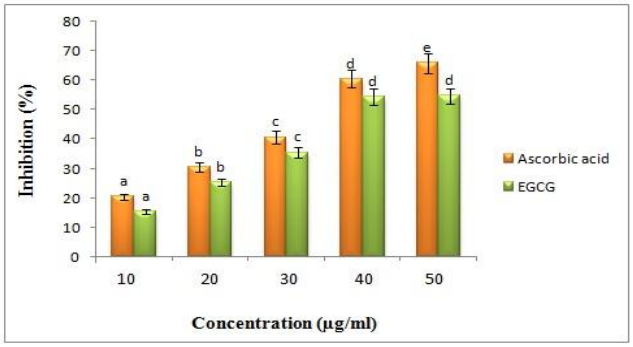


Fig 4. Hydroxyl radical scavenging assay

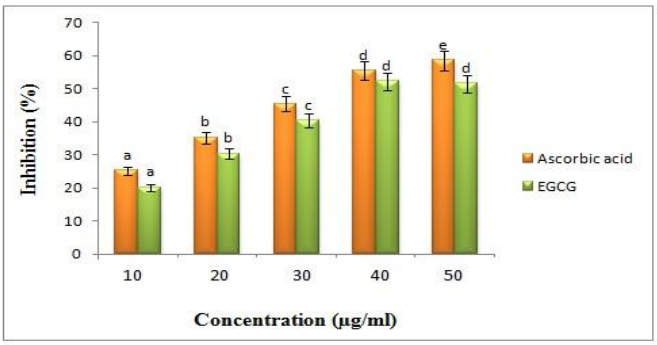


Fig 5. Free radical scavenging assay

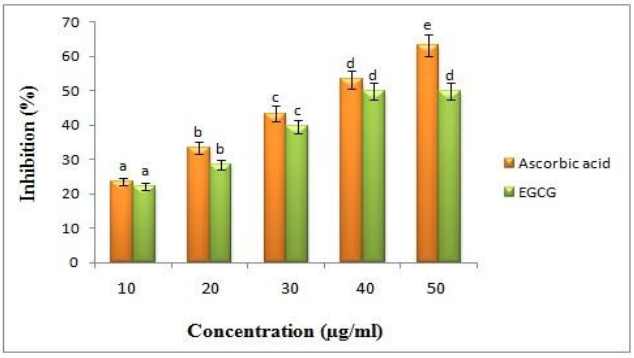


Fig 6. Total antioxidant assay

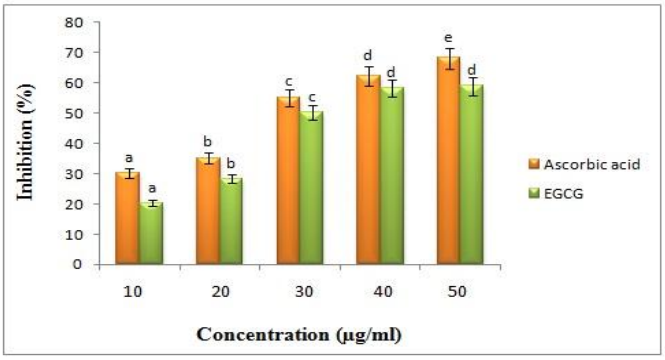


Fig 7. Metal chelating assay

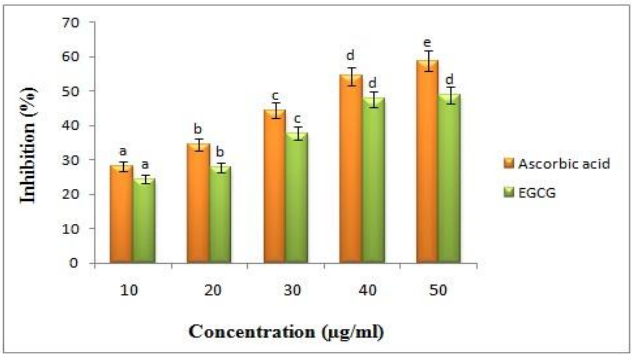


Fig 8. Reducing power assay

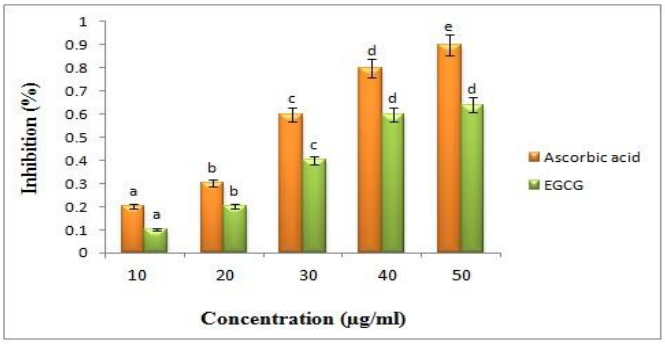
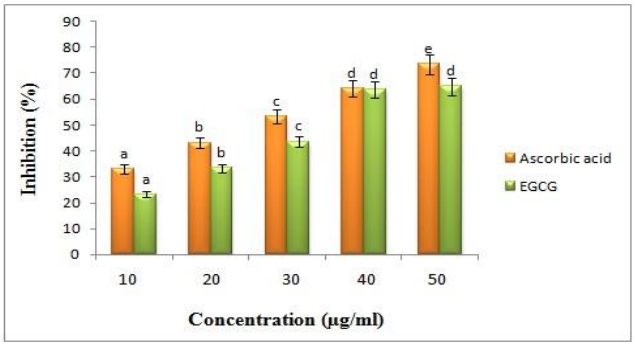


Fig 9. Nitric oxide scavenging assay



DISCUSSION

Fluoride is also one of the main environmental and occupational pollutants in industrialized countries and induces a wide array of toxicological effects, biochemical dysfunctions in various organ systems posing a serious threat to health. Fluoride induces oxidative damage by producing reactive oxygen species and decreasing the levels of antioxidants such as vitamins C, E and GSH which play an important role in the elimination of free radicals (Shivarajashankara *et al*., 2001). Fluoride has also been reported to cause damage to lipids by lipid peroxidation. Cell membranes are phospholipid bilayers with extrinsic proteins and are the direct target of lipid peroxidation which leads to a number of deleterious effects such as increased membrane rigidity, osmotic fragility, cell membrane destruction and cell damage. The observed increase in the level of plasma TBARS and LOOH in fluoride toxicity is generally thought to be the consequence of increased production and liberation of

tissue lipid peroxides into circulation due to the pathological changes in tissues.

Pei Feng, *et al*., (2012) also observed that treatment with fluoride increased the lipid peroxide concentration in blood. Increase in lipid peroxidation in plasma and tissues have been implicated in fluoride - induced organ damage and dysfunction. Treatment with EGCG significantly reverted the fluoride induced peroxidative damage in plasma which is evidenced from the lowered levels of TBARS and LOOH. This may be due to the antioxidative effect of EGCG. Vitamin C is a primary antioxidant, water-soluble vitamin that can directly scavenge singlet oxygen, superoxide and hydroxyl radicals. Numerous reports have shown the positive effect of vitamin C as an antioxidant and scavenge of free radicals (Das *et al*., 2001). Vitamin E (α-tocopherol) is a well-known antioxidant: it acts as a free radical scavenger, more exclusively within cell membranes by preventing the oxidation of polyunsaturated lipids by free radicals such as the hydroxyl radical. The antioxidant potential of vitamin E is no longer disputed. Most *in vivo* studies have shown that vitamin E improves various parameters of oxidative stress in both animals and human beings (Golestani *et al*., 2006; Martin *et al*., 1996).

GSH is a powerful tripeptide antioxidant and a powerful nucleophile, critical for cellular protection such as detoxification of ROS, conjugation and excretion of toxic molecules and control of inflammatory cytokine cascade (Brown *et al*., 2004). Depletion of GSH in tissues leads to impairment of the cellular defence against ROS and may result in peroxidative injury. In our study we observed decreased concentration of vitamins C, E and GSH in plasma in fluoride-induced rats. Administration with EGCG brought normal levels of these antioxidants. It might be due to excellent antioxidant activity of EGCG with mechanism involving both free radical scavenging and metal chelation (Yang *et al*., 1994).

Free radicals have been implicated in a number of diseases including asthma, cancer, cardiovascular disease, cataract, diabetes, gastrointestinal inflammatory diseases, liver diseases, muscular degeneration and other inflammatory processes (Wilson, 1998). Radical scavenging activities are very important due to the deleterious role of free radicals in biological systems. *In vitro* antioxidant activity of the EGCG was investigated in the present study by DPPH, hydroxyl radical scavenging, superoxide anion, total antioxidant, reducing power, and metal chelating assays. These methods have proven the effectiveness of the EGCG in comparison to that of the reference standard antioxidant, ascorbic acid.

Superoxide anion is a precursor to active free radicals that have potential of reacting with biological macromolecules and, thereby, inducing tissue damage. Yen and Duh (1994) have reported that superoxide anions damage biomolecules directly or indirectly by forming H_2O_2, OH, and peroxy nitrite or singlet oxygen during aging and pathological events. Na and Surh, (2008); Sriram *et al*., (2009) has been reported that epigallocatechin- 3-gallate (EGCG), known to be most abundant in green tea, is an effective scavenger of ROS *in vitro*. In the present study, using different concentration of EGCG shows the highest percentage scavenging effect on superoxide radical scavenging activity with previous findings of Bing tian *et al*., (2007) was observed. The maximum percentage scavenging effect of EGCG on superoxide radicals was 54.5% observed at the concentration of 40 µM. This observable fact due to the presence of EGCG quinone molecules, and they are produced after EGCG molecules are oxidized. Then the EGCG quinone molecules react with EGCG molecules via phenolic coupling reactions (SN2) to form EGCG dimmers (20,200-gallyl adduct). Each EGCG dimer retains its reactive hydroxyl groups and exhibits a powerful antioxidant capacity (Bors *et al*., 2009).

The hydroxyl radical, ($^{\bullet}$HO) is the neutral form of the hydroxide ion (HO^-). Hydroxyl radicals are highly reactive and consequently short-lived; however, most notably hydroxyl radicals are produced from the decomposition of hydro peroxides. The hydroxyl radical can damage virtually all types of macromolecules: carbohydrates, nucleic acids (mutations), lipids (lipid peroxidation) and amino acids (e.g. conversion of Phe to m-Tyrosine and o-Tyrosine). Hydroxyl radical will react with other elements to produce free radical which is very dangerous to the organism (Reiter *et al*., 2007). In the present exploration, we have used different concentration of EGCG and the highest percentage scavenging effect of EGCG on hydroxyl radicals was 52.3% observed at the concentration of 40 µM.

Free radical scavenging activity (DPPH) is a very useful method as it is highly sensitive and rapid assay (Nishanthini *et al*., 2012). This assay is independent on substrate polarity where DPPH can accept an electron or hydrogen radical to become a stable diamagnetic molecule (Murugan and Mohan, 2012). When antioxidant scavenges the free radical by hydrogen donation, the purple color of DPPH in assay solution turns to yellow, which can be monitored spectrophotometrically at 517 nm (Yen *et al*., 2008). Chang *et al*., (2007) proved that Phellinus merrilli extracts shows the effective scavenging activity on DPPH radicals. In the present investigation, our antioxidant EGCG also scavenge DPPH• radical in dose dependent manner. The highest percentage scavenging effect of EGCG 40.2% on 2,2′-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) radical was observed at the concentration of 40 µM. This is due to the prevention of free radical species by EGCG from damaging biomolecules such as lipoproteins, polyunsaturated fatty acids (PUFA), DNA, amino acids,

proteins in a biological system by its natural hydrogen donating ability (Devika and Prince, 2008).

The reduction of the 2,2'-azinobis (3-ethylbenzothiazoline sulphonate) radical cation (ABTS) has been widely used to measure the antioxidant capacity of natural extracts (Kalpana devi and Mohan, 2012). ABTS is a relatively stable free radical which involves in the direct generation of ABTS radical monocation without any involvement of intermediary cation. In the present study, the total antioxidant activity of EGCG was investigated *in vitro* by 2,2′-azinobis- (3-ethyl-benzothiazoline-6-sulfonic acid) cation which forms the basis of one of the spectrophotometric (734 nm) methods that have been applied to the total antioxidant activities of solutions of pure substances. In this investigation, EGCG scavenges 2,2′- azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) radical shows dose dependently. A similar result has been reported by Leelavinothan, and Kalist, (2011) with hesperetin. The highest percentage scavenging effect of EGCG (75.3%) on 2,2′- azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) radical was observed at the concentration of 40 µM. This is mainly due to the strong antioxidant property of EGCG and the presence of its vicinal trihydroxy structure in which oxygen atoms act as electron donors to from bonds with electrophilic ions and thereby helps in the recoupment of antioxidant defense system (Higdon and Frei, 2003).

The presence of transition metal ions in a biological system could catalyse the Haber-Weiss and Fenton type reactions, resulting in the generation of hydroxyl radicals (OH). However, these transition metal ions could form chelates with the antioxidants, which results in the suppression of OH generation and inhibition of peroxidation processes of biological molecules (Chew *et al*., 2009). Weisburg *et al*., (2004) reported that, administration of EGCG exhibit effective abrogation of metal iron by increasing with increasing concentration. In the present investigation also in agreement with the previous findings, reveals that the EGCG abrogates highest metal iron with increasing concentrations. The maximum level of metal chelating efficacy by EGCG (45.5%) at the concentration of 40 µM. The predomination of metal chelating power on iron ions over ROS scavenging activity of EGCG at high concentrations might result in the prooxidant effect of EGCG on DNA.

A reducing power is an indicative of reducing agent having the availability of atoms which can donate electron and react with free radicals and then convert them into more stable metabolites and terminate the radical chain reaction (Ganu *et al*., 2010). In the present exploration, EGCG has potent free radical reducing agent, accordingly, EGCG might contain a sizable amount of reductants which may react with the free radicals to stabilize and terminate free radical chain reaction. The maximum level of reducing power of EGCG (0.06%) at the concentration of 40 µM. These is due to the presence of 4 ring structure with 8hydroxyle group of EGCG, which easily donating hydrogen atoms and stabilize the free radicals (Elena *et al*., 2013).

Nitric oxide is a free radical produced in mammalian cells, involved in the regulation of various physiological process including neurotransmission, vascular homeostasis, antimicrobial and antitumor activities. However, excess production of NO is associated with several diseases (Hepsibha *et al*., 2010). Rajamanikandan *et al*., (2011) has reported that administration of ethanolic extracts Mollugo nudicaulis exhibits increasing abrogation of nitric oxide with increasing concentrations. In the present investigation also in agreement with the previous findings that EGCG shows increasing abrogation of nitric oxide with increasing concentrations. The maximum levels of nitric oxide reducing ability of EGCG (64.5%) at the concentration of 40 µM. This is due to potent nitric oxide radical scavenging activity of EGCG, which competes with oxygen to react with nitric oxide and thus inhibits the generation of nitrite.

CONCLUSION

In conclusion, our study suggests that EGCG has a significant protective effect against NaF induced oxidative damage in rat plasma, as well as possesses a greater free radical scavenging and antioxidant properties through *in vivo* and *in vitro* studies, which could be mainly due to its antioxidant, free radical scavenging and metal chelating properties. Recently, much attention has been focused on the protective functions of naturally occurring antioxidant phytochemical entities in biological systems against toxic effects fluoride. This study therefore provides biological evidence supporting the beneficial role of EGCG, in protecting NaF induced toxic effects in rats.

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REFERENCES

Bing T, Zongtao S, Zhenjian X, Yuejin H. Chemiluminescence analysis of the prooxidant and antioxidant effects of epigallocatechin-3-gallate. *Asia Pac J Clin Nutr*, 16 (supp 1), 2007, 153-157

Bors W, Michel C, Stettmaier K. Electron paramagnetic resonance studies of radical species of proanthocyanidins and gallate esters. *Archives of Biochemistry and Biophysics*, (374), 2009, 347–355.

Brown LA, Harris FL, Ping XD, Gauthier TW. Chronic ethanol ingestion and the risk of acute lung injury: a role for glutathione availability, *Alcohol*, (33), 2004, 91–7.

Chang HY, Ho YL, Sheu MJ, Lin YH, Tseng MC, Wu SH, Huang GJ, Chang YJ. Antioxidant and free radical scavenging activities of *Phellinus merrillii* extracts. *Botanical Studies*, (48), 2007, 407-417.

Chew YL, Goh JK, Lim YY. Assessment of *in vitro* antioxidant capacity and polyphenolic composition of selected medicinal herbs from Leguminosae family in Peninsular Malaysia. *Food Chem*, 116, 2009, 13-18.

Chinoy NJ. Effects of sodium fluoride on physiology of some animals and human beings. *Indian J. Environ. Toxicol*, 1, 1991, 17-32.

Chlubek D. NaF and oxidative stress. *Fluoride*, 36, 2003, 217–228.

Das KK, Das SM, Dasgupta S. The influence of ascorbic acid on nickel induced hepatic lipid peroxidation in rats, *J. Basic. Clin. Physiol. Pharmacol*, 12, 2001, 187–195.

Desai ID. Vitamin E analysis methods for animal tissues, *Meth Enzymol*, 105, 1984, 138–143.

Devika PT, Prince SM. (−)Epigallocatechin-gallate (EGCG) prevents mitochondrial damage in isoproterenol-induced cardiac toxicity in albino Wistar rats: A transmission electron microscopic and *in vitro* study. *Pharmacological Research*, 57, 2008, 351–357.

Dinis TCP, Madeira VMC, Almeida LM. Action of phenolic derivatives (Acetoaminophen, Salycilate, and 5-aminosalycilate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Arch Biochem Biophys*, 315, 1994, 161-169.

Droge W. Free radicals in the physiological control of cell function. *Physiol Rev*, 82, 2002, 47-95.

Duncan BD. multiple range test for correlated and heteroscedastic means. *Biometrics*, 13, 1957, 359-364.

Elena L, Yves MD, Raymond M, Claude P. Green tea polyphenol epigallocatechin-3-gallate (EGCG) as adjuvant in cancer therapy. *Clinical Nutrition*, 2013, 1-10.

Ellman, GL. Tissue sulphydryl groups, Arch. *Biochem. Biophys*, 82, 1959, 70–77.

Ganu GP, Jadhav SS, Deshpande AD. Antioxidant and antihyperglycemic potential of methanolic extract of bark of Mimusops elengi L in mice. *Res J Pharm Biol Chem Sci*, 1, 2010, 67-77.

Golestani AR, Rastegar A, Shariftabrizi S, Khaghani, SM, Payabvash AH, Salmasi ARD, Pasalar P. Paradoxical dose- and time-dependent regulation of superoxide dismutase and antioxidant capacity by vitamin E in rat, *Clinica. Chimica. Acta*, 365, 2006, 153–159.

Halliwell B, Gutteridge JMC, Aruoma OI. The deoxyribose methods a simple assay for determination of rate constants for reaction of hydroxyl radicals. *Anal Biochem*, 165, 1989, 215-219.

Hepsibha BT, Sathiya S, Babu CS, Premalakshmi V, Sekar T. *In vitro* studies of antioxidant and free radical scavenging activities of Azima tetracantha. Lam leaf extract. *Indian J Sci Technol*, 3, 2010, 571-577.

Higdon JV, and Frei B. Tea catechins and polyphenols: health effects, metaboloism, and antioxidant functions. *Critical Review in food Science and Nutrition*, 43(1), 2003, 89-143.

Jiang ZY, Hunt JY, Wolff SP. Detection of lipid hydroperoxides using the "fox method". *Anal Biochem*, 202, 1992, 384–389.

Kalpana DV, Mohan VR. *In vitro* antioxidant studies of *Begonia malabarica* Lam. and Begonia floccifera Bedd. *Asian Pacific Journal of Tropical Biomedicine*, (3), 2013, S1572-S1577.

Leelavinothan P, Kalist S. Beneficial effect of hesperetin on cadmium induced oxidative stress in rats: an *in vivo* and *in vitro* study. *European Review for Medical and Pharmacological Sciences*, (15), 2011, 992-1002.

Ling LU, Tan KB, Lin H, Chiu GNC. The role of reactive oxygen species and autophagy in safingol-induced cell death. *Cell Death & Disease*, (2), 2011, 1-12.

Martin A, Wu DY, Baur W, Meydani SN, Blumberg, JB, Meydani, M. Effect of vitamin E on human aortic endothelial cell responses to oxidative injury, *Free Radical Biol Med*, (21), 1996, 505–511.

Mensor L, Menezes FS, Leitao GG, Reis AS, Dos Santos TC, Coube CS, Leitao SG. Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. *Phytother Res*, (15), 2001, 127-130.

Miller NJ, Castelluccio C, Tijburg L, Rice-Evans C. The antioxidant properties of the flavins and their gallate esters-radical scavengers or metal chelators. *FEBS Lett*, 392, 1996, 40-44.

Murphy MP, Holmgren A, Goran Larsson N, Halliwell B. Unraveling the biological roles of reactive oxygen species. *Cell Metab*, 13(4), 2011, 361-366.

Murugan M, Mohan VR. *In vitro* antioxidant studies of Dioscorea esculenta (Lour). Burkill. *Asian Pacific Journal of Tropical Biomedicine*, (3), 2012, S1620- S1624.

Na HK, Surh YJ. Modulation of Nrf 2-mediated antioxidant and detoxifying enzyme induction by the green tea polyphenol EGCG. *Food and Chemical Toxicology*, 46, 2008, 1271–1278.

Nakagawa T, Yokozawa T, Terasawa K, Shu S, Juneja LR. Protective activity of green tea against free radical- and glucose-mediated protein damage. *Journal of Agricultural and Food Chemistry*, 50, 2002, 2418–2422.

Niehaus WG, Samuelsson B. Formation of malonaldehyde from phospholipid arachidonate during microsomal lipid peroxidation. *Eur J Biochem*, 6, 1968, 126–130.

Nishanthini A, Ruba A, Mohan VR. Total phenolic, flavonoid contents and *in vitro* antioxidant activity of leaf of Suaeda monoica Forssk ex. Gmel (Chenopodiaceae). *International Journal of Advanced Life Science*, 5, 2002, 34-43.

Nishimiki M, Rao Na, Yagi K. The occurrence of superoxide anion in the reaction of reduced phenazine methosulphate and molecular oxygen. *Biochem Biophys Res Comm*, 46, 1972, 849-853.

Omaye ST, Turbull TD, Sauberlich HC. Selected method for the determination of ascorbic acid in animal cells, tissues and fluids. *Methods in Enzymology*, 62, 1979, 1–11.

Oyaizu M. Studies on product of browning reaction prepared from glucose amine. *Jap J Nutr*, 44, 1986, 307-315.

Pari L, Amudha K. Antioxidant effect of naringin on nickel induced toxicity in rats: an *in vivo* and *in vitro* study. *Int J Pharm Sci Res*, 2(1), 2012, 151-158.

Pei F, Jun-ren W, Zi-gui Z. Influence of selenium and fluoride on blood antioxidant capacity of rats. *Experimental and Toxicologic Pathology*, 64, 2012, 565– 568.

Peng KT, Hsu WH, Shih HN, Hsieh CW, Huang TW, Hsu RWW. The role of reactive oxygen species scavenging enzymes in the development of septic loosening after total hip replacement. *J Bone Joint Surg*, 93(B), 2011, 1201-1209.

Rajamanikandan S, Sindhu T, Durgapriya D, Sophia D, Ragavendran P and Gopalakrishnan VK. Radical Scavenging and Antioxidant Activity of Ethanolic Extract of Mollugo nudicaulis by *Invitro* Assays. *Ind J Pharm Edu Res*, 45(4), 2011.

Reiter RJ, Carneiro RC, Oh CS. Melatonin in relation to cellular antioxidative defense mechanisms. *Horm. Metab. Res*, 29(8), 2007, 363–72.

Sakat SS, Juvekar AR, Gambhire MN. *In vitro* antioxidant and antiinflammatory activity of methanol extract of Oxalis corniculata Linn. *Int J Pharm Pharm Sci*, 2(1), 2010, 146-155.

Sen S, Chakraborty R, Sridhar C, Reddy YSR. Free radicals, antioxidants, diseases and phytomedicines: current status and future prospect. *Int J Pharmaceutical Sci Rev Res*, 3(1), 2010, 91-100.

Shao HB, Chu LY, Lu ZH, Kang CM. Primary antioxidant free radical scavenging and redox signaling pathways in higher plant cells. *Int J Biolog Sci*, 4, 2008, 8-14.

Shivarajashankara YM, Shivashankara AR, Gopalakrishnan Bhat P, Hanumanth RS. Effect of NaF intoxication on lipid peroxidation and antioxidants system in rats. *Fluoride*, 34, 2001, 108-113.

Sriram N, Kalayarasan S, Sudhandiran G. Epigallocatechin-3-gallate augments antioxidant activities and inhibits inflammation during bleomycin-induced experimental pulmonary fibrosis through Nrf2-Keap1 signaling. *Pulmonary Pharmacology & Therapeutics*, 22(3), 2009, 221–236.

Thambiraj J, Paulsamy S, Sevukaperumal R. Evaluation of *in vitro* antioxidant activity in the traditional medicinal shrub of western districts of Tamilnadu, India, Acalypha fruticosa Forssk. (Euphorbiaceae) *Asian Pacific Journal of Tropical Biomedicine*, 2012, S127-S130.

Thangapandiyan S, Miltonprabu S. Epigallocatechin gallate effectively ameliorates fluoride induced oxidative stress, DNA damage in the liver of rats. *Canadian journal of physiology and pharmacology*. 91, 2013, 1-10.

Venkatesh S, Deecaraman M, Kumar R, Shamsi MB, Dada R. Role of reactive oxygen species in the pathogenesis of mitochondrial DNA (mt DNA) mutations in male infertility. *Indian J Med Res*, 129, 2009, 127-137.

Weisburg JH, Weissman DB, Sedaghat T, Babich H. *In vitro* cytotoxicity of epigallocatechin gallate and tea ex-tracts to cancerous and normal cells from the human oral cavity. *Basic Clin Pharmacol Toxicol*, 95, 2004, 191-200.

Whitford GM. Metabolism and Toxicity of Fluoride, *Karger, Basel*, 1996.

Wilson RL. Free radicals and tissue damage, mechanistic evidence from radiation studies. Biochemical mechanism of liver injury. *Academic Press*. New York, 1998, 123.

World Health Organization, *Fluorides*, 2002. Available from: <http:// inchem.org/documents/ehc/ehc/ehc227.htm#ref/> (accessed on 25.07.09).

Yang X, Sheng S, Hou J, Zhao B, Xin W. Mechanism of scavenging effects of (_)-epigallocatechin gallate on active oxygen free radicals. *Acta Pharmacologica Sinica*, 15, 1994, 350–353 (in Chinese).

Yen FL, Wua TH, Lin LT, Chan TM, Lin CC. Concordance between antioxidant activities and flavonoids contents in different extracts and fractions of Cuscuta chinensis. *Food Chemistry*, 108, 2008, 455-462.

Yen GC, Duh PD. Scavenging effect of methanolic extract of peanut hulls on free radical and active oxygen species. *J Agric Food Chem*, 42, 1994, 629-632.