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AFLATOXIN-INDUCED BIOCHEMICAL CHANGES IN LIVER OF MICE AND ITS MITIGATION BY BLACK TEA EXTRACT

ANAMIKA JHA [1], KOMAL SHAH [2] and RAMTEJ J. VERMA [2]*

[1] Department of Biochemistry, St. Pious X PG College for Women, Nacharam, Hyderabad 500076, India
[2] Department of Zoology, University School of Sciences, Gujarat University, Ahmedabad 380009, India

Abstract: Aflatoxin belongs to the class of naturally occurring mycotoxins, food contaminants having potent carcinogenicity. We have evaluated the ameliorative role of black tea extract on aflatoxin-induced biochemical changes in the liver of albino male mice. Adult male mice were orally administered with 750 and 1500 μg of aflatoxin in 0.2 mL olive oil/kg b.w./day for 30 days. Oral administration of aflatoxin caused, as compared with controls, significant, dose-dependent reduction in DNA, RNA, protein and glycogen contents; however, cholesterol content and phsphorylase activity were significantly increased. Black tea is one of the most potent antioxidants containing numerous bioactive phytonurtients having therapeutic applications. Aflatoxin-induced changes in the liver of mice were significantly ameliorated on co-treatment of black tea extract (2% infusion in water).

Keywords: aflatoxin, black tea, liver

Aflatoxins are among the most common mycotoxins to which humans are exposed. They are highly substituted coumarin derivatives containing a fused dihydrofurofuran moiety. Human beings and mammals are exposed to aflatoxin through food/feed-stuffs, milk, meat and eggs. Consumption of aflatoxins in many parts of the world varies between 0–30,000 ng/kg/day. Epidemiological and experimental studies have shown that aflatoxins are hepatotoxic (1), hepatocarcinogenic (2), mutagenic (3) and teratogenic (4). From the carcinogenic point of view, AFB_1,-2,3-oxide is a highly reactive substance which can combine with DNA bases such as guanine to produce alterations in DNA (5). The presence of AFB_1-DNA adduct was identified both *in vivo* and *in vitro* (6). Acute aflatoxin poisoning caused hepatocellular necrosis and derangement of hepatic functions (7–9). Subacute or chronic aflatoxicosis caused fatty changes in the liver, enlargement of the gall bladder and periportal fibrosis with proliferative changes in bile duct epithelium (10).

Tea has one of the highest total flavonoid contents of all plants at 15% of the leaf by dry weight (11). In the manufacture of black tea, the "fermentation" process causes green tea catechins to oxidize and form oligomeric flavonols, including theaflavins, thearubigin and other oligomers. Multiple biological effects of flavonoids have been described, among them anti-inflammatory, anti-allergic, anti-hemorrhagic, anti-mutagenic, anti-neoplastic and hepatopreventive activities (12). Several studies have found that black tea and green tea offered protection against oxidative damage to red blood cells induced by a variety of inducers. Fenton et al. (3) showed that black tea theafulvins protect against aflatoxin-induced mutagenesis. Black tea extracts were also found to reduce lipid peroxidation in aflatoxin treated animals (13). Studies by Matsumoto et al. (14) demonstrated that tea catechins, black tea extract and oolong tea extracts had an inhibitory effect on the development of hepatocarcinogenesis in rats. Tea polyphenols sharply decreased the mutagenicity of a number of aryl and heterocyclic amines, of aflatoxin B_1, benzo[a]pyrene, 1,2-dibromoethane, and more selectively, of 2-nitropropane in *Salmonella typhimurium* (15).

The present study was an attempt to evaluate aflatoxin-induced biochemical changes in the liver of mice and its possible amelioration by black tea extract.

* Corresponding author: e-mail: ramtejverma2000@yahoo.com; anamikajha22@gmail.com; phone: +919825323077, +919966202281

MATERIALS AND METHODS

A toxigenic strain of *Aspergillus parasiticus var. globosus* (MTCC 411), obtained from Institute of Microbial Technology, Chandigarh, India, was grown on sucrose-magnesium sulfate-potassium nitrate-yeast extract (SMKY) liquid medium at 28 ± 2°C for 10 days (16). Ten day old culture filtrates were extracted with chloroform and aflatoxin content was determined using UV-Vis spectrophotometer (17). Young adult inbred Swiss strain male albino mice (*Mus musculus*) weighing approximately 32–35 g were obtained from Alembic Ltd., Baroda, India. Animals were provided with animal feed and water *ad libitum* and maintained under 12 h light/dark cycles at 26 ± 2°C. Guidelines for care and use of animals in Scientific Research 1991 published by Indian National Science Academy, New Delhi, India, was followed.

Seventy animals were randomly divided into seven groups and caged separately. Group 1 (untreated control) animals were maintained without any treatment. Animals of Group 2 and 3 received olive oil (0.2 mL/animal/day) and black tea extract (2% in drinking water), respectively, for 30 days and served as vehicle and antidote controls, respectively. Animals of Group 4 and 5 were orally administered with aflatoxin in 25 (LD-low dose) and 50 (HD-high dose) µg/0.2 mL olive oil/animal/day (750 and 1500 µg/kg b.w.), respectively, for 30 days. Group 6 and 7 animals were orally treated with aflatoxin as mentioned for Group 4 and 5 animals and given 2% black tea infusion instead of drinking water for 30 days.

Olive oil was obtained from Figaro, Madrid, Spain. Eighty grams of black tea solids (Lipton Yellow Label of Hindustan Lever Ltd., Mumbai, India) and 4 litres of deionized water were used to produce a 2% tea infusion. Aflatoxin was dissolved in olive oil; hence it was administered as a vehicle alone in Group 2 animals. As different isomers of aflatoxin exist together in the food-stuffs, we preferred to carry out the experiment with mixed aflatoxins. The dose of aflatoxin was based on LD_{50} value of aflatoxin, i.e., 9 mg/kg b.w. for male mice (18). The effective dose of black tea was based on earlier work on male mice (19). All the treatments were given orally using a feeding tube attached to a hypodermic syringe for 30 days. All chemicals used in the present study were of analytical grade.

On completion of the treatment, the animals were sacrificed by cervical dislocation. The liver of all experimental groups of animals were quickly isolated, blotted free of blood and utilized for biochemical analysis.

Biochemical estimations

Protein content

Protein content was measured in liver tissue by the method of Lowry et al. (20) using bovine serum albumin as a standard. Reaction of protein with Folin Ciocalteau reagent results in blue color, which is due to two reactions occurring simultaneously i.e., the reaction of alkaline copper sulfate solution with peptide bonds and reduction of phosphomolybdic and phosphotungstic acids by aromatic amino acids present in the protein. Resulting blue color was measured at 540 nm.

Nucleic acid content

A known weight of liver was homogenized in 5 mL of cold 5% trichloroacetic acid (TCA) and homogenate was kept at 0–4°C for 30 min. The precipitates obtained after centrifugation (10 min at 1,000 × g) were dissolved again in 5 mL of cold 5% TCA and left for 30 min at 0–4°C. Thereafter, centrifugation (10 min at 1,000 × g) was carried out and precipitates obtained were dissolved in ethyl alcohol : ether (1:3, v/v) mixture and left for 30 min at 50°C. This process was repeated once again. The tubes were centrifuged at 1,000 × g for 10 min and the supernatant was discarded. The pellet obtained finally, which was lipid free, was dissolved in 5 mL of 0.1 M KOH and incubated at 37°C for 16–18 h. Then 0.17 mL of 6 M HCl and 5 mL of 10% TCA were added to the incubated suspension and precipitates were allowed to be formed at 4°C for 30 min. After centrifugation at 1,000 × g for 10 min the supernatant and the pellet were separated. The supernatant was used for RNA estimation. The pellet containing DNA and protein was heated at 90°C for 15 min after adding 5 mL of 5% TCA. The supernatant was then separated by centrifugation (10 min at 1,000 × g) and used for DNA estimation.

Deoxyribonucleic acid

The estimation of deoxyribonucleic acid (DNA) was carried out by the method of Giles and Meyer (21). The DNA in the supernatant reacts with diphenylamine to give blue colored complex whose optical density was read at 620 nm. The concentration of DNA was expressed as µmoles/100 mg tissue weight.

Ribonucleic acid

The estimation of ribonucleic acid (RNA) was carried out by the method of Mejboum (22). The RNA content in the supernatant reacts with orcinol reagent to give a greenish color, which was read at 670 nm. The concentration of RNA was expressed as µmoles/100 mg tissue weight.

Glycogen content

Method described by Seifter *et al*. (23) was used to estimate tissue glycogen content. Anthrone reagent reacts with tissue glucose coming from glycogen breakdown to give green color. The color was read at 620 nm and was directly proportional to glycogen content of liver.

Cholesterol content

Cholesterol content was estimated in the liver by the method of Zlatkis et al. (24). Cholesterol forms a colored complex with ferric chloride ($FeCl_3$) in the presence of concentrated sulfuric acid and glacial acetic acid, which can be measured at 540 nm.

Phosphorylase activity

Phosphorylase activity in liver tissue was estimated according to the method described by Cori et al. (25). Known amount of tissue was homogenized in sodium citrate buffer (0.1 M), potassium fluoride (0.15 M) and glucose-1-phosphate. After incubation, reaction was terminated by addition of TCA to the tubes. The enzyme phosphorylase hydrolyzes the substrate glucose-1-phosphate into glucose and inorganic phosphorus.

Inorganic phosphorus

Liberated inorganic phosphorus was estimated by the method of Fiske and Subbarow (26). When inorganic phosphorus reacts with ammonium molybdate and amino-2-naphthol-4-sulfonic acid, blue color develops which was measured colorimetrically at 660 nm.

Statistical analysis

Results are expressed as the mean ± SEM. The data were statistically analyzed using one way analysis of variance (ANOVA) followed by Tukey

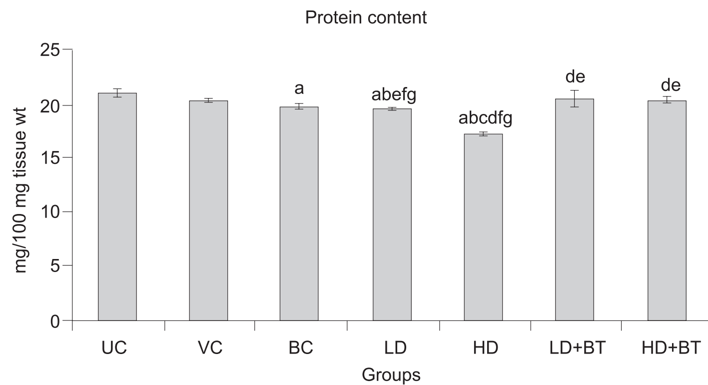

Figure 1. Effect of aflatoxin and black tea on protein content in mice liver. Values are expressed as the mean ± SEM, where n = 10. [a] as compared to group 1: $p < 0.05$, [b] as compared to group 2: $p < 0.05$, [c] as compared to group 3: $p < 0.05$, [d] as compared to group 4: $p < 0.05$, [e] as compared to group 5: $p < 0.05$, [f] as compared to group 6: $p < 0.05$, [g] as compared to group 7: $p < 0.05$. UC – untreated control, VC – vehicle control, BC – black tea control, LD – low dose of aflatoxin, HD – high dose of aflatoxin, LD + BT – low dose of aflatoxin + black tea, HD + BT – high dose of aflatoxin + black tea

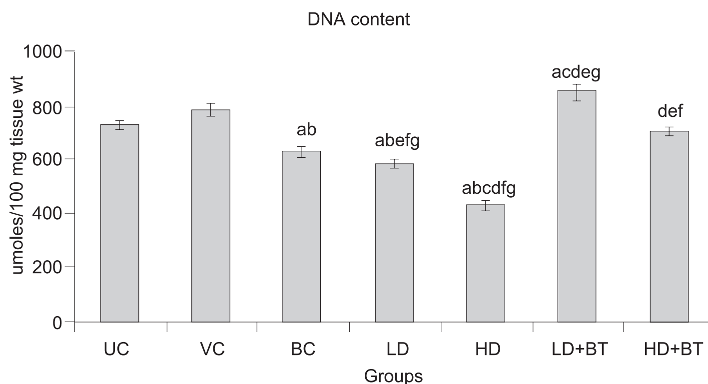

Figure 2. Effect of aflatoxin and black tea on DNA content in mice liver. Values are expressed as the mean ± SEM, where n = 10. [a] as compared to group 1: $p < 0.05$, [b] as compared to group 2: $p < 0.05$, [c] as compared to group 3: $p < 0.05$, [d] as compared to group 4: $p < 0.05$, [e] as compared to group 5: $p < 0.05$, [f] as compared to group 6: $p < 0.05$, [g] as compared to group 7: $p < 0.05$. Bars designations as in Fig. 1

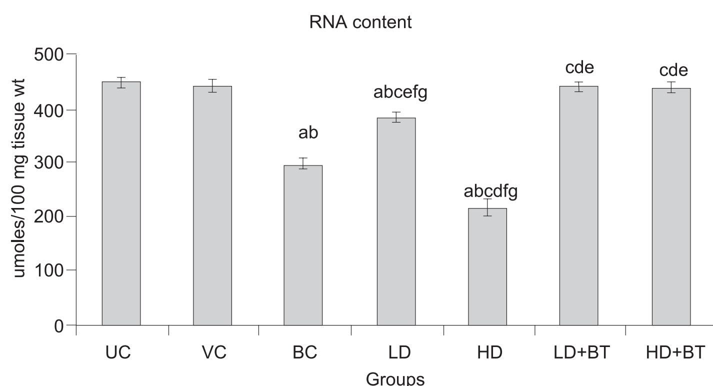

Figure 3. Effect of aflatoxin and black tea on RNA content in mice liver. Values are expressed as the mean ± SEM, where n = 10. [a] as compared to group 1: $p < 0.05$, [b] as compared to group 2: $p < 0.05$, [c] as compared to group 3: $p < 0.05$, [d] as compared to group 4: $p < 0.05$, [e] as compared to group 5: $p < 0.05$, [f] as compared to group 6: $p < 0.05$, [g] as compared to group 7: $p < 0.05$. Bars designations as in Fig. 1

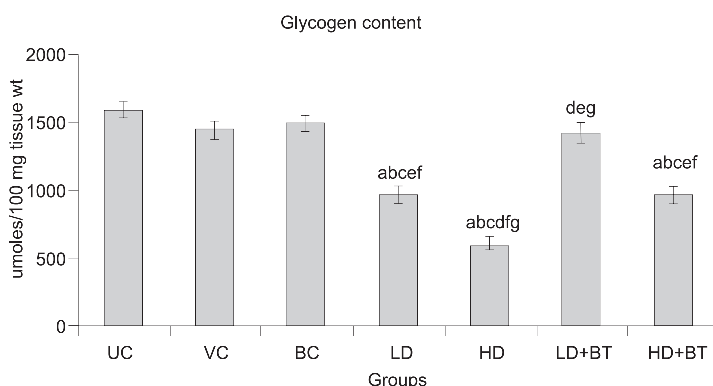

Figure 4. Effect of aflatoxin and black tea on glycogen content in mice liver. Values are expressed as the mean ± SEM, where n = 10. [a] as compared to group 1: $p < 0.05$, [b] as compared to group 2: $p < 0.05$, [c] as compared to group 3: $p < 0.05$, [d] as compared to group 4: $p < 0.05$, [e] as compared to group 5: $p < 0.05$, [f] as compared to group 6: $p < 0.05$, [g] as compared to group 7: $p < 0.05$. Bars designations as in Fig. 1

test. The level of significance was accepted with $p < 0.05$. Comparisons of p-values between different groups were performed.

RESULTS

No significant alteration in protein, DNA, RNA, glycogen, cholesterol content and phosphorylase activity were noted between untreated, vehicle and antidote control groups of animals. Oral administration of aflatoxin for 30 days in mice had resulted in significant reduction in protein content (LD: –4.42%, HD: –15.37%; Fig. 1). Both the doses of aflatoxin (LD: –25.33%, HD: –44.99%; Fig. 2) had significantly reduced levels of DNA content in mice liver as compared to control. Low dose and high dose of aflatoxin had also reduced RNA content in mice liver upon 30 days treatment up to 13.14% and 50.82%, respectively (Fig. 3). Glycogen content of aflatoxin treated animals was also found to be reduced significantly and in a dose-dependent manner (LD: –33.16%, HD: –58.68%; Fig. 4). On the other hand, cholesterol content (LD: 16.28%, HD: 27.97%; Fig. 5) and activities of phosphorylase (LD: 85.79%, HD: 166%; Fig. 6) were significantly higher as compared to controls, in the liver of aflatoxin-treated mice. The effect was dose-dependent.

Concurrent addition of black tea with low and high dose of aflatoxin resulted in significant increase in protein content (LD + BT: 4.98%, HD + BT: 17.93%; Fig. 1). In a same manner significant increase in DNA (LD + BT: 45.43%, HD + BT: 63.59%; Fig. 2) and RNA (LD + BT: 14.62%, HD + BT: 101.25%; Fig. 1) content was also achieved with treatment of black tea along with aflatoxin. Oral administration of black tea with aflatoxin had resulted in increased levels of glycogen (LD + BT: 46.97%, HD + BT: 61.66%; Fig. 4). Reduced levels of cholesterol content (LD + BT: 11.76%, HD + BT: 13.38%; Fig. 5) and phosphorylase activities (LD + BT: 46.26%, HD + BT: 56.09%; Fig. 6) were also recorded with black tea administration.

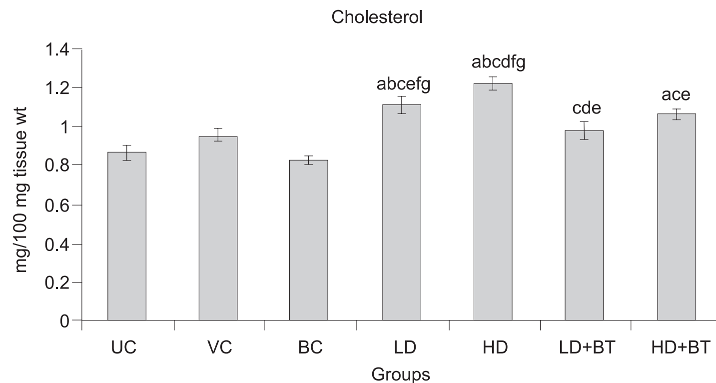

Figure 5. Effect of aflatoxin and black tea on cholesterol content in mice liver. Values are expressed as the mean ± SEM, where n = 10. [a] as compared to group 1: $p < 0.05$, [b] as compared to group 2: $p < 0.05$, [c] as compared to group 3: $p < 0.05$, [d] as compared to group 4: $p < 0.05$, [e] as compared to group 5: $p < 0.05$, [f] as compared to group 6: $p < 0.05$, [g] as compared to group 7: $p < 0.05$. Bars designations as in Fig. 1

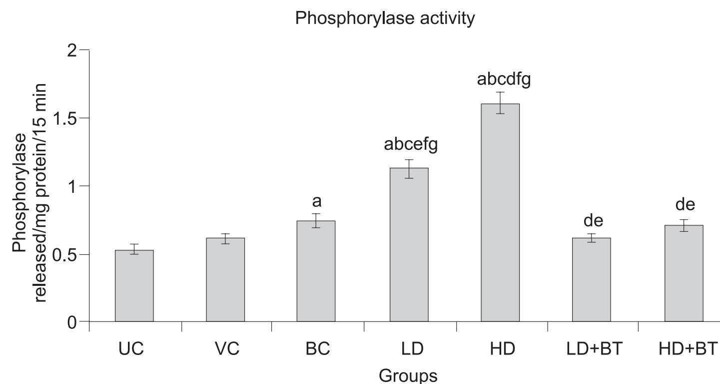

Figure 6. Effect of aflatoxin and black tea on phosphorylase activity in mice liver. Values are expressed as the mean ± SEM, where n = 10. [a] as compared to group 1: $p < 0.05$, [b] as compared to group 2: $p < 0.05$, [c] as compared to group 3: $p < 0.05$, [d] as compared to group 4: $p < 0.05$, [e] as compared to group 5: $p < 0.05$, [f] as compared to group 6: $p < 0.05$, [g] as compared to group 7: $p < 0.05$. Bars designations as in Fig. 1

DISCUSSION

Aflatoxin treatment caused significant dose-dependent reduction in concentration of DNA, RNA and protein content in the liver of mice, which could be due to formation of adducts with DNA, RNA and protein. Aflatoxin is known to impair protein biosynthesis by forming adducts with DNA, RNA and proteins (27), inhibits RNA synthesis, DNA-dependent RNA polymerase activity and causes degranulation of endoplasmic reticulum (28–30). Reduction in protein content could also be due to increased hepatocellular necrosis. Thus, reduction in protein biosynthesis as well as increased necrosis could be responsible for a decrease in protein. Many other investigators reported a decrease in protein concentration in skeletal muscle (31, 32), heart (33), liver and kidney (34–36) of aflatoxin-fed animals.

Our results showed that black tea extract treatment (2%) along with aflatoxin significantly mitigates aflatoxin-induced changes in DNA, RNA and protein contents in the liver of mice. The amelioration in DNA, RNA and protein contents might be due to increased DNA synthesis and reduction in harmful adduct formation. Biosynthesis of protein becomes normalized as no necrotic changes were observed in histopathological study of liver of aflatoxin plus black tea-treated mice.

Studies have reported the effects of tea polyphenols on cell growth and apoptosis of primary cultured rat skin keratinocytes (37). It was seen that tea polyphenols stimulated the cell growth from G_1/G_0 to G_2/M phase, enhanced the synthesis of DNA (increased S phase) and increased the proliferative index from 18.17% to 25.62%. It was concluded that tea polyphenols could stimulate the growth of skin keratinocytes through stimulating the synthesis of cell DNA and inhibiting apoptosis. Cheng et al. (38) investigated the inhibitory effects of curcumin, garlic squeeze, grape seed extract, tea polyphenols, vitamin C and vitamin E on nicotine-DNA addiction *in vivo*. They

suggested that these dietary constituents are beneficial to prevent the harmful adduct formation and thus to block the potential carcinogenesis induced by nicotine. Lin and his colleagues (39) have also found that an aqueous extract of green and black tea, mixture of green and black tea polyphenols, as well as purified polyphenols could strongly inhibit the DNA binding of N-acetoxy-PhIP, a putative ultimate carcinogen of PhIP formed *in vivo via* metabolic activation.

The present study clearly indicates significant increase in cholesterol concentration in the liver of aflatoxin-treated mice. Cholesterol is the principal sterol found in all tissues and body fluids of animals and human beings. In addition to dietary sources, cholesterol can be biosynthesized actively and gets distributed all over the body through blood. Exact mechanism for a significant rise in cholesterol content in liver is not clearly understood. This might be due to fatty infiltration and degeneration of hepatocytes during aflatoxicosis as toxin is fat soluble. Once brought to the liver through hepatic portal system, fat present in the liver cells might dissolve toxin and retain it. Verma et al. (40) have reported significant rise in testicular cholesterol in aflatoxin-fed rabbits. Rastogi et al. (41, 42) also reported increased cholesterol in rat liver. Also, Tung and his colleagues (43) reported, on the basis of their experiments in chicks, that lipid transport is altered leading to significant reduction in serum concentrations of triglycerides, total phospholipids as well as free and esterified cholesterol.

Black tea extract alone treatment did not have any significant effect on cholesterol content in liver of mice. However, black tea extract along with aflatoxin treatment caused significant amelioration in cholesterol content in mice. This could be due to lesser deposition of lipid in the liver, as histopathological studies revealed absence of fatty infiltration and fatty degeneration. Zhang et al. (44) reported that the lipase activity in liver with fatty degeneration was lower than that in normal liver. Tea polyphenols can increase hepatic lipase activity in hepatic tissue and protect hepatocytes from fatty degeneration in rabbits. Hasegawa et al. (45) revealed that the hypocholesterolemic activity of powdered green tea might be due to inhibition of synthesis of cholesterol in the liver. Maron et al. (46) also showed that theaflavin-enriched green tea extract is an effective adjunct to a low-saturated fat diet to reduce LDL-C in hypercholesterolemic adults and is well tolerated. Green tea may have an antilipogenic effect due to its radical scavenging activity (47).

Oral administration of aflatoxin for 30 days caused significant, concentration-dependent decrease in glycogen content, which could be due to significant, concentration-dependent increase in phosphorylase activity in the liver of mice. Phosphorylase is a key glycogenolytic enzyme which helps to accelerate the breakdown of glycogen. Earlier studies have also shown significant reduction in glycogen content in the liver (34), skeletal muscle (32) and heart (33) of rabbits and rats (41, 42) fed with aflatoxin-contaminated diet. Accelerated breakdown of glycogen through increased phosphorylase activity along with increased gluconeogenesis, might cause hyperglycemia. Hyperglycemia induced during aflatoxicosis has been reported in rabbits (34, 40) and mice (48). Increased blood glucose might be due to alterations in hormones or due to stress condition, which prevails during aflatoxicosis and is known to increase blood sugar by altering insulin action (48). Administration of black tea extract along with aflatoxin for 30 days caused the significant increase in glycogen content and the decrease in phosphorylase activity in the liver. It was reported by Hosoda and his colleagues (49) that oolong tea markedly lowered concentrations of plasma glucose and fructosamine. They said that oolong tea may be an effective adjunct to oral hypoglycaemic agents in the treatment of type 2 diabetes. Han (50) has revealed that epigallocatechin gallate is a possible therapeutic agent for the prevention of diabetes mellitus progression. Kim et al. (51) have reported that in rats treated with (–)-epicatechin and streptozotocin (STZ) hyperglycemia and weight loss were not observed and islet morphology was well preserved compared with STZ treatment alone; insulin release was increased and nitrite production was decreased in EC + STZ treated islets.

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