



Ameliorative effect of epigallocatechin gallate on sodium fluoride induced oxidative stress mediated metabolism in rat

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Abstract

Background: The present study was designed to examine the ant hyperglycemic and antihyperlipidemic effect of Epigallocatechin gallate (EGCG) on fluoride induced oxidative changes in the metabolisms of rats.

Methods: The control group received the vehicles only. EGCG alone treated group received the EGCG orally at a dose of 40mg/kg in 10% tween 80 for 4 weeks. The FI treated group received NaF (25 mg/kg) orally in saline for 4weeks. EGCG + FI treated group received the EGCG at a dose of 40mg/kg/bw along with NaF.

Results: The levels of serum SGOT, SGPT, ALP, ACP and plasma glucose, G6PD, cholesterol, triglycerides, free fatty acids, phospholipids, low density lipoprotein, very low density lipoprotein were significantly increased in fluoride intoxicated rats. Furthermore, a significant increase HMG CoA with decreased glycogen, hexokinase, total protein, albumins, high density lipoprotein, lipoprotein lipase, and lecithin cholesterol acyl transferase was observed in fluoride treated rats with altered histological changes. Pre-oral administration of EGCG at a dose of 40 mg/kg bw to fluoride intoxicated rats for a period of 28 days resulted in a significant reduction in the cholesterol, triglycerides, free fatty acids, phospholipids, low density lipoprotein, very low density lipoprotein, and elevation of high density lipoprotein with hexokinase. Moreover, lipoprotein lipase, lecithin cholesterol acyl transferase, total protein, and albumins were also increased with decreased serum markers and HMG CoA after EGCG treatment. The histological findings of liver also significantly improved with EGCG pretreatment.

Conclusions: These results indicate that the natural dietary antioxidant EGCG showed significant protective effect against FI- induced oxidative stress mediated metabolic alternations in rats. This may be due to its antihyperlipidemic and ant hyperglycemic property; it will provide an accessible and cheap traditional medicine source for treatment of FI mediated environmental and occupational ailments.

Keywords: EGCG, Fluoride, Metabolisms, Lipids, Anti-Hyperlipidemia ROS, Rat.

1. Introduction

Fluorosis is the phenomenon caused by excessive ingestion of fluoride for a prolonged period of time. Intrinsic geological resources and more recently the arrival of increased industrialization and attendant ecological contaminated waste have contributed seriously to the increasing incidence of fluoride-related human health problem. High stratum of fluoride in drinking water has become a future health hazard all over the world, approximately with 66.62 million victims in India alone. Fluoride enters the human and animal body through drinking water and to a slight extent through food (Susheela 2007). High quantity of fluoride ingestion causes metabolic anarchism, by interacting with a variety of cellular processes such as gene expression, cell cycle, proliferation and migration, respiration, metabolism, ion transport, secretion, endocytosis, apoptosis, necrosis and oxidative stress, and disrupts the antioxidant security system in the body (Strunecka et al. 2007; Barbier et al. 2010). Numerous studies have shown that fluorosis may induce nucleotide damage, inhibition of protein synthesis and alters mitochondrial functions resulting in the energy deficient state of the cell (Jeng et al. 1998). Fluoride has already been reported to be hyperglycemic, hyperlipidemic, in fasting blood glucose levels in laboratory animals, which was attributed to lower

Insulin levels (Rupal et al. 2010; Gracia-Montalvo et al. 2009). Philips and Hart (1935) first demonstrated that lipid metabolism was altered by toxic levels of fluoride given to rats. Fluoride exposure is resulting in generation of superoxide anion (O₂⁻), and its downstream consequences such as hydrogen peroxide, peroxynitrite and hydroxyl radicals, which are important in mediating the toxic effects of fluoride (Barbier et al. 2010). Oxidative stress mediated free radical generation is known to be one of the most important mechanisms of fluoride toxicity (Nabavi et al. 2012). Fluoride has the ability to initiate respiratory burst and stimulate the generation of free radicals, which change the structure and permeability of cell membranes and impair the cell function (Chlubek 2003). Numerous studies have indicated an increased oxidative stress in the serum, liver and brain of animals exposed to fluoride (Grucka-Mamczar et al. 2009). Moreover, fluoride affects the activity of enzymes constituting the cell antioxidant system whose role is to protect against free radicals (Chinnoy 2003).

Recently, the search for appropriate hypoglycemic and hypolipidemic agents has been focused on plants based traditional medicines because, natural products that may be better treatments than currently used drugs (Rates 2001). Therefore, numerous studies have been carried out to evaluate natural products, including plant materials, as alternative treatments for oxidative stress induced diseases. Plants are rich sources of hypoglycemic,



hypolipidemic and antioxidant agents such as flavonoids, ellagic acids, phenolic acids, phytosterols, gallotannins, and other related polyphenols (Muruganandan et al. 2005). Tea is the second most commonly drunk liquid on earth after water. Tea is an infusion of the leaves of the *Camellia sinensis* plant. Epigallocatechin gallate (EGCG) (Fig.1), the major bioactive constituent of *C.sinensis* has been reported to exhibit many pharmacological effects, including anti-cancer, anti-hyperlipidemic and anti-hyperglycemic activities against a broad spectrum of research [Li et al. 2006; Singh et al. 2011]. EGCG has been reported to possess strong antioxidant properties (Zhong & Shahidi 2011). Oral administration of EGCG is capable of protecting several organs such as liver, kidney, testes, heart and brain against oxidative stress induced by free radicals (Thangapandiyan & Miltonprabu 2013; Thangapandiyan & Miltonprabu 2014; May Al-Maghrebi et al. 2012; Devika & Stanely Mainzen Prince 2008; Puneet K & Anil 2008). However, the role of EGCG against fluoride altered carbohydrates, protein, lipid profiles and lipid enzymes in rats have not so far been studied. Therefore, the present study was carried out to investigate the metabolic alterations in the carbohydrate, protein, lipid profiles and lipid enzymes on fluoride induced oxidative stress mediated metabolism in rats.

2. Materials and methods

2.1. Chemicals

Sodium fluoride (NaF), Epigallocatechin gallate (EGCG), 1, 1', 3, 3'- tetramethoxy propane, bovine serum albumin was purchased 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 Himedia Laboratories Pvt. Ltd., Mumbai, India. Reagent kits were obtained from span Diagnostics, Mumbai, India. Fig.1 Chemical structure of EGCG.

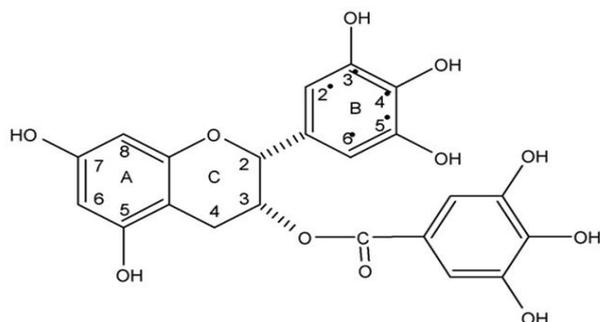


Fig. 1: Chemical Structure of EGCG

2.2. Animals and diet

Healthy adult male albino rats of Wistar strain, bred and reared in Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College, and Annamalai University were used for the experiment. Males were preferred to avoid complications of the estrous cycle. Animals of equal weight (160–180 g) were selected and housed in polypropylene cages lined with husk and kept in a semi natural light/dark condition (12 h light/12 h dark). The animals had free access to water and were supplied with standard pellet diet (Pranav Agro Industries Ltd., Bangalore, India), constitution of protein (22.21%), fat (3.32%), fiber (3.11%), balanced with carbohydrates (> 67%), vitamins and minerals. Animal handling and experimental procedures were approved by the Institutional Animal Ethics Committee, Annamalai University (Registration Number: 952/2012/CPCSEA) and the animals were cared in accordance with the "Guide for the care and use of laboratory animals" and "Committee for the purpose of control and supervision on experimental animals".

2.3. Experimental design

In the present study, fluoride (Fl) was administered orally (gastric intubation) as sodium fluoride (NaF) at a dose of 25 mg/kg body weight/day for 4 weeks, which was 1/10 of the oral LD₅₀ value in rats (Chinnoy 1991). Control (Group 1) received the vehicles only, experimental rats were subdivided into two groups (3 and 4). Drug control group (Group 2) received the EGCG (40mg/kg bw) alone. In the experiment, a total of 24 rats were used. The rats were randomly divided into 4 groups of 6 animals in each.

Group 1: (*n* = 6) Control rats received the normal saline and vehicle.

Group 2: (*n* = 6) Rats administered with EGCG (40 mg/kg, b.w/day) dissolved in Tween 80

Group 3: (*n* = 6) Rats administered with NaF (25 mg/kg BW) dissolved in normal saline

Group 4: (*n* = 6) Rats exposed NaF and pre-administered with EGCG (40 mg/kg, b.w/day) (Thangapandiyan & Miltonprabu 2013).

At the end of the experimental period, rats were fasted overnight and anesthetized with pentobarbital sodium (35 mg/kg, IP) and euthanized by cervical decapitation. Blood samples were drawn from the caudal vena cava, and collected in test tubes and centrifuged at 1500xg for 10 min to obtain the serum. Tissue (liver) were dissected out, washed in ice-cold saline and stored at -20°C until used. The tissues were weighed and 10% tissue homogenate was prepared with 0.025 M Tris-HCl buffer, pH 7.5. After centrifugation at 2000 rpm for 10 min, the clear supernatant was used for biochemical assays.

3. Biochemical assays

3.1. Estimation of serum marker enzymes SGOT, SGPT, ACP, and ALP

Serum glutamate oxaloacetate (SGOT) and pyruvate (SGPT) transaminases, and alkaline phosphatases (ACP, ALP) levels were determined using standard kits (Eve's Inn Diagnostics, Baroda, India).

3.2. Estimation of plasma glucose, liver glycogen, hexokinase and G6PD activities

Plasma glucose levels were measured by standard kit (Eve's Inn Diagnostics, India). Hepatic glycogen was extracted with 30 % KOH, and the yield was estimated by anthrone -sulphuric acid method of Seifter et al. (1950). Hepatic hexokinase (EC 2.7.1.1) was determined based on reduction of nicotinamide adenine dinucleotide through a coupled reaction with glucose-6-phosphate dehydrogenase (Brandstap et al. 1957). Glucose-6-phosphatase (EC 3.1.3.9) activity was assayed by measuring the inorganic phosphate liberated from glucose- 6-phosphate (G-6-P) (Baginsky et al. 1974).

3.3. Estimation of serum protein markers

The concentration of total protein in the serum was assayed by the method of Lowry et al. (1951) and albumin concentrations were assayed according to the method of Doumas et al. (1971).

3.4. Estimation of plasma lipid profile

Plasma total cholesterol, triglycerides, HDL-cholesterol and LDL-cholesterol were determined using Diagnostic Kit-Beacon Diagnostics, Kabilpore, Navsari, India. The high density lipoprotein cholesterol (HDL-C) content in plasma was estimated by using a reagent kit (Qualigens Diagnostics, Mumbai, India). Very low density lipoprotein cholesterol (VLDL-C) and low density lipoprotein cholesterol (LDL-C) fractions were calculated as VLDL-C = TG/5 and LDL-C = total cholesterol - (HDL-C + VLDL-C), respectively.

3.5. Estimation of lipid marker enzymes

The activity of HMG-CoA reductase in the liver was assayed by the method of Philipp and Shapiro (1970). In brief, equal volumes of fresh 10% tissue homogenate and diluted perchloric acid were mixed, kept for 5 min and centrifuged at 2000 x g for 10 min. To 1.0 ml of filtrate, 0.5 ml of freshly prepared hydroxylamine reagent (alkaline hydroxylamine in the case of HMG-CoA) was added, mixed and after 5 min, 1.5 ml of ferric chloride was added and shaken well. Reading's were taken after 10 min at 540 nm against a similarly treated saline-arsenate blank. The ratio of HMG-CoA to mevalonate was calculated. Lower ratio indicates higher enzyme activity and higher ratio indicate slower enzyme activity.

Plasma lipoprotein lipase activity was assayed by the method of Korn (1955). The incubation mixture contained 0.3 ml of ammonium chloride (0.025 M)–ammonia buffer (0.25 M, pH 8.5), 0.1 ml of calcium chloride (1 M), and 0.4 ml of substrate and 0.2 ml of plasma. The contents were incubated at 37 °C for 60 min. At the end of the incubation period, 1.0 ml of cold 0.2 N H₂SO₄ was added to arrest the reaction. In case of control, plasma was added after the addition of 1.0 ml of 0.2 N H₂SO₄. The liberated glycerol was estimated in aliquot of the incubation mixture.

Plasma lecithin cholesterol acyl transferase activity was assayed by the method of Hitz et al. (1983). 0.6 ml of the substrate was mixed with 0.6 ml of the enzyme. 0.2 ml of this mixture was mixed with 1.0 ml of isopropanol while the remaining mixture was incubated at 27 °C for 90 min. The precipitate was removed by centrifugation and the supernatant was taken for the estimation of free cholesterol (Zlatkis et al. 1953). This represented the amount of free cholesterol present in the test sample at zero time. After 90 min, 0.2 ml of the incubated mixture was mixed with 1.0 ml of isopropanol and the remaining mixture was incubated at 27 °C for a further period of 90 min. At the end of 180 min, 0.2 ml of the incubated mixture was treated with 1.0 ml of isopropanol to arrest the reaction. The precipitated protein in all the tubes were separated by centrifugation and the free cholesterol content in the supernatant were estimated by Zlatkis et al. (1953). Control tubes containing only the substrate were treated similarly to check for complete in activation of plasma during substrate preparations. Lecithin cholesterol acyl transferase activity was expressed as a function of the disappearance of free cholesterol during the incubation period.

3.6. Estimation of cholesterol

Lipids were extracted from tissues by the method of Folch et al. (1957) using chloroform–methanol mixture (CHCl₃: MeOH) (2:1, v/v). The total cholesterol was estimated by the method of Zlatkis et al. (1953). To 0.1 ml of the lipid extract/serum, 9.9 ml of ferric chloride–acetic acid reagent was added and allowed to stand for 15 min and then centrifuged. To 5 ml of the supernatant, add 3 ml of conc. H₂SO₄. The colour developed was read after 20 min at 560 nm against a reagent blank. A set of standards (cholesterol 200 mg/dl) was also performed in the similar manner. Values were expressed as mg/100 g tissue and mg/dl serum.

3.7. Determination of triglycerides

Triglycerides were estimated by the method of Fossati and Lorenzo (1982). To an aliquot of plasma/lipid extract, evaporated to dryness. Volumes of 0.1 ml of methanol, 4 ml of isopropanol were added. To this mixture 0.4 g of alumina were added and shaken well for 15 min. Centrifuged and then 2 ml of the supernatant was taken for estimation. The tubes were placed in a water bath at 65 °C for 15 min for saponification after adding 0.6 ml of the saponification reagent (5 g of potassium hydroxide was dissolved in 60 ml of distilled water and 40 ml of isopropanol was added to it) followed by 0.5 ml of acetyl acetone reagent (0.75 ml of acetyl acetone was dissolved in 60 ml of distilled water and 40 ml of isopropanol was added to it). After mixing, the tubes were kept in

a water bath at 65 °C for 1 h, the contents were cooled. A series of standards of concentrations 8–40 lg triolein (1 g of triolein was dissolved in 100 ml of isopropanol) were treated similarly along with a blank containing only the reagents and absorbance was read at 420 nm. The triglyceride content was expressed as mg/100 g tissue and mg/dl serum.

3.8. Determination of phospholipids

Phospholipids content was determined by the method of Zilversmit and Davis (1950). To 0.1 ml of plasma/lipid extract, added 1 ml of 5 N H₂SO₄ and 1 ml of concentrated nitric acid and digested to a colorless solution. The phosphorus content in the extract was determined by the method of Fiske and Subbarow (1925). The values were expressed as g/100 g tissue and mg/dl serum.

3.9. Determination of free fatty acids

Free fatty acids were estimated by the method of Falholt et al. (1973). 0.1 ml of plasma/lipid extract was evaporated to dryness. 1 ml of phosphate buffer, 6 ml of extraction solvent and 2.5 ml of copper reagent (10 ml of copper solution was mixed with 10 ml of triethanolamine and 6 ml of sodium hydroxide and diluted to 100 ml, then 33 g of sodium chloride was added and the pH was adjusted to 8.1) were added. All the tubes were shaken vigorously. 200 mg of activated silicic acid was added and left aside for 30 min. The tubes were centrifuged and 3 ml of the copper layer was transferred to another tube containing 0.5 ml of diphenyl carbazide (1.5 M in ethanol) and mixed carefully. A solution containing 2 mg/ml of palmitic acid in chloroform was used as the standard. The absorbance was read at 550 nm immediately. The amount of free fatty acids was expressed as mg/100 g tissue and mg/dl serum.

3.10. Estimation of faecal cholesterol and bile acid content

The faecal cholesterol and bile acids were extracted using alkaline-methanol medium, and cholesterol was estimated by Kaiek et al. (1984). A portion of the extract was acidified and used for bile acid estimation by the method of Snell and Snell (1953).

3.11. Histological examination

A portion of the liver from control and experimental rats were taken and fixed in 10% buffered neutral formal saline and dehydrated by passing successfully in different mixtures of ethylalcohol–water, cleaned in xylene and embedded in paraffin. Sections of the tissue (5–6 mm thick) were prepared by using a rotary microtome and stained with haematoxylin and eosin (H&E) dye, which was mounted in a neutral deparaffinated xylene. The structural changes in the sections were examined under a light microscope.

3.12. Statistical analysis

Data are presented as the mean ± SD and were statistically analyzed by one-way analysis of variance (ANOVA) using SPSS version 16.0 (SPSS Inc., Cary, North Carolina, USA) and the individual comparisons were obtained by Duncan's multiple range test. Values for P < 0.05 were considered statistically significant.

4. Results

4.1. Effect of EGCG on serum SGOT, SGPT, ACP, and ALP

The Fluoride administration caused abnormal liver function, in rats. Hence, the activities of serum liver marker enzymes such as

SGOT, SGPT, ALP and ACP were significantly ($P < 0.05$) increased when compared with control rats. Whereas EGCG (40mg/kg bw) pre-oral supplemented rats significantly ($P < 0.05$) decreased the activities of serum hepatic markers. However, oral administration of EGCG alone to normal rats did not show any significant ($P < 0.05$) effect on serum hepatic markers (Table 1).

4.2. Effect of EGCG on plasma glucose, liver glycogen, hexokinase and G6PD activities

Table 2 shows the levels of plasma glucose, liver glycogen, hexokinase and G6PD in control and experimental rats. Fluoride administered rats significantly increased ($P < 0.05$) the levels of plasma glucose and hepatic G6PD and decreased hepatic glycogen and hexokinase activity when compared with control. EGCG supplementation resulted in a significant ($P < 0.05$) decrease in the plasma glucose and hepatic G6PD levels with significant ($P < 0.05$) increase in the hepatic glycogen content and hexokinase activity in fluoride treated rats. Normal rats treated with EGCG also showed a significant ($P < 0.05$) difference in the lipids profiles as compared with control group.

4.3. Effect of EGCG on serum total proteins and albumin

Results about the effect of fluoride and EGCG on serum total proteins and albumin are shown in Fig. 2. Fluoride administration at a dose of 25 mg/kg for 4 weeks evoked a significant decrease ($P < 0.05$) in total protein as evidenced by decrease in hepatic serum albumin level compared to the control group. EGCG pre-administration of fluoride-intoxicated rats enhanced the mitigated total proteins as evidenced by significant ($P < 0.05$) increase in

serum albumin level. EGCG-treated group did not show any significant change compared to the control group.

4.4. Effect of EGCG on plasma lipid profile

Table 3 shows the effect of treatment with the EGCG on fluoride induced plasma cholesterol, triglycerides, phospholipids, free fatty acids and lipoproteins in the control and experimental group of rats. The levels of serum cholesterol, triglycerides, phospholipids, free fatty acids, LDL-cholesterol (LDL-C) and VLDL-cholesterol (VLDL-C) were significantly ($P < 0.05$) increased, whereas levels of HDL-cholesterol (HDL-C) significantly decreased in fluoride intoxicated rats when compared with control rats. Pre-oral administration of EGCG to fluoride intoxicated rats significantly ($P < 0.05$) reversed all these changes into normal levels. Normal rats treated with EGCG alone showed no significant difference in the lipid profiles.

4.5. Effect of EGCG on plasma lipid marker enzymes

Figure 3 exemplify the changes in the activities of lipoprotein lipase and lecithin cholesterol acyl transferase in the control and experimental rats. A significant decrease ($P < 0.05$) in the activities of lipoprotein lipase (Fig.3A) and lecithin cholesterol acyl transferase (Fig.3B) were observed in plasma of fluoride intoxicated rats as compared to the normal rats. Pre-administration of EGCG to fluoride rats significantly ($P < 0.05$) increased the activities of lipoprotein lipase and lecithin cholesterol acyl transferase when compared to fluoride untreated rats.

Table 1: Effect of EGCG on Fluoride Induced Changes in the Serum Hepatic Markers of Control and Experimental Rats.

Parameter	Control	EGCG	Fl	EGCG+Fl
SGOT (U/I)	48.06 ± 0.09 ^a	50.3 ± 0.45 ^a	67.02 ± 0.25 ^c	50.25 ± 0.15 ^d
SGPT (U/I)	31.11 ± 0.72 ^a	33.43 ± 0.56 ^{ab}	43.51 ± 0.96 ^c	30.96 ± 0.54 ^{cd}
ALP (U/I)	61.22 ± 0.57 ^a	63.1 ± 0.42 ^{ab}	82.31 ± 0.32 ^c	62.52 ± 0.1 ^d
ACP (U/I)	40.3 ± 0.1 ^a	42.05 ± 0.65 ^a	71.43 ± 0.26 ^c	50.03 ± 0.14 ^{cd}

Values are given as mean ± SD for six rats in each group. Values with different superscript letter. (a–d) in the same row or column differ significantly at $p < 0.05$ (DMRT).

- a) Values not differ significantly from control group at ($P < 0.05$).
- b) Values differ significantly from control group at ($P < 0.05$).
- c) Values differ significantly from EGCG group at ($P < 0.05$).
- d) Values differ significantly from Fluoride group at ($P < 0.05$).

Table 2: Effect of EGCG on Fluoride Induced Changes in the Plasma Glucose, Liver Glycogen, Hexokinase and G6PD Activities of Control and Experimental Rats

Parameters	Control	EGCG	Fl	EGCG + Fl
Glucose (mg/dl)	93.44 ± 1.31 ^a	120.04 ± 1.60 ^b	188.72 ± 0.88 ^c	150.43 ± 1.23 ^d
Glycogen (mg/g)	16.97 ± 0.21 ^a	15.11 ± 0.18 ^b	10.09 ± 0.31 ^c	13.10 ± 0.27 ^d
Hexokinase (U/mg /protein/min)	8.32 ± 0.17 ^a	6.33 ± 0.20 ^b	3.70 ± 0.10 ^c	5.04 ± 0.16 ^d
G6PD (U/mg protein/min)	0.17 ± 0.03 ^a	0.33 ± 0.02 ^b	0.48 ± 0.02 ^c	0.28 ± 12 ^d

Values are given as mean ± SD for six rats in each group. Values with different superscript letter.

(a–d) in the same row or column differ significantly at $p < 0.05$ (DMRT).

- a) Values not differ significantly from control group at ($P < 0.05$).
- b) Values differ significantly from control group at ($P < 0.05$).
- c) Values differ significantly from EGCG group at ($P < 0.05$).
- d) Values differ significantly from Fluoride group at ($P < 0.05$).

Table 3: Effect of EGCG on the Level of Plasma Cholesterol, Triglycerides, Phospholipids, Free Fatty Acids and Lipoproteins Concentrations in Control and Experimental Rats.

Parameters	Control	EGCG	Fl	EGCG + Fl
Cholesterol (mg/dl)	96.8 ± 4.01 ^a	95.5 ± 3.7 ^a	248.9 ± 9.4 ^b	121.2 ± 4.4 ^c
Triglycerides(mg/dl)	78.5 ± 2.9 ^a	76.1 ± 2.7 ^a	257.1 ± 8.9 ^b	101.6 ± 3.4 ^c
Phospholipids (mg/dl)	87.6 ± 2.8 ^a	85.2 ± 2.7 ^a	179.4 ± 5.5 ^b	134.2 ± 4.2 ^c
Free fatty acids (mg/dl)	80.5 ± 2.3 ^a	77.1 ± 2.1 ^a	175.1 ± 4.7 ^b	80.8 ± 2.1 ^{ac}
VLDL-C (mg/dl)	20.7 ± 0.5 ^a	21.4 ± 0.7 ^a	58.3 ± 2.0 ^b	28.3 ± 0.9 ^c
LDL-C (mg/dl)	44.4 ± 1.5 ^a	42.8 ± 1.3 ^a	138.1 ± 5.3 ^b	61.6 ± 2.3 ^c
HDL-C (mg/dl)	29.9 ± 1.0 ^{abc}	30.6 ± 1.2 ^{abc}	20.3 ± 5.1 ^b	25.3 ± 1.3 ^{abc}

Values are given as mean ± SD for six rats in each group. Values with different superscript letter.

(a–c) in the same row or column differ significantly at $p < 0.05$ (DMRT).

- a) Values not differ significantly from control and EGCG group at ($P < 0.05$).
- b) Values differ significantly from control and drug control group at ($P < 0.05$).

c) Values differ significantly from FI group at ($P < 0.05$).

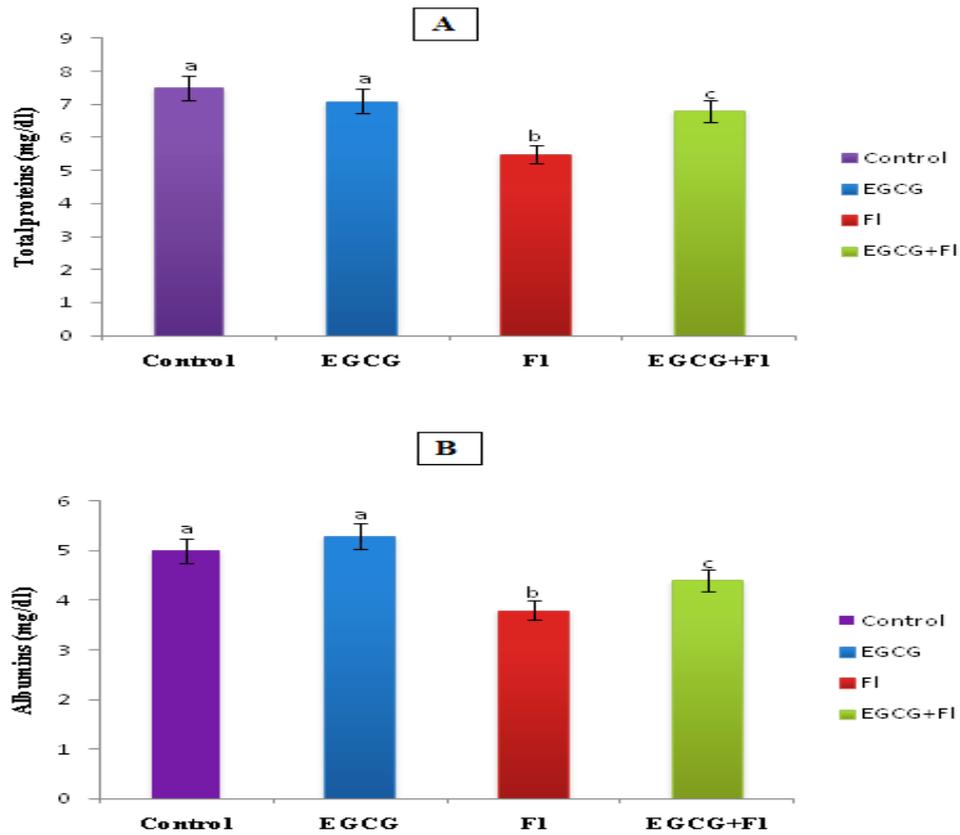


Fig. 2: Effect of EGCG on Fluoride Induced Changes in the Levels of Total Protein (A) and Albumins (B) in Control and Experimental Groups. Values are Mean \pm SD for Six Rats in Each Group. Values Not Sharing a Common Superscript Letters (A, B And C) Differ Significantly at $P < 0.05$ (DMRT).

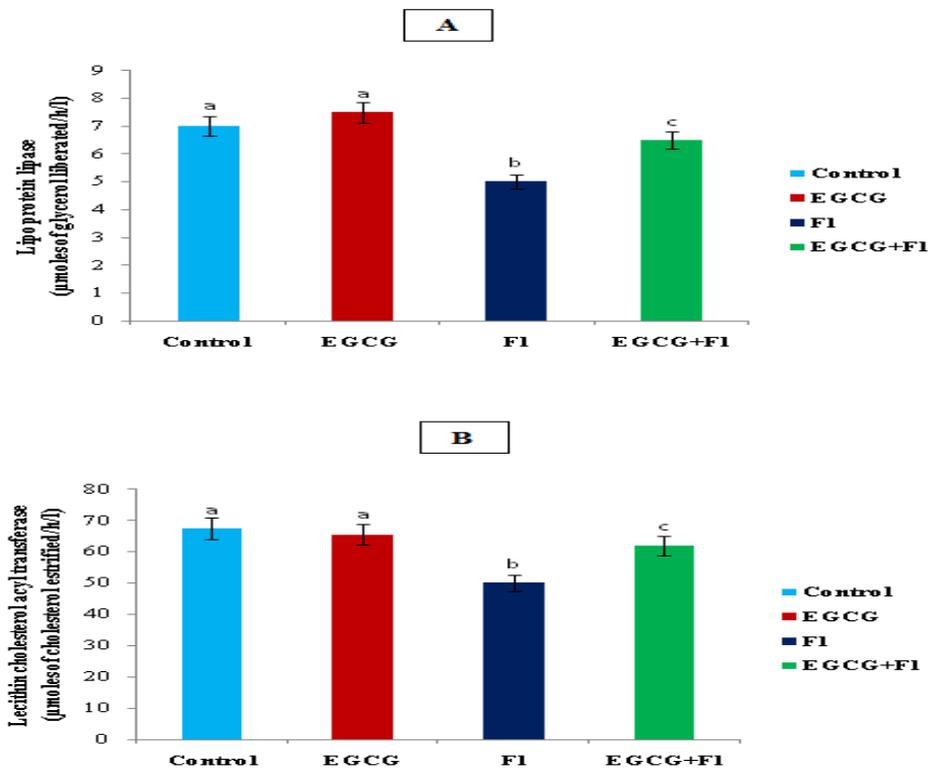


Fig. 3: Effect of EGCG on Fluoride Induced Changes in the Lipoprotein Lipase (A) and Lecithin Cholesterol Acyltransferase (B) in Control and Experimental Groups. Values are Mean \pm SD for Six Rats in Each Group. Values Not Sharing a Common Superscript Letters (A, B and C) Differ Significantly at $P < 0.05$ (DMRT).

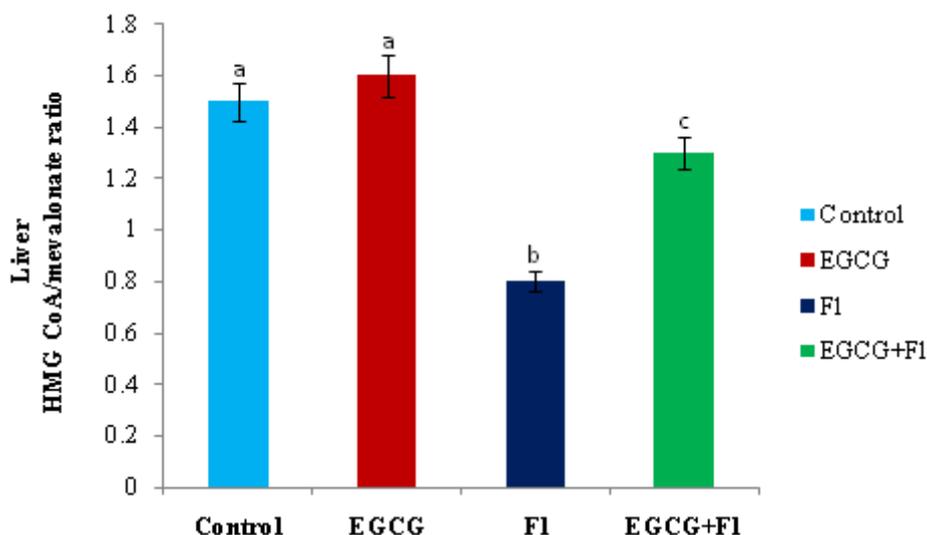


Fig. 4: Effect of EGCG on Fluoride Induced HMG Coa in Liver of Control and Experimental Rats. Values are Mean \pm SD for Six Rats in Each Group. Values Not Sharing A Common Superscript Letters (A, B and C) Differ Significantly at $P<0.05$ (DMRT).

4.6. Effect of EGCG on liver marker enzyme

Figure 4 illustrate the changes in the activities of liver HMG-CoA reductase in the control and experimental rats. A significant ($P<0.05$) increase in the activity of HMG-CoA reductase was observed in the liver of fluoride intoxicated rats. Pre-oral treatment of EGCG to fluoride rats significantly ($P<0.05$) decreased the activity of HMG-CoA reductase in the liver tissue in comparison with fluoride alone treated rats. EGCG alone treated rats showed no significant difference in the HMG-CoA reductase.

4.7. Effect of EGCG on liver lipid profiles

Table 4 depicts the levels of cholesterol, triglycerides, phospholipids and free fatty acids in the liver of control and experimental groups of rats. There was a significant ($P<0.05$) increase in the cholesterol, triglycerides, phospholipids and free fatty acids in the liver of fluoride intoxicated rats. Following the pre-oral administration of EGCG significantly ($P<0.05$) decreased these levels were found to be similar to those in control rats.

Table 4: Effect of EGCG on Fluoride Induced Cholesterol, Triglycerides, Phospholipids, and Free Fatty Acids in the Liver of Control and Experimental Rats.

Parameters	Control	EGCG	FI	EGCG + FI
Cholesterol (mg/dl)	12.6 \pm 1.1 ^a	11.6 \pm 1.3 ^a	20.7 \pm 1.3 ^b	15.4 \pm 2.5 ^c
Triglycerides (mg/dl)	12.3 \pm 1.4 ^a	11.2 \pm 1.2 ^a	18.5 \pm 2.4 ^b	13.4 \pm 1.3 ^c
Phospholipids (mg/dl)	20.9 \pm 2.4 ^a	21.7 \pm 1.8 ^a	30.3 \pm 3.5 ^b	24.6 \pm 4.4 ^c
Free fatty acids (mg/dl)	13.9 \pm 1.5 ^a	13.5 \pm 1.3 ^a	19.1 \pm 1.4 ^b	16.4 \pm 3.4 ^c

Values are given as mean \pm SD for six rats in each group. Values with different superscript letter.

(a-c) in the same row or column differ significantly at $p<0.05$ (DMRT).

a) Values not differ significantly from control and EGCG group at ($P<0.05$).

b) Values differ significantly from control and drug control group at ($P<0.05$).

c) Values differ significantly from FI group at ($P<0.05$).

4.8. Effect of EGCG on faecal cholesterol and bile acid content

Figure 5 demonstrate the effect of EGCG on fluoride induced changes in the fecal cholesterol and bile acid content in control and experimental rats. Fluoride intoxicated rats showed higher

levels of faecal cholesterol (Fig 5A) and bile acid (Fig 5B) content when compared to the control group. However, oral administration of EGCG significantly ($P<0.05$) increased the fecal cholesterol and bile acid contents when compared to control and fluoride intoxicated group. While EGCG administrated rat also showed increased levels of fecal cholesterol and bile acid content when compared to control.

4.9. Effect of EGCG on liver histology

Fig.6A–E depicts the photomicrographs of hematoxylin–eosin stained liver tissues of control and experimental animals. Liver section from normal and drug control rats showed normal histoarchitecture with well-defined manner (Figs. 6A and B). The liver cells of animals exposed to fluoride for 4 weeks suffered several pathological changes, such as extensive degeneration of hepatocytes with focal necrosis, bridging necrosis, inflammation, inflammatory cell infiltration, portal inflammation, and fatty degenerative changes, etc., (Figs. 6C and 5D). Pre-administration of EGCG effectively attenuated the histopathological abnormalities evoked by exposure to fluoride (Fig. 6E).

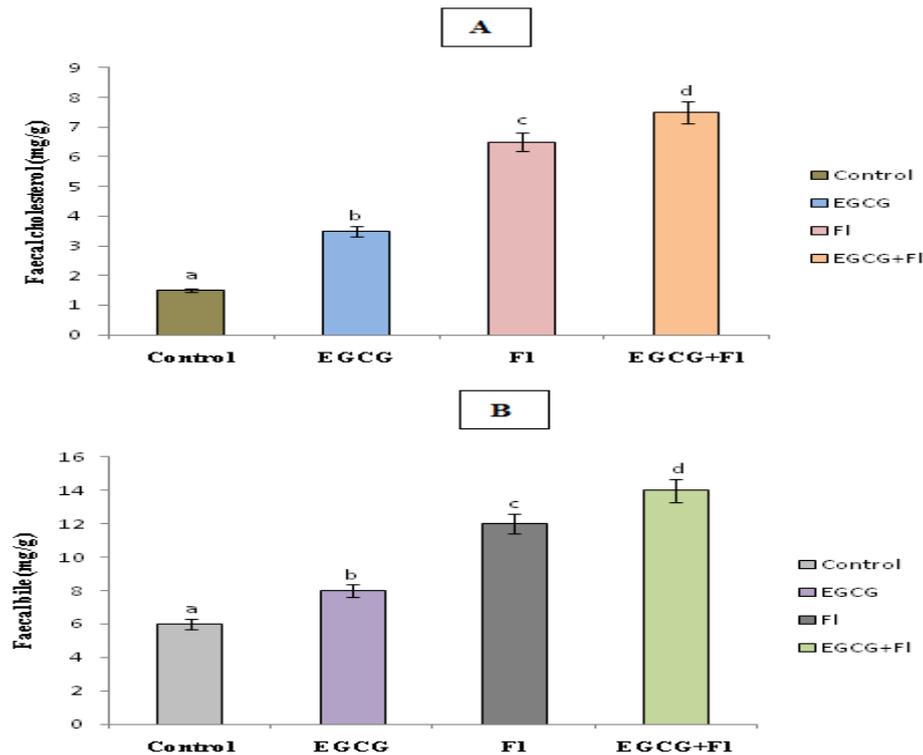


Fig. 5: Effect of EGCG on Fluoride Induced Serum Faecal Cholesterol and Faecal Bile Acid in Control and Experimental Rats. Values are Mean \pm SD for Six Rats in Each Group. Values Not Sharing A Common Superscript Letters (A, B C And D) Differ Significantly at $P < 0.05$ (DMRT).

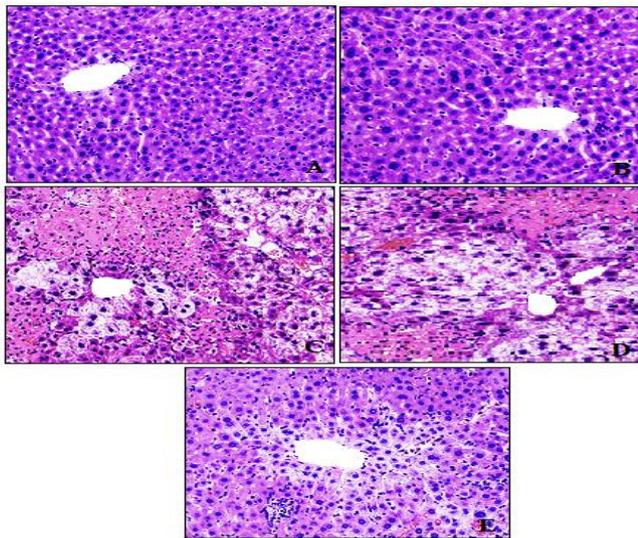


Fig. 6: Representative Photomicrographs from Liver Sections of Control and Experimental Male Wistar Rats Stained with Haematoxylin and Eosin. (A) Section of Control Liver Showing Normal Arrangement of Sinusoids and Hepatocytes. (B) Section of Liver Treated with Epigallocatechin Gallate (EGCG) Alone Showing the Normal Histoarchitectural Pattern. (C & D) Section of Liver Exposed to FI Showing the Extensive Inflammation, Dilated Sinusoids, Degeneration of Hepatocytes with Necrosis, Vacuolization, and Inflammatory Cell Infiltration, Fatty Degeneration of Hepatocytes (E) Section of Liver Treated with EGCG and FI Showing Significant Improvement of Hepatic Histoarchitecture with Mild Infiltration.

5. Discussion

Liver is an important organ for metabolism and detoxification of any substances. Leakage of hepatospecific enzymes into blood serum has been considered an indicator of hepatic damage and dysfunction. Fluoride is known to cause hepatic tissue injury as reflected by a significant increase in serum hepatic marker enzymes indicating the cellular leakage and loss of functional integrity of the hepatic membrane architecture (Thangapandiyan & Miltonprabu 2013). In the present study, Fluoride intoxication

caused a significant increase in the activities of SGOT, SGPT, ALP and ACP, due to hepatocyte membrane damage. Reactive oxygen species (ROS) elicited by fluoride are implicated as important pathological mediators in many types of disorders. Their presence is reflected by increased oxygen radical generation and caused lipid peroxidation in the liver (Chinoy & Memon 2001). Rzeuski et al. (1998) reported that excessive ROS persuaded by fluoride can induce lipid peroxidation, which reduced activities of antioxidant enzymes, which may result in the damage of membrane integrity that leads to leakage of liver specific enzymes into blood. Administration of EGCG remarkably improved the alterations in hepatic functions induced by fluoride. Because, EGCG has already been reported to exhibit membrane-stabilization properties against fluoride induced ROS-mediated oxidative hepatocellular injury (Thangapandiyan & Miltonprabu 2013).

In the present study fluoride-exposed rats exhibited a significant elevation in blood glucose and G6PD levels with decreased hepatic glycogen and hexokinase activity. Administration of EGCG resulted in a substantial lowering of blood glucose levels and hepatic G6PD activity along with improvements in hepatic glycogen content and hexokinase activity in fluoride-exposed animals. The decline in hexokinase activity in fluoride administered animals could be due to lowered insulin levels (Garcia-Montalvo et al. 2009). The increase in hexokinase activity could be due to an insulin restoratory potential of EGCG (Lin & Lin 2008). These antihyperglycaemic activities of EGCG could be ascribed to the secondary metabolites (polyphenols and flavonoids) present in green tea leaves. Polyphenols are known to inhibit glucose absorption in the gut, inhibit peripheral tissue glucose uptake by glucose transporters (Pandey & Rizvi 2009), protect pancreatic β cells and inhibit insulin resistance (Zunino & Storms 2007). Flavonoids are reported to possess antidiabetogenic potential. This purported antidiabetogenic activity of flavonoids was attributed to an increase in pancreatic secretion of insulin from β cells or its release from bound insulin (Sharma et al. 2003). Therefore, the phytometabolites present in EGCG may have contributed individually or synergistically to the declined levels of glucose and hepatic G-6-Pase while enhancing the activity of hexokinase and improving the glycogen content in the liver.

Irregular protein metabolism is considered a sign of hepatic dysfunction. In the present study, the administration of fluoride resulted in a significant decrease in the attention of total protein and albumin as compared to the control group. Earlier studies reported a similar reduction in protein content in fluoride treated animals and related it to inhibition of decarboxylation of branched chain amino acids and simultaneously promoting protein breakdown (Shashi et al. 1992). Fluoride affects cellular protein synthesis mainly due to the impairment of peptide chain initiation (Godehaux & Atwood 1976). Fluoride spawned free radicals down-regulate the activity of enzymes important in the polymerization of amino acids, thus inhibiting the process of elongation of peptides (Hordyjewska & Pasternak 2004). Furthermore, the observed decrease in protein content may be explained in part by the reduction in insulin level since insulin has an anabolic effect on protein metabolism in that it stimulates protein synthesis and retards protein degradation (Murray et al. 1999). Previous reports have shown that protein synthesis is decreased in all tissues due to absolute or relative deficiency of insulin (Chatterjea & Shinde 1994). EGCG Supplementation improved the reduced levels of total proteins and albumin of fluoride intoxicated rats. This tendency to increase the level of protein contents could be ascribed to suppression of fluoride induced oxidative stress and liver damage with the subsequent improvement of natural antioxidant in the body against free radicals.

A high intake of fluoride result in various biochemical changes, including alternation in lipid metabolism in the liver (Thangapandiyan & Miltonprabu 2013). In the present study, a significant increase level of serum lipid profiles (cholesterol, triglycerides, phospholipids, free fatty acids and phospholipids) and lipoproteins were observed. The changes in the serum lipid profiles and other lipid compounds noted in the fluoride treated rats may be explained by the increased activity of HMG-CoA via accumulation of ROS releasing inflammatory cytokines in the liver (Afolabi et al. 2013). The changes in the activity of HMG-CoA reductase may depress LDL receptor gene expression. Defects in LDL receptor interfere with cholesterol uptake from the blood stream, which in turn causes excess cholesterol synthesis in liver and high levels of plasma cholesterol and LDL-C (Rupal et al. 2012). Lipase enzyme responsible for the controlling of triglyceride accumulation in the liver, but the buildup of ROS induced by fluoride inhibit the lipase enzyme and increase the triglycerides (TG) level in the tissue and serum. Increasing levels of TG leads to elevation of VLDL-C in the serum because VLDL particles are the main transporters of TG in plasma [52]. The overproduction of hepatic VLDL-C and impaired catabolism of TG-rich particles may lead to hypertriglyceridemia. Lowered level of plasma HDL-C implies the altered metabolism of the major HDL apoprotein A-I in the liver (Suttie & Phillips 1960). Phospholipids (PL) and free fatty acids (FF) are consisting of rich polyunsaturated compounds, which are easily susceptible to reactive oxygen species (Doroshov 1983). Formations of reactive oxygen species (ROS) induced by fluoride may affect the membrane-bound enzymes, and permeability of ions by which increase the PL levels in serum and liver tissue. Pre-treatment of EGCG in fluoride treated rats significantly has normalized the serum lipid profiles (cholesterol, triglycerides, phospholipids and free fatty acids) and lipoproteins through their antilipoperoxidative and antioxidant actions, which inhibit the extensive accumulation of cholesterol and oxidized lipid components as well as prevent hypercholesterolemia.

HMG-CoA reductase is the rate-limiting enzyme in cholesterol biosynthesis. An increase in the activity of HMG-CoA reductase leads to the excessive production and accumulation of cholesterol resulting in the formation of foam cells, a pre-requisite step in the development of atherosclerosis (Easterbauer et al. 1992). Rising evidence indicates that the activity of HMG-CoA reductase is increased in fluoride intoxicated rats (Rupal et al. 2012) due to deficiency of insulin is associated with increased HMG-CoA reductase activity. Fluoride induced ROS has the ability to inhibit lipoprotein lipase and lecithin cholesterol acyl transferase which was also responsible for the elevated HMG-CoA levels in the liver

tissue. In the present study, significantly increased HMG-CoA activity was observed in fluoride intoxicated rats. Our results of elevated HMG-CoA were corroborated with the existing previous reports (Miltonprabu et al. 2010; Nareshkumar et al. 2013). Pre-administration of EGCG to fluoride intoxicated rats had a positive effect on the HMG-CoA enzyme of lipid metabolism. This is due to the activities of lipoprotein lipase and lecithin cholesterol acyl transferases were increased in fluoride rats treated with EGCG. Restoration of the lipid profile in fluoride rats could be the lipid lowering activities of EGCG could also due to its insulinotropic effect (Chih-Li et al. 2008).

From the present observation fluoride treated rats showed decreased levels of lipoprotein lipase in the plasma. Our findings of decreased lipoprotein lipase by fluoride are in tune with the streptozotocin (45mg/kg/BW) treated rats for 30days (Nareshkumar et al. 2013). It is one of the lipid metabolizing enzymes and its activity was controlled by the insulin. The decreased lipoprotein lipase activity may contribute by the fluoride induced free radicals, which were actively oxidize the lipid molecules and significantly increased HMG-CoA and triglyceride levels in the liver and plasma of fluoride intoxicated rats by which lowered lipoprotein lipase (Bhardwaj & Shashi 2013). In this circumstance, the pre-oral administration of EGCG was significantly increased the lipoprotein lipase activity by the direct reduction of glucose in the blood of fluoride intoxicated rats. This may be due to the enhanced free radicals quench and activation of β -cells to secrete insulin for decreasing the mobilization of free fatty acids and triglycerides from the fat depots (Suqing et al. 2014).

Lecithin cholesterol acyl transferase is one of the key enzymes involved in HDL-C metabolism. It is esterifies free cholesterol to cholesteryl esters in serum (Chtubek et al. 2003). The formation and accumulation of cholesteryl esters in the core of HDL results from the removal of cholesterol from the surface lipoprotein with simultaneous efflux through cell membranes into HDL-C (Suqing et al. 2014). In the present study, the decreased activity of lecithin cholesterol acyl transferase was observed in the plasma of fluoride intoxicated rats, which was in accordance with the previous reports (Chtubek et al. 2003; Nareshkumar et al. 2013). Fluoride is a well-known inhibitor of lipases, phosphatases, esterases, and acetyl Co-A synthetase. It interferes with fatty acid oxidation which results in decreased synthesis of lecithin cholesterol acyl transferase in the plasma (Machoy-Mokrzynska et al. 1994). Pre oral administration of EGCG to fluoride treated rats had a positive effect on the marker enzyme of lecithin cholesterol acyl transferase. The lipid lowering activities of EGCG could be due to its strong antioxidants and hydrogen donating property to stabilize the lipid membranes.

In the present study, faecal cholesterol and bile were significantly increased in fluoride intoxicated rats, which was similar to the previous report of Rupal et al. (2012). The effect of fluoride on serum fecal cholesterol levels could be explained by several mechanisms: (1) an increased effectiveness of the feedback mechanism for cholesterol synthesis, (2) a decreased absorption of cholesterol from the intestine, (3) an increased conversion of cholesterol to bile salts, and (4) a decreased absorption of bile salts from the intestine resulting in an increased conversion of cholesterol to bile acids. The lack of response to the increased levels of fluoride in the absence of exogenous cholesterol suggests, that fluoride does not influence endogenous cholesterol metabolism [64]. The fecal bile acid increased due to fluoride induced ROS, which is responsible for the inhibition of HDL-C synthesized in the liver and intestinal cells. However, pathophysiological accumulation of bile acids can elicit cytotoxicity, and elevated concentrations are associated with liver injury (Dewayntownsen & Dleonsing 1977). Pre-administration of EGCG to fluoride intoxicated rats showed a significant increase of fecal cholesterol and fecal bile. This could be due to the rich polyphenolic fiber contents of green teas contained dietary EGCG found to increase cholesterol excretion by interfering with the enterohepatic circulation of cholesterol and bile acid. The presence of polyphenol and flavonoids in

EGCG also could be responsible for the antihypercholesterolaemic effects on fluoride intoxicated rats.

The histological results also strongly support our biochemical findings that EGCG protects against the fluoride-induced oxidative stress mediated hepatic (Fig. 5) injury. The histological findings from the liver of fluoride treated rats showed the necrosis, inflammatory cell infiltration, dilation of sinusoidal spaces, and inflammation (Fig. 5C&D). This could be due to the accumulation of free radicals as a consequence of increased lipid peroxidation by free fluoride- ions. The increased formation of lipid peroxides and protein carbonyls leads to the damage of membrane integrity and other pathological alterations in the fluoride treated liver tissue. Pre-treatment with EGCG markedly lessened the pathological changes provoked by fluoride, which is quite appreciable. It can be attributed to the antiradical, antioxidant, anti-inflammatory and chelating efficacy of EGCG, which significantly reduced the oxidative stress, leading to the reduction of pathological alterations and restoration of normal physiological state of an organism. Further, the membrane stabilizing properties of EGCG might be helpful to improve the pathological alterations caused by fluoride in the both tissues of rats.

Scientific reports showed that catechins possess many of the structural components that contribute to their antioxidant property. Catechins have a gallate moiety esterified at the 3rd position on the C ring, the catechol groups (3,4,5-trihydroxyl groups) on the B ring and the hydroxyl groups at the 5th and 7th positions on the A ring (Fig. 1). The potent free radical scavenging activity of EGCG was attributed to the presence within the C ring gallate group. The observation was also made that the more hydroxyl groups the catechin (EGCG) possesses the more effective for scavenging free radicals (Zhao et al. 2001).

6. Conclusion

In conclusion, the present investigation depicts that the administration of EGCG to fluoride intoxicated rat's decreases the ROS mediated metabolisms in liver tissue lipid profiles and restored the regulatory enzymes involved in the carbohydrates, protein, lipids and lipoprotein metabolism and maintains at a normal level. Therefore, in light of our observations, we conclude that EGCG could be used as a component in foods to promote the health of people living in endemic fluorosis areas as a means to ameliorate fluoride-induced ailments.

7. Conflicts of interest

The author declares that there are no conflicts of interest.

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