Available online at www.pelagiaresearchlibrary.com

Pelagia Research Library

Asian Journal of Plant Science and Research, 2011, 1 (4):61-69

ISSN : 2249 – 7412

Biochemical and histopathological analysis of aflatoxin induced toxicity in liver and kidney of rat

G. Devendran and U. Balasubramanian

P.G. and Research Department of Zoology and Biotechnology, A.V.V.M. Sri Pushpam College (Autonomous), Poondi, Thanjavur District, Tamil Nadu, India

---

ABSTRACT

The present investigation was an attempt to evaluate the effect of aflatoxin induced toxicity in liver and kidney of albino rats. Aflatoxin was obtained by growing Aspergillus flavus in PDA liquid medium. Young adult albino rat were administered aflatoxin through intraperitoneal route by different concentrations viz., 20 ppm, 40 ppm, 60 ppm, 80 ppm and 100 ppm for 8 days. On 9th day the animals were sacrificed by cervical dislocation. Liver and kidney were removed and Homogenates were prepared for measuring lipid peroxidation, lactate dehydrogenase, superoxide dimutase, catalase, Glutathione peroxidase, glutathione reductase, glutathione s-transferase, alkaline phosphatase, glucose 6-phosphatase, Fructose 1, 6-bisphosphate, vitamin C, vitamin E, sodium, potassium and uric acid. The results revealed concentration dependent increase in lipid peroxidation and alkaline phosphatase along with reduction in enzymatic and non-enzymatic antioxidants. Hence they have shown that concurrent infection during aflatoxin exposure increase the risk of hepatocellular carcinoma.

Key words: aflatoxin, lipid peroxidation, liver, kidney, *Aspergillus flavus*.

INTRODUCTION

Presently the world is facing a drastic problem regarding diseases. Every now and then we come to know about a new disease and its related causes. But the base of many of the diseases in food/feed stuff contamination which can be caused at any stage of production and storage. These contamination are basically due to fungi such as aflatoxins. Aflatoxins are secondary toxic fungal metabolites produced as Aspergillus flavus and A. parasiticus. There are four naturally occurring aflatoxins, the most hepatotoxic being aflatoxin B_1 (AFB_1), and three structurally similar compounds namely aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG1) and Aflatoxin G_2 (AFG2). Aflatoxins not only contaminate our food stuffs but are also found in ediple tissues, milk and eggs after consumption of contaminated feed by farm animals [1].

Aflatoxin, a potent hepatotoxic and hepatocarcinogenic mycotoxin, induce lipid peroxidation in rat liver and associated with various diseases such as aflatoxicosis and hepatocellular carcinoma [2]. Epidemiological survey indicates that occurrence of hepatic and kidney disorder are increasing as life style changes causing serious problem in the area of public health. Swine are highly susceptible to aflatoxins. Extreme effects can lead to dead, but the greatest impact comes from reduced reproductive capability, suppressed immune function, reduced productivity capability and various pathological effects on organs and tissues [3]. The objective of this study were to determine the effects of aflatoxin induced toxicity in liver and kidney of albino rats.

MATERIALS AND METHODS

Aflatoxigenic organism

Aspergillus flavus was used for the production of Aflatoxin in this study. The samples were inoculated into Potato Dextrose Agar (PDA) plate and incubated at 28 ± 2°C for 3-5 days. After incubation the fungal species were used for further use.

Preparation of aflatoxin

The potato dextrose broth (PDB) was prepared to culture the fungi for aflatoxin production. The pH was adjusted to 6 and the medium was distributed in 2 liters conical flask was cooled and then inoculated with spore suspension of A. flavus and incubated at 28 ± 2°C for 2-3 weeks.

After incubation, the mycelia were removed from the medium and the liquid was filtered through Whatmann No.1 filter paper. The culture filtrate was concentrated under reduced in an evaporator on a water bath. The concentrated culture filtrate was shaken repeatedly with 100ml volume of chloroform and the extracts were combined and filtered through Whatmann No.1 filter paper. From the filtered chloroform extract, the toxin was extracted using sodium bicarbonate solution by shaking the chloroform extract several times with 0.5 molar sodium bicarbonate solution. All the lipid materials were removed by filtration after keeping the sodium bicarbonate extract over night in a separating funnel. Finally the pH of the solution was brought down to 2 and the toxin was extracted from the concentrate into chloroform by repeated extraction with aliquots of chloroform. The extract was pooled and concentrated thus the crude toxin was isolated.

Detection of aflatoxin by thin layer chromatography

Silica gel was coated on TLC plates and dried at 60°C for 1 hour. 1ml of the concentrate of the chloroform extract was spotted in the form of a thin line on the chromatographic plates and developed with chloroform ethyl acetate formic acid toluene (50:40:10:2v/v) solvent system in a closed chamber. After drying the plate, portion of the place was sprayed with 1% paradimethyl amino benzaldehyde in n-butanol dried with warm air and placed in a tank containing hydrochloric acid vapors for 15 minutes, a bright blue colour reaction to find out the presence of aflatoxin B_1. The mobility of extracted aflatoxin B_1 and authentic aflatoxin was compared.

Detection of aflatoxin by High performed liquid chromatography

Samples were analysed for aflatoxins using a model 1100 HPLC system consisting of a degasser, autosampler and quaternary pump and a fluorescence detector (Agilent) equipped with a 250 mm × 4.6 mm i.d., 5 µm, Inertsil ODS-3 column (GL Sciences, Inc., Torrance, CA). A starting

mobile phase of 100% H_2O/CH_3CN/MeOH (45:25:30, v/v/v) was held for 2 min after injection, followed by a gradient to 100% MeOH over the next 8 min, with 100% MeOH held for 1 min. The column was re-equilibrated with the starting solvent for 4 min before the next injection. The injection volume was 20 µL, and the flow rate was 1.0 mL/min. Fluorescence detection at 365 nm excitation and 455 nm emission was enhanced with a post column photochemical reactor for enhanced detection. Aflatoxin retention time were 12.1 min for G_2, 14.5 min for G_1, 15.4 min for B_2 and 18.8 min for B_1. See Figure 1.

Figure 1. HPLC separation of Aflatoxin using Fluorescence Detection

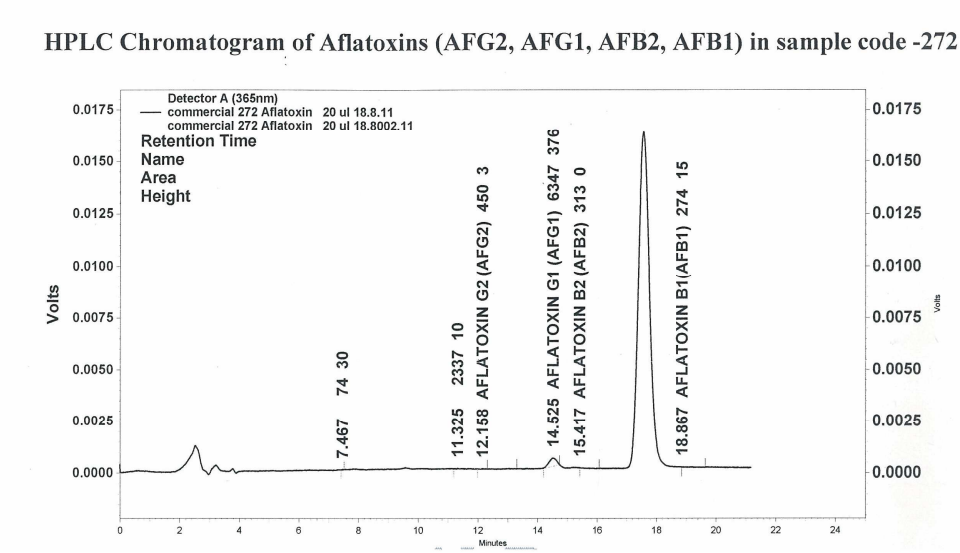

Table 1. Statistical result for determination of Aflatoxin in *Aspergillus flavus* extract by using HPLC

Detector A (365 nm)				
Retention time	Area	Height	Concentration (µg/mg of extracted sample)	Name
12.158	460	3	6.11	Aflatoxin G_2
14.525	6347	376	3.16	Aflatoxin G_1
15.417	313	0.1	2.86	Aflatoxin B_2
18.867	274	15	0.80	Aflatoxin B_1

Experimental Design

The study was carried out on mixed sex of albino rats (100-150g). They are fed with a standard pellet (Lipton India Ltd., Calcutta) and water and libitum. The rats were kept in standard environmental conditions (temperature 25-28°C and 12h light/12h dark cycle). There were 36 animals into six groups and caged separately. Group I (untreated control) animals were maintained with out any treatment. Animals of Group 2 to 6, the Aflatoxin were administered though intraperitoneally route by different concentrations viz., 20 ppm, 40 ppm, 60 ppm, 80 ppm and 100 ppm respectively, for 8 days on completion of the treatment, the rat were sacrificed by cervical dislocation. The liver and kidney were isolated, blotted free of blood, rinsed in ice-cold physiological saline and homogenized in Tris-Hcl buffer (0.1M, pH 7.4) to give a 10% homogenate. Aliquots of the tissue homogenate were suitably processed for the assessment of following biochemical parameters.

The activity of lactate dehydrogenase (LDH), alkaline phosphatase (ALP) in liver and kidney were estimated by the method of Kings [4] lipid peroxidase (LPO) was determined by the procedure of Hogberg *et al*. [5]. Superoxide dismutase (SOD) was assayed according to the method of Marklund and Marklund [6], Glutathione peroxidase (GPx) was assayed by the method of Rotruck *et al*. [7]. Glulathione reductase (GR) that utilizes NADPH to converts oxidized glutathione (GSSG) to the reduced from was assayed by the method of Staal *et al*. [8]. Gluththione-s-transferase (GST) was assayed by the method of Habig *et al*. [9]. Catalase (CAT) was assayed by the method of Sinha [10]. Vitamin C was estimated by the method of Omaye *et al*. [11]. Vitamin E was estimated according to the procedure of Desai [12]. Glucose-6-phosphatase activity was assay by method of Harper [13]. Fructose-1, 6 bisphosphatase activity was determined by method of Gancedo and Gancedo [14]. The sodium and potassium was estimated by using flame photometer.

RESULTS AND DISCUSSION

Table 2 and 4 reveal the abnormal level of liver and kidney oxidative stress biomarkers in rats that indicate the cellular damage caused by AF treatment. The level of lipid peroxidation was significantly increased as compared with control groups. Aflatoxin treatment was caused significant reduction in the activities of catalase, superoxide dimutase and glutathione peroxidase as well as glutathione reductase and total vitamin C and vitamin E content in the liver and kidney of rats. The effect was almost dose dependent. Thus aflatoxin treatment caused dose-dependent decrease in lipid perioxidation by decreasing the antioxidative defense mechanisms of the cell. The activities of enzyme, lactate dehydrogenase, were decreased respectively, in group 2-6 animals when compared with control. Activities of these marker enzymes were significantly ($p<0.001$) decreased in the liver of AF administered animals.

Table 2 and 4 shows the effect of different concentration of Aflatoxin induced toxicity in liver and kidney of rat. A significant decrease ($p<0.001$) in the activities of Glutathione s-transferase, Glucose-6-phosphatase, and Fructose1, 6-bisphosphatase was seen in the Aflatoxin treated animals (group 2-6). The biochemical analysis showed a significant increase in activity of Alkaline phosphatase and an elevation in the value of uric acid with a reduction of sodium and potassium.

Histophathological analysis

In liver degenerative reversible lesion were present, from mildest of severest degree with various distributions in test groups mild parenchymatous degeneration characterized by granular appearance of hepatocytes cytoplasm was observed, severe hydrophilic and vacuolar degeneration. The vast majority of hepatocytes had significant cytoplasmic visualization with disseminated necrotic cells were observed in the experimental groups.

In Kidney, moderate parenchymatous tubular degeneration, predominantly of the distal tubules, manifested by epithelial swelling and fine granular appearance of cytoplasm was most prominent in aflatoxin treated rats. In the experimental animals, hydropic and vacuolar degeneration was also noted, but the severe degree characterized by desquamation of epithelial tubular cells was present in almost all cells when compared to control groups.

Intraperitoneal route of aflatoxin for 8 days caused significant increase in lipid peroxidation in liver and kidney of aflatoxin treated rats, as compared to controls, lipid peroxidation is regarded as one of the primary key events in cellular damage [15] and the relationship between GSH levels, lipid peroxidation and cell lysis has been reported [16]. Carcinogens like aflatoxin B_1, which generate epoxides, have been found to conjugate readily with GSH [17] liver cells, which are lethally injured by several toxins, exhibit marked alternation in intracellular Ca^{2+} homeostasis after excessive accumulation of Ca^{2+} [18]. During hepatocellular necrosis, excessive intracellular Ca^{2+} is known to thrust the metabolism in an unmanageable disorder, which leads to mitochondrial dysfunction inhibition of enzymes and denaturation of structural proteins [19].

Oxidative stress was originally defined as the disequilibrium between prooxidants and antioxidants in biological systems [20]. Therefore an increase in lipid peroxidation could be due to significant reduction in the activities of enzymatic antioxidant such as catalase, superoxide dimutase and glutathione peroxidase as well as non enzymatic antioxidant such as total ascorbic acid and α-tocopherol contents in the liver and kidney of aflatoxin, treated rat; as compared to the controls. Superoxide dimutase protects cells from oxidative damage by breaking down a potentially hazardous free radical superoxide (O_2^-) to H_2O_2 and O_2. The H_2O_2 produce can then be decomposed enzymatically by catalase and glutathione peroxidase. Glutathione peroxidase not only decomposes H_2O_2 but can also interact with lipid peroxidation [21]. Thus significant reduction in these enzyme activities (Table 2&4) could be responsible for increased lipid peroxidation observed during aflatoxicosis.

Present study shows significant reduction in ascorbic acid and α-tocopherol content in the liver and kidney of aflatoxin treated rat (Table 3&5). During free radical scavenging action, ascropic acid is transformed into L-dehydroascorbate [22]. Reduced glutathione is required for the conversion of L-dehydroascorbate back to ascorbate [22]. The fall in the level of reduced glutathione decreases the conversion of L-dehydroascorbate to ascorbate and this probably explains the lowered level of ascorbic acid in aflatoxin treated animals.

The enzymes are markers of liver injury since liver is the major site of metabolism. The marked decrease in the activity of hepatic LDH with AF treatment indicates impaired liver function. ALP is a membrane bound enzyme and its alteration is likely to affect the membrane permeability and produce derangement in the transport of metabolites [23]. The uric acid, the metabolic and product of purine metabolism. The reduced level of uric acid into hepatocellular carcinogenic conditions may be due to the increased utilization of uric acid against increased production of the free radicals, which is a characteristic features of cancer condition.

The inhibition of activities of gluconeogenic enzymes glucose-6-phosphatase and fructose-1, 6-bisphophatase in group II AFB1 – induced rats was in accordance with the earlier report [24]. Glucose-6-phosphatase is also reduced in residual liver tissue of Novikoff hepatoma [25]. Degreased rate of glucose-6-phosphatase mediated dephosphorylation is also reported in malignant cells [26]. Decreased activity of Fructose-1, 6-bisphosphatase, the key regulatory enzyme for the synthesis glucose-6-phosphate from pyruvic acid observed in liver of group II rats is supported earlier report [27]. Which reported that in Novikoff hepatoma, there appears to be an absence of fructose-1, 6-bisphosphatase in the tumour and consequently a block in the pathway, leading to the synthesis of glucose-6-phosphate from pyruvate.

Fig.2. Histopathological analysis of Aflatoxin induced toxicity in liver and kidney of albino rats

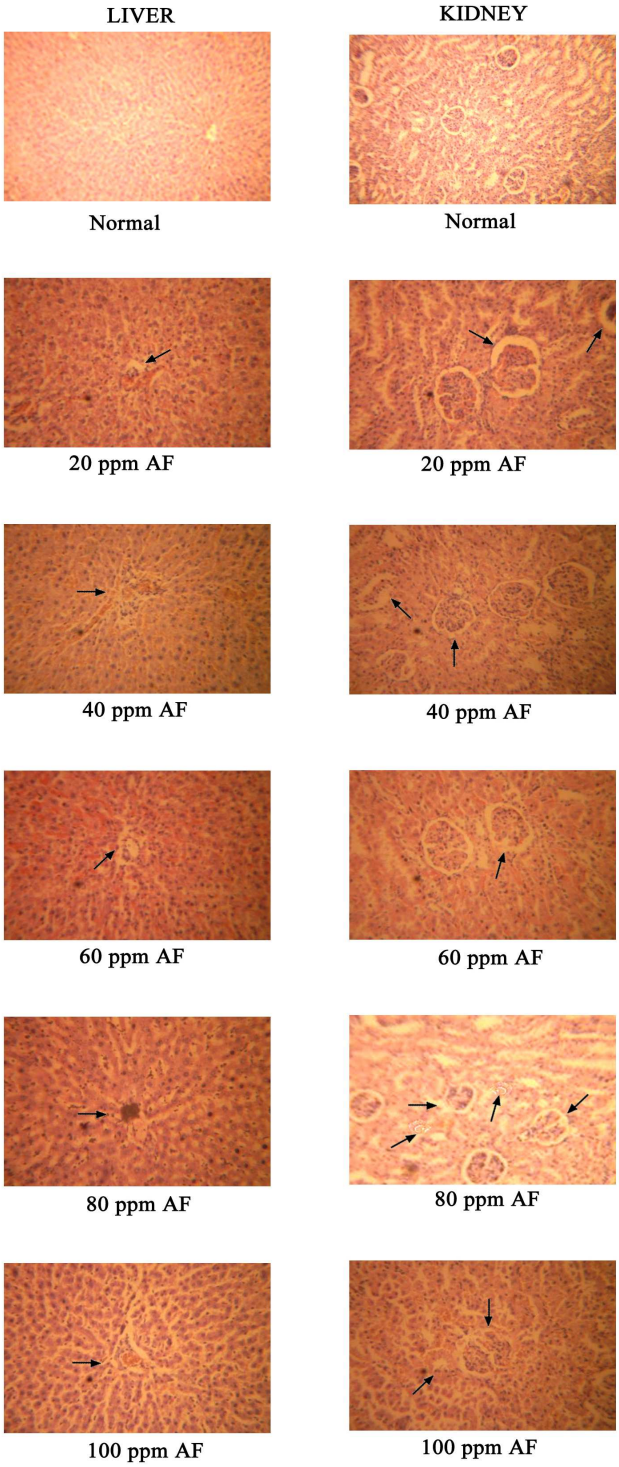

Table 2. Effect of aflatoxin induced changes in the enzyme parameters of rat liver

Groups	LDH	SOD	CAT	GPX	GST	GR	LPO	ALP	G-6-ph	Fru-1-6 bisphos
Normal	20.0 ± 1.46	4.80 ± 0.09	36.90 ± 0.46	4.00 ± 0.014	0.90 ± 0.008	0.80 ± 0.009	1.60 ± 0.008	160	12.10	11.90
20 ppm	18.0 ± 1.80	3.60 ± 0.08	30.92 ± 0.49	4.00 ± 0.018	0.82 ± 0.007	0.72 ± 0.004	1.49 ± 0.08	190	10.00	11.00
40 ppm	18.5 ± 0.89	3.40 ± 0.06	26.90 ± 0.56	2.91 ± 0.016	0.65 ± 0.006	0.60 ± 0.006	2.33 ± 0.005	200	9.60	11.00
60 ppm	12.0 ± 0.42	2.40 ± 0.07	20.85 ± 0.38	2.90 ± 0.026	0.60 ± 0.004	0.40 ± 0.008	2.25 ± 0.004	260	8.00	10.50
80 ppm	10.9 ± 1.02	2.20 ± 0.01	16.80 ± 0.28	1.60 ± 0.019	0.50 ± 0.006	0.20 ± 0.007	4.19 ± 0.06	300	6.00	9.00
100 ppm	10.5 ± 1.03	1.10 ± 0.05	10.70 ± 0.26	0.50 ± 0.011	0.45 ± 0.005	0.10 ± 0.005	3.08 ± 0.003	290	6.20	9.00

Enzyme unit: LDH; μ mol $\times 10^{-1}$ of pyruvate laberated/min, SOD: μ/mg protein, CAT: μ mol of H_2O_2 consumed/min/mg protein, GPX: μg of GSH utilized/min/mg, GST: nmol of 1-chloro-2, 4-dinitrobenzene-GSH conjugate formed/min/mg protein, GR: nmol of NADPH oxidized/min/mg/protein, LPO: nmol of MDA formed/mg protein, ALP: μ mol $\times 10^{-2}$ of phenol liberated/min, G-6-P; n moles of inorganic phosphorus liberated min/mg protein, Fru-1,6-bisphos; n moles of inorganic phosphorus liberated min/mg protein.

Table 3 Vitamins and mineral content from liver of control and experimental rats

Group	Vitamin E (mg/dl)	Vitamin C (mg/dl)	Sodium (mmol/L)	Potassium (mmol/L)	Uric acid (mg/dl)
Normal	7.0 ± 0.68	0.80 ± 0.02	140 ± 26.4	10.0 ± 0.58	3.9 ± 0.09
20 ppm	5.5 ± 0.48	0.80 ± 0.03	169 ± 36.5	11.2 ± 0.38	4.5 ± 0.08
40 ppm	4.5 ± 0.26	0.60 ± 0.01	160 ± 28.6	11.0 ± 0.46	5.1 ± 0.05
60 ppm	3.0 ± 0.32	0.40 ± 0.05	110 ± 33.2	9.8 ± 0.42	5.0 ± 0.03
80 ppm	1.0 ± 0.13	0.10 ± 0.04	124 ± 25.6	10.0 ± 0.56	5.9 ± 0.06
100 ppm	1.0 ± 0.10	0.10 ± 0.02	105 ± 22.01	8.9 ± 0.41	6.1 ± 0.04

Table 4. Effect of aflatoxin induced changes in the enzyme parameters of rat kidney

Groups	LDH	SOD	CAT	GPX	GST	GR	LPO	ALP	Glu-6-phos	Fru-1, 6-bisphos
Normal	16.5 ± 1.09	3.80 ± 0.07	30.70 ± 0.24	4.10 ± 0.001	1.80 ± 0.001	1.00 ± 0.005	1.49 ± 0.004	120 ± 9.64	10.40 ± 0.48	12.50 ± 0.33
20 ppm	13.0 ± 1.04	2.20 ± 0.05	20.80 ± 0.18	3.10 ± 0.002	1.82 ± 0.004	0.62 ± 0.006	1.50 ± 0.006	110 ± 6.48	9.80 ± 0.56	12.10 ± 0.38
40 ppm	12.5 ± 1.01	2.00 ± 0.06	20.70 ± 0.13	2.92 ± 0.004	0.65 ± 0.003	0.59 ± 0.005	1.80 ± 0.008	130 ± 6.47	8.50 ± 0.38	12.00 ± 0.39
60 ppm	10.0 ± 0.08	1.60 ± 0.09	18.65 ± 0.16	2.60 ± 0.011	0.66 ± 0.005	0.40 ± 0.004	2.28 ± 0.009	140 ± 8.42	6.00 ± 0.46	12.00 ± 0.48
80 ppm	11.0 ± 0.09	1.40 ± 0.05	10.60 ± 0.09	1.40 ± 0.001	0.50 ± 0.006	0.10 ± 0.001	3.20 ± 0.008	140 ± 7.48	4.00 ± 0.33	10.00 ± 0.42
100 ppm	9.5 ± 0.09	1.30 ± 0.04	8.50 ± 0.21	0.39 ± 0.003	0.59 ± 0.005	0.18 ± 0.001	4.10 ± 0.009	150 ± 8.46	3.20 ± 0.48	8.00 ± 0.49

Enzyme unit: LDH; μ mol $\times 10^{-1}$ of pyruvate laberated/min, SOD: μ/mg protein, CAT: μ mol of H_2O_2 consumed/min/mg protein, GPX: μg of GSH utilized/min/mg, GST: nmol of 1-chloro-2, 4-dinitrobenzene-GSH conjugate formed/min/mg protein, GR: nmol of NADPH oxidized/min/mg/protein, LPO: nmol of MDA formed/mg protein, ALP: μ mol $\times 10^{-2}$ of phenol liberated/min, G-6-P; n moles of inorganic phosphorus liberated min/mg protein, Fru-1,6-bisphos; n moles of inorganic phosphorus liberated min/mg protein.

Histopathological analysis reveals that lesions were observed in tissues of liver and kidney. The liver is very important organ of our human system having various multifunctional activities like metabolism and excretion. It is involved with almost all the biochemical pathways to growth, fight against disease, nutrient supply, energy provision and reproduction [28, 29]. This result indicates the significant damage of vital organs in rats. These findings are coinciding with previous findings. The biochemical and histopathological analysis of rat showed that decrease of enzyme alternation and histological changes of important organs in rats.

Based on the present research it is, concluded that the clinical signs of aflatoxicosis in rats, where it acts as an aflatoxin enterosorbant that tightly and selectively binds the poison in the metabolic reaction. Hence the present study has shown that concurrent infection during aflatoxin exposure increase the risk of hepatocellular carcinoma.

Table 5. Vitamin and mineral content from kidney of control and aflatoxin treated rats

Group	Vit-E (mg/dl)	Vit-C (mg/dl)	Sodium (mmol/L)	Potassium (mmol/L)	Uric acid (mg/dl)
Normal	8.5 ± 0.62	1.10 ± 0.06	155 ± 28.4	9.5 ± 0.42	4.5 ± 0.10
20 ppm	7.2 ± 0.61	0.90 ± 0.03	150 ± 26.8	9.0 ± 0.46	3.4 ± 0.12
40 ppm	7.0 ± 0.56	0.90 ± 0.05	140 ± 27.4	8.5 ± 0.52	5.0 ± 0.06
60 ppm	4.6 ± 0.48	0.60 ± 0.01	130 ± 29.8	8.9 ± 0.58	4.8 ± 0.09
80 ppm	2.8 ± 0.58	0.40 ± 0.03	120 ± 24.6	7.5 ± 0.59	4.0 ± 0.08
100 ppm	2.0 ± 0.46	0.20 ± 0.02	100 ± 22.8	7.0 ± 0.49	4.1 ± 0.07

REFERENCES

[1] Fink-Gremmels J, Mycotoxins: their implications for human and animal health. Vet Q. **1999**, 21: 115-20.
[2] Premalatha B and Sachdanandam P, Semecarpus anacardium L. *J. Ethanopharmacol*. **1999**, 66: 131-139.
[3] Hoerr FJ and D' Andrea GH, Biological effect of aflatoxin in swine In: Diener, U., Asquith R., Dickens. J. [Eds.], Aflatoxins an *Aspergillus flavus* in corn. Anburn University, Anburn, *Ala*. **1983**, pp.51-55.
[4] King J, The transferases – alanine and aspartate transaminases In: King J [Ed.], Practical Clinical Enzymology. Van Nostrand Company Ltd, London. **1965c**, pp.121-138.
[5] Hogberg J, larson RE, Kristoferson A and Orrenius S, *Biochem. Biophy. Res. Comm.* **1973**, 56: 836-842.
[6] Markland S and Marklund G, *Eur. J. Biochem.* **1974**, 47: 469-74.
[7] Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG and Hoekstra WG, Selenium: *Science,* **1973**, 179: 588-590.
[8] Staal GE, Visser J and Veeger C, *Biochem. Biophys. Acta.* **1969**, 185: 39-48.
[9] Habig WH, Pabst MJ and Jakoby WB, *J. Biol. Chem.* **1974,** 249: 7130-39.
[10] Sinha AK, Colorimetric assay to catalase. *Anal. Biochem.* **1972**, 47: 389-94.
[11] Omaye ST, Turnbull JD and Sauberlich HE, *Met. Enzymol.* **1979**, 62: 3-11.
[12] Desai ID, *Met. Enzymol.* **1984**, 105: 138-147.
[13] Harper AE, In methods of Enzymatic Analysis [Bergermer HU. ed.], Academic Press, New York. **1965**, p.788.
[14] Gancedo JM and Gancedo C, *Arch Microbiol.* **1971**, 76: 132-138.

[15] Mead JF, In: Free Radicals in Biology Pryer WA (ed.), Academic Press, New York. **1976**, p.51.
[16] Annundi I Kristoferson A and Hogberg J, Excerpta Med. Int. Cong. Series. **1978,** 440, 270.
[17] Degan GH and Neumann HG, *Chem. Biol. Interact.* **1978**, 22**,** 239.
[18] Nicotera P, Bellomo G and Orrenius S, *Ann. Rev. Pharmacol. Toxicol.* **1992,** 32, 449.
[19] Fagian MM, Pereira-da-silva L, Martins IS and Vercesi AE, *J. Biol. Chem.* **1990,** 265**:** 19955.
[20] Sies H, Oxidative stress: Oxidants and Antioxidants, Academic Press, San Diego, California, **1991**.
[21] Venkateshwaran PS, Millman I and Blumberg BS, *Proc. Natl. Acad. Sci.* USA. **1987**, 84: 274.
[22] Breimer LH, *Mol. Carcinog.* **1990,** 3, 188.
[23] Plaa GL and Hewitt WR, Detection and evolution of chemically induced liver injury. In: Hayes, AW (ed.), Principles and Methods of Toxicology. Raven Press, New York. **1989**, pp.401-41.
[24] Weber G and Morris HP, *Cancer Res.* **1961**, 21: 933-937.
[25] Mc Fadzean AJS and Youn RTT, *Am. J. Med.* **1969**, 47: 200-235.
[26] Graham MM, Spence AM, Muzi M and Abbot GL, *J. Careb. Blood flow Metabol.* **1989**, 9: 315-322.
[27] Weber G and Cantero A, *Acta. Unio Int. Contra. Cancrum*, **1960**, 16: 1002.
[28] Ward FM and Dally MJ, Hepatic disease. In: Clinical pharmacy and therapeutic (Walker R and C Edwards eds.).Churchill living stone, New York, **2002**, pp.195-212.
[29] Ahsan MR and Islam KM, *European J of Scientific Research*, **2009**, 27(2): 302-310.