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Full Length Research Paper

Copper (I)-Nicotinate Complex Exhibits More Prophylactic Effect than Butylated hydroxytoluene Against Nephrotoxicity in Chronically Aflatoxicosed Rats

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This submitted work was designed in order to evaluate the prophylactive efficacy of the synthetic copper (I)-nicotinate complex against nephrotoxicity by aflatoxin B1 (AFB1) with regard to the highly accepted antioxidant agent butylated hydroxytoluene (BHT). Aflatoxin B1 fraction was obtained by growing Aspergillus flavus in potato dextrose agar (PDB) liquid medium. Healthy young males albino rats (n=45) were exposed to AFB1 day after day for five weeks (20 µg/kg body weight). One third of them was co-treated with BHT (0.05 g/kg body weight) and the second third by the copper complex (400 µg/kg body weight) while the third was considered as only intoxicated control group. Such intoxication resulted into the histopathological (light and electron transmission) characteristic features of nephrotoxicity. The sever degenerative changes swelling of the cells even rapture of the membrane and cellular organelles in the tubular lumen as well as fibrocytic reaction and congestion of the vasculature in addition to glomerular reaction manifested by atrophy of both vesiral (bodocyte) and parital shirnked cells. The co-treated BHT group did not eliminate all such features of degenerative characters in intoxicated tissue kidney while the co-treated copper complex group mostly appeared normal. The enzyme level of phase II of body detoxificating GST was significantly increased than that improved by BHT. Conclusively, the food additive probable use of the copper nicotinate complex could be a promising agent against nephrotoxicosis.

Keyword: Aflatoxin B1, copper (I)-nicotinate complex, Butylated hydroxytoluene, Nephrotoxicity, Antioxidant, Food additive.

INTRODUCTION

Since, the role of the kidney as an organ of excretion, reabsorption and general homeostasis make it the most subjected one for aflatoxicosis. The load of accumulated toxins within the tubules enhances mycotoxins nephrotoxicity that encourages many workers to focus on determining the toxic mode of action whatever in vivo and in vitro, to emphasis characters of AFB1-mediated renal toxicity (Evelyn and Daniel 2004). In feeding study Rati et al., in 1991 found that 7mg/kg body weight was the LD_{50}

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for rats. Moreover, the species and strain differences appear to arise due to genetic variability in the expression levels of the cytochrom P 450 mixed function oxidases. There are at least five families and sub-families involved in the conversion of AFB1 to give DNA or RNA adducts with the reactive metabolite AFB1-8,9-epoxide (Bbosa et al., 2013; Autrup et al., 1996; Eaton and Gallagher 1994).

Three fold higher concentrations of adducts was formed in murine kidney than liver following AFB1 exposure (Autrup et al., 1996). The first study on renal toxicity AFB1 in rats showed decreased tubular reabsorption of several compounds following a single intraperitoneal dose AFB1 of about 100 µg/kg body weight (Grosman et al., 1983). The authors interpreted that the observed nephrotoxicity was possibly due to degenerative effects of glomerular basement membrane. This interpretation was supported by Valdiva and coworkers (2001) who reported that a thickening of the glomerular basement membrane was observed after AFB1 exposure in chicks. Detoxification phase II enzymes such as isoforms of glutathione-s-transferases can conjugate the electrophilic AFB1-8,9-epoxide (AFBO) with GSH is an alternative fate for binding to nucleophilic center in cellular macromolecules. The enzymes that play a crucial role in protection of the exposed tissue against the deleterious effects of oxidative bioactive AFB1 metabolites (Chen et al., 2010; Klein et al., 2003). Noticeably, CYP 1A homologues also metabolize AFB1 to produce the detoxified metabolite AFM1 whereas CYP 3A enzymes produce another detoxified metabolite AFQ1 from the parent AFB1 molecule (Guengerich et al., 1996). Glutathione-S-Transferases are inducible by many compounds including some antioxidants such as BHT (Hayes et al., 2000; Pickett and Liu, 1989) that significantly increased activities of several isoforms of hepatic cytosolic GST (Klein et al., 2003).

In a previous work (Shatat et al., 2013) the copper (I)-nicotinate complex was reported to exhibit more antioxidant power effect than the highly accepted food additive antioxidant BHT in aflatoxicosed rats. Moreover to determine the efficacy of such a complex against AFB1 intoxication in rats (unpublished data) which showed more significantly potentiation to direct AFB1 metabolism towards the less toxic metabolites AFM1 and AFQ1 than that of BHT (Nassar et al., 2014).

Basically, this work has been designed to recognize to what extent this complex can protect the kidney tissue as well as its GST activity against the deleterious effects of chronica aflatoxicosis.

MATERIALS AND METHODS

Chemicals and Kit

The phenolic BHT was purchased from Oxford Laboratory – Mumbai- 400002, CAS No. (128-73-0), The [Copper (I)-(nicotinic acid)$_2$]$^+$Cl$^-$ complex was synthesized as described by Gohar and Dratoviscky (1975). GST kit was purchased Sigma Chemical Co. (Saint Louis, Missouri 63103, Catalog Number CS410, USA).

Preparation of AFB1 for the biological use

Aflatoxin B1 was harvested from fungal growth from an isolated Aspergillus flavus (generously received from research center of fungi, Assiut University, Egypt), according to Potato Dextrose Broth (PDB) method of Booth (1972). This medium was prepared according to El-Kady and Moubasher (1982). Deharvested growth media from each flask (n=50) extracted in chloroform where the organic layer was treated with anhydrous Na_2SO_4. The collected anhydrous chloroform was dried on rotator. In silica gel column chromatography the AFB1 fraction was isolated and purified. The toxin was suspended in maize oil and checked on TLC against authentic sample in chloroform: methanol (97:3). Collected solid residue of AFB1 was suspended in maize oil for biological use (1 mg/450 ml).

Experimental design

Forty five healthy male Wister albino rats (120–150 g B.W.) intoxicated day after day for five weeks by a dose rate of 20 µg/kg body weight. One third of them was co-treated with BHT G_{II} (0.05 g/kg body weight) according to Williams et al., (1996), the second third by the copper (I)-nicotinate complex G_{III} (400 µg/kg body weight) according to Shatat et al., (2013), the third one was considered as only intoxicated control group G_I and 15 rats were taken as reference control group G_0. The animals were scarified at the end of the time course of the experiment and kidney tissue samples were collected for tissue GST assay and histopathological examination by light and electron transmission.

Light and transmission electron microscope

Samples for light and electron microscope were taken and processed in electron microscope unite (E.M.U) of Assiut University where, 5-10 small pieces 1 x 1 mm in size were taken from each specimen and fixed in 5% cold glutaraldehyde immedialy after dissecting the animal for 24-48 hr. the specimens were then washed in cacodylate buffer (pH 7.2) 3-4 times for 20 minutes every time and post fixed in 1% O_4S_4 for 2 hr, after that washed in the same buffer four times. Dehydration by ascending grades of alcohol (30 – 50 – 70 – 90 and 100% for 2 hr) were done and were embedded in epon –araldite mixture according to Bozzpla and Russell (1991). From the embedded blocks semithin section by LKB ultramicrotom

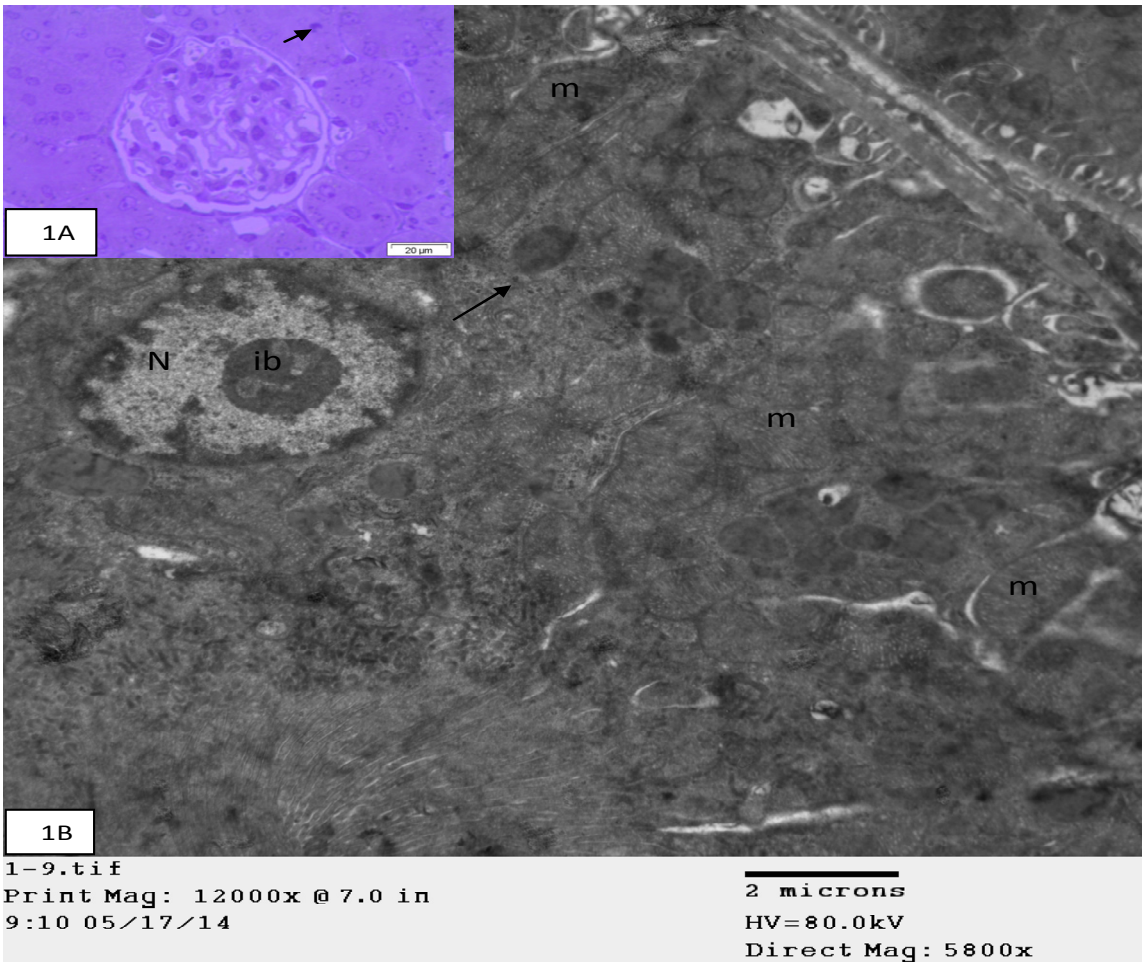

Figure 1A. Semithin section of kidney belonging to aflatoxicosed rats showing degeneration of the tubular epithelium of hydropic type and presence of darkly stained granules in its cytoplasm (arrow), with markedly thinning of the parietal and vesiral epithelium of the glomeruli (T.B. stain).
Figure 1B. T.E. micrograph of aflatoxicosed rats of tubular epithelium showing presence of inclusion body (ib) in the nucleus (N), electron dens lysosoms (arrow) in the cytoplasm and swelling of the mitochondria (m) with disintegration of its criste.

thickness of 0.5M were prepared for orientation of the tissue and photographed by sc30 olympus camera and the ultrathin section in thickness of 500 – 700 A were made using leica AG ultramicrotome and contrasted in uranyl acetate and lead citrate, as usual, examined by T E M 100 CXII electron microscope at 80 kvand photographed by CCD digital camera model XR-41.

Biochemical parameter

The enzyme activity of renal tissue GST was determined by diagnostic GST kit.

Statistical analysis

Data are expressed as the mean ±S.E. (standard error) Statistical analysis was done using analysis of variance (ANOVA) followed by Tukey ’s multiple comparison test using the Statistical Package for the Social Science (S.P.S.S. 11). The level of significant was set at $p<0.05$.

RESULTS

Generally, light microscopic examination and ultrastructural investigation clarify the harmful AFB1 effect on the chronically intoxicated renal tissue. In the light microscopy the tubular epithelium was swollen with narrowing lumen that was containing cellular depress or cast and proteinatous materials as well as numerous darkly stained granules. The glomerular system was mostly appeared with markedly decrease in mesangial cells as well as the matrix, while the visceral and parital layer of the epithelium was atrophied. The capillary tuft was dilated Figure (1A). The ultrastructural findings presented chronic intoxication of the kidney tubules that

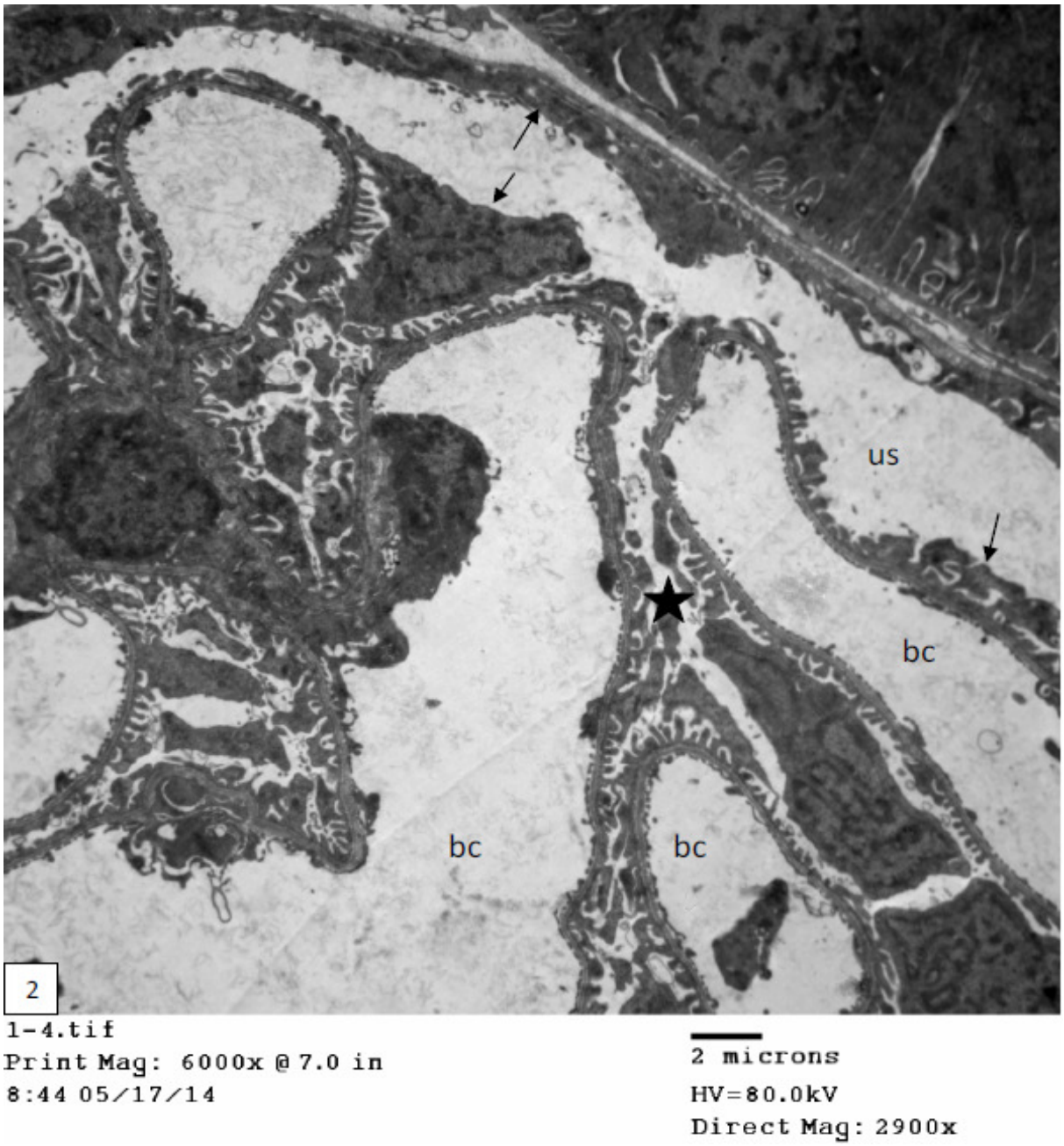

Figure 2. T.E. micrograph of aflatoxicosed rats showing atrophy of the parietal and vesiral epithelium of the glomeruli (arrow) and decreases of mesingial cell and martex (star). Notice dilation of the blood capillary (bc) of glomerular tuft and urinary space (us).

Histopathological and ultrastructural examination of co-treated with BHT rats group:

was associated with severs degenerative changes in the tubular epithelium of hydropic type manifested by swelling of the cells even rupture of the cell membrane and presence of cellular organelles in the tubular lumen. In addition some electron dens lysosomes in the cytoplasm of the cell and prominent spherical electron dens inclusion were apparent in the nucleus Figure (1B). The interstitium fibrocytic reaction and congenstion of the vasculature was recognized. The glomerular reaction was also manifested by atrophy of both vesiral (podocyte) and parietal epithelium that appeared shrinked and more electron dens. The mesingial cells and the matrix were markedly decreased Figure (2).

The degenerative characters of kidney intoxication were still recognized by using the co-treating antioxidant BHT but to a lesser extent than the other untreated one otherwise the tubular epithelium was swollen, contained numerous vacuoles and darkly stained granules. The

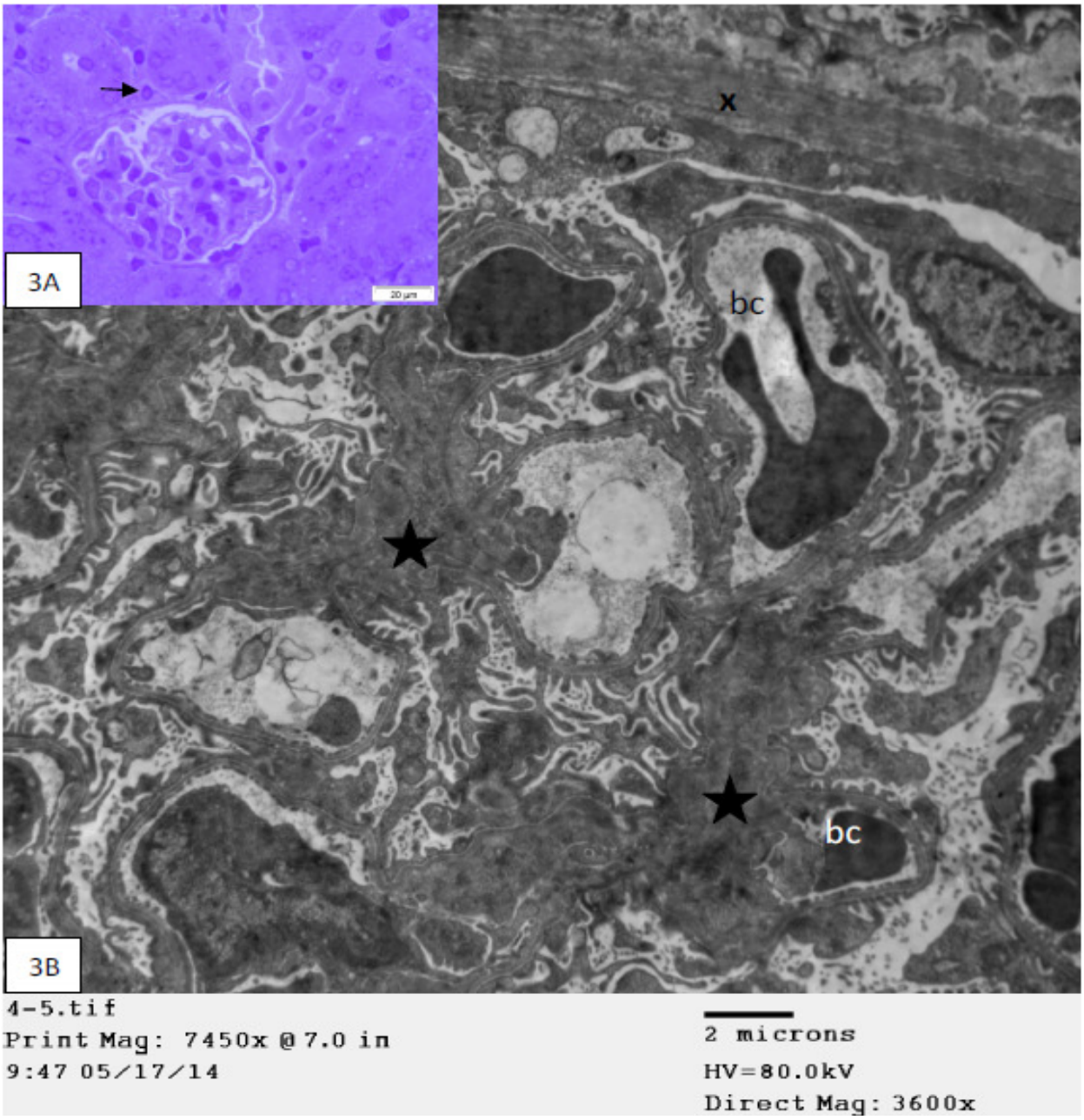

Figure 3A. Semithin section of kidney of co-treated with BHT showing swelling of the tubular epithelium and containing numerous vacuoles and darkly staind granules (arrow). The glomeruli showing thickening of the glomerular capsule (T.B. stain).
Figure 3B. T.E. micrograph of the glomeruli of the kidney of co-treated with BHT showing thickening of the fibrous layer of the capsule (x) and slightly increases of the mesingial cell and matrix (star) and congestion of the bold capillary of the glomerular tuft (bc).

glomeruli showing thickening of the glomerular capsule Figure (3A). The fine ultrastructural features of such BHT co-treated kidney showed thickening of the fibrous layer of the glomeruli and mesangial cell and matrix that was slightly increased and the podocyte with its processes surrounding the capillary tufts shoed normal morphological appearance Figure (3B). The tubular epithelium with numerous electron dens lysosoms, while the nucleus was containing small spherical inclusion bodies. The tubular lumen was containing protienatous material, cellular debris and vacuoles. Furthermore the mild fibrocytic cellular reaction in the interstitium was still recognized Figure (4).

Comparatively, the co-treated intoxicated kidney with copper nicotinate complex showed normal morphological appearance of the glomeruli under light microscopic

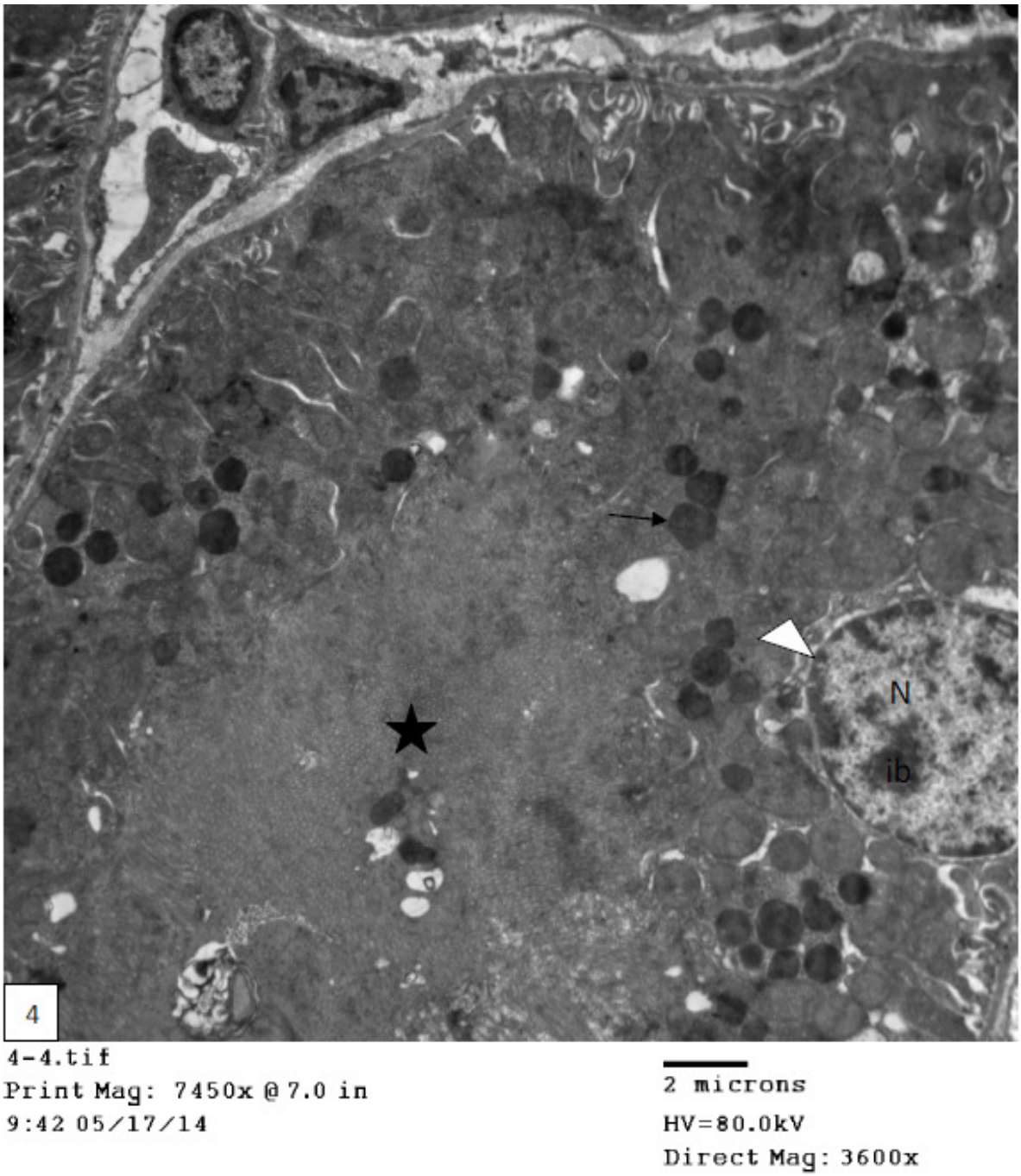

Figure 4. T.E. micrograph of tubular epithelium of co-treated with BHT showing the nucleus (N) contain small inclusion body (ib) with dilatation of perinuclear space (arrow head) and the cytoplasm contain large amount of electron dens lysosom (arrow). The lumen of the tubule contain proteinatous material and cellular depress (star).
Histopathological and ultrastructural examination of co-treated with copper complex rats group:

examination Figure (5A). The ultrastructur investigation frankly illustrated that the tubular epithelium was containing large vesicular nucleus, normal structure of mitochondria, free ribosoms, few electron dens lysosoms, numerous vacuoles and fat globules (Figure 5B). Obviously, the glomeruli showed presence of the podocyte of normal morphological appearance. In addition the capillary tufts that were slightly dilated Figure

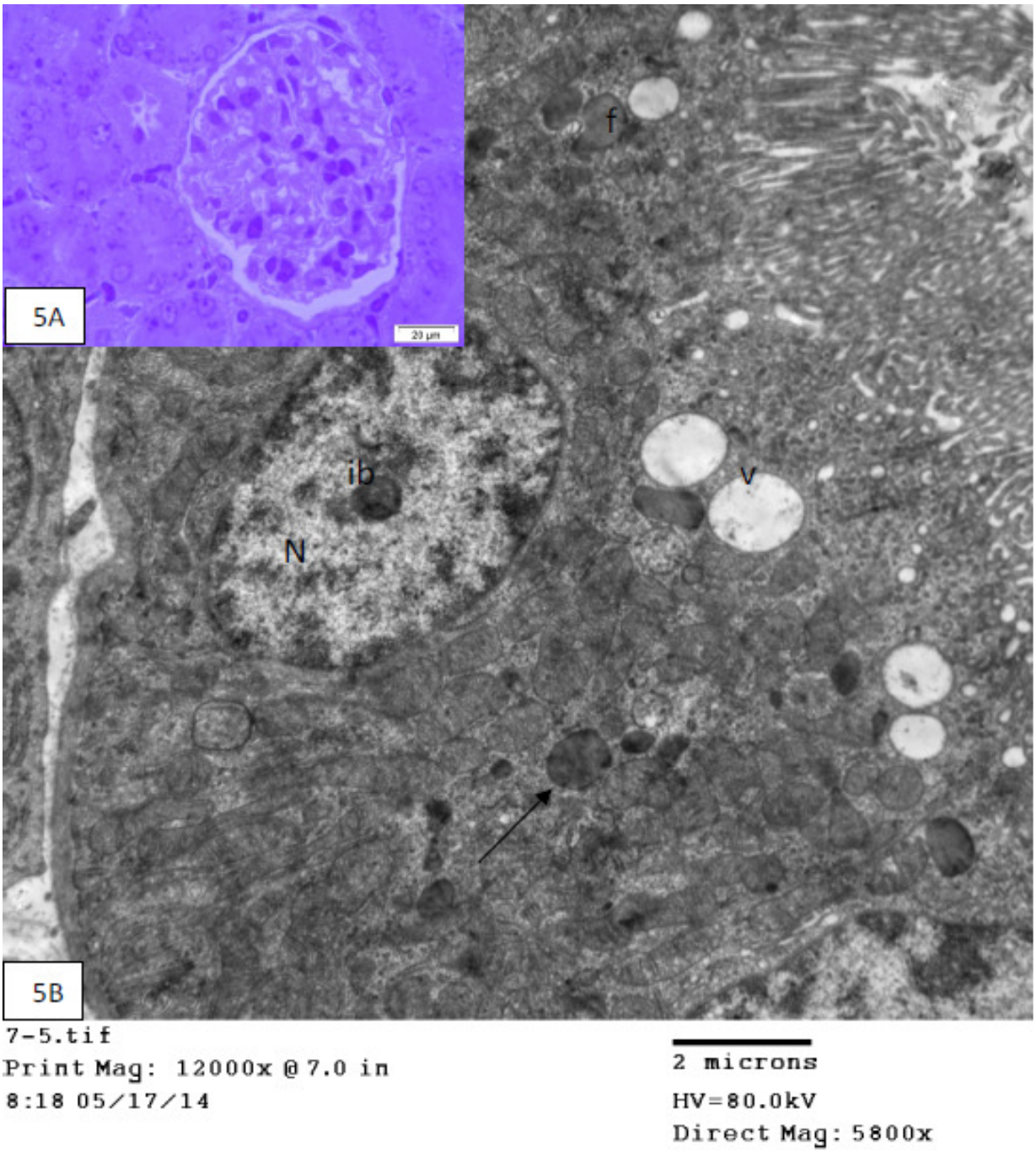

Figure 5A. Semithen section of kidney belonging to co-treated with copper complex showing milled cellular reaction in the interstitium, slightly swelling of the tubular epithelium with presence of few darkly stain granules in the cytoplasm and the glomeruli with normal morphological appearance.(T.B. stain).
Figure 5B. T.E. micrograph of kidney tubule of co-treated with copper complex showing the tubular epithelium having small inclusion body (ib) in the nucleus (N), numerous vacuoles (v), lysosoms (arrow) and fat globule (f) in the cytoplasm.

(6). The parietal layer of the glomerular epithelium was larged, flattened containing microvilli and the urinary space was slightly dilated (Figure 7).

The GST which is engaged in the phase II of body

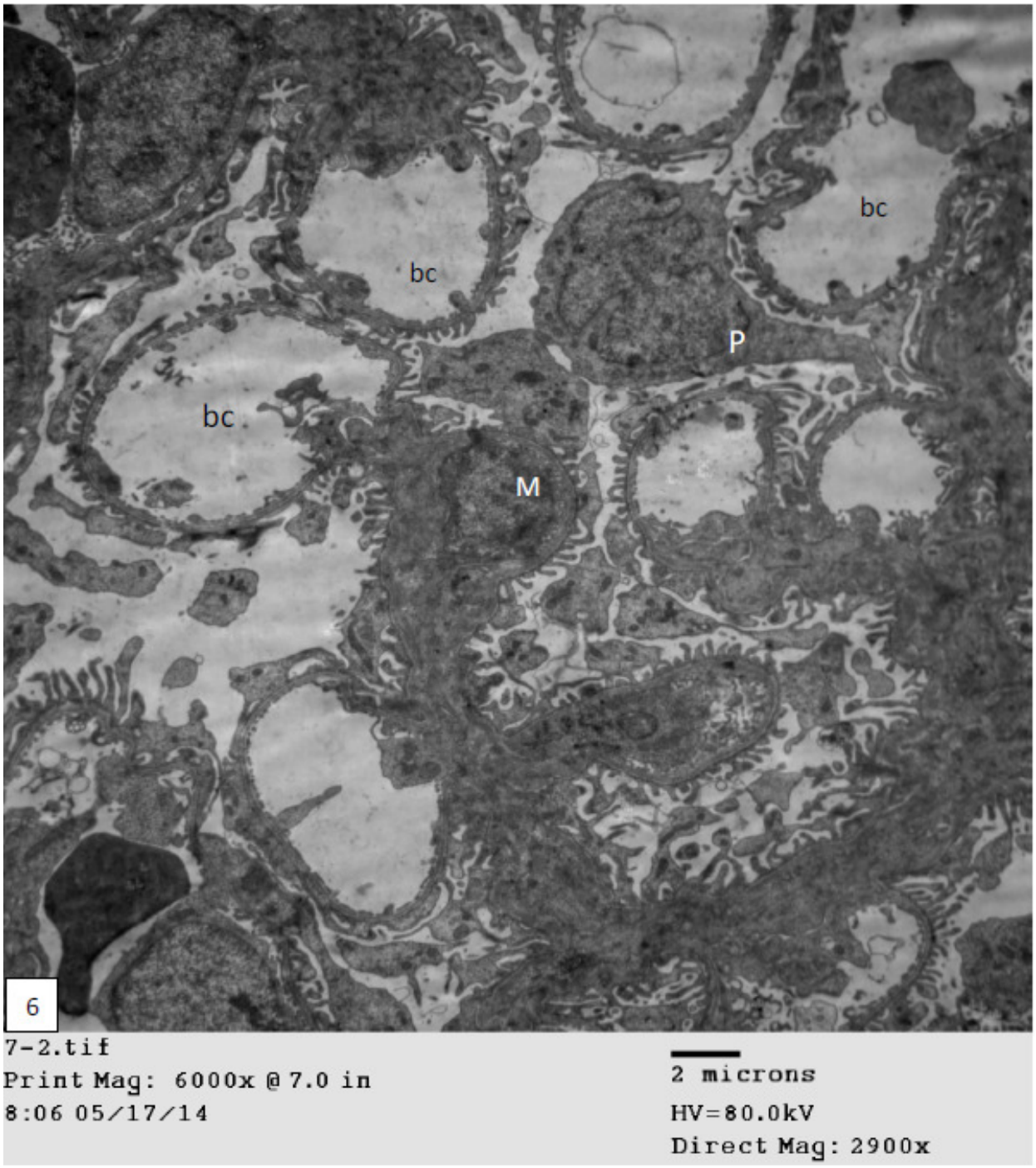

Figure 6. T.E. micrograph of the glomeruli of co-treated with copper complex showing the normal morphological appearance of the mesingial (M) cell and matrix as well as (the vesiral layer of the glomerular epithelium) podocyte (p), blood capillary (bc).

detoxification mechanisms was found to be highly reduced by aflatoxicosis in the kidney tissue to reach its half value after chronic exposure to AFB1 table (1). The co-treating antioxidant BHT dramatically increased its level but was still significantly reduced from the level of normal kidney but the co-treated rats by the copper chelating complex under the same conditions promoted its level to exactly the normal one, in other words the detoxification enhancing effect of the copper complex was significantly greater than that of the antioxidant agent BHT Figure (8).

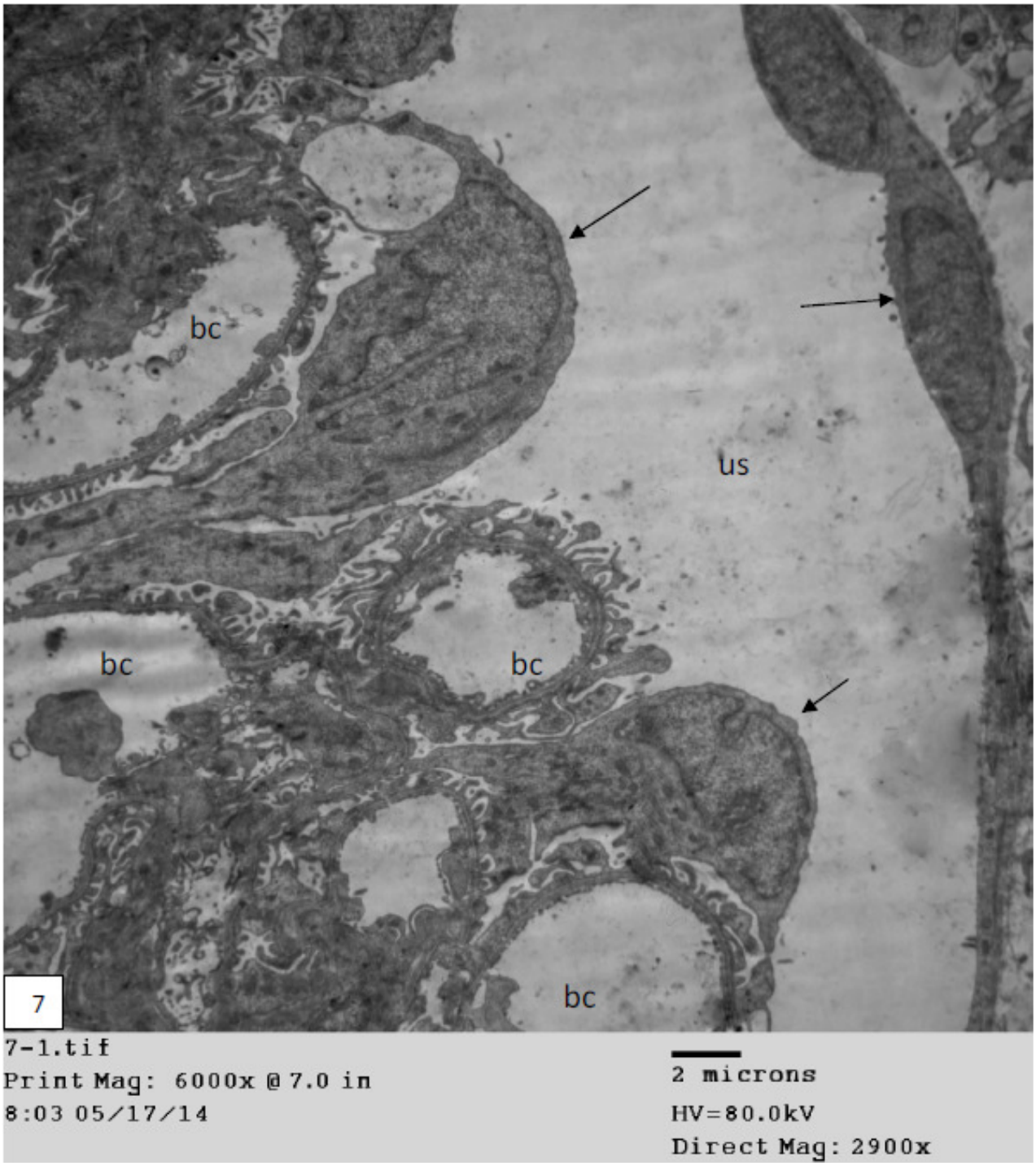

Figure 7. T.E. micrograph of kidney glomeruli of co-treated with copper complex showing the parietal and vesiral layer (arrow) of the glomerular epithelium, the urinary space (us) of the glomeruli and the blood capillary of the glomerular tuft (bc).

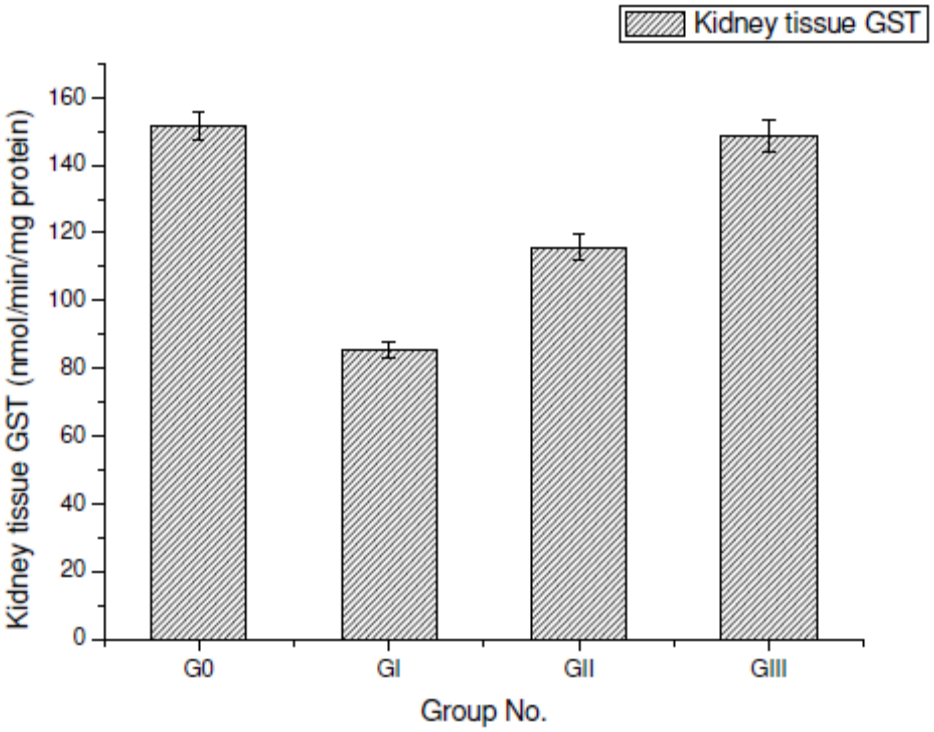

Figure 8. Kidney tissue enzymatic activity of GST (nmol/min/mg protein) of reference control G0, aflatoicosis GI, co-treated with BHT GII and co-treated with copper complex GIII.

Table 1. Changes in renal tissue GST activity (nmol/min/mg protein) in reference control G0, aflatoxicosis GI, co-treated with BHT GII and copper nicotinate complex GIII.

	G_0	G_I	G_{II}	G_{III}
Kidney tissue GST activity (nmol/min/mg protein)	151.66±3.50	75.31±2.32	115.81±2.38	147.73±3.98
P		***	*,a	NS

Data are expressed as (M ± SE) for n= 15 rats, P probability for significance have been designated as the following: *p<0.05; **p<0.01; ***p<0.001 for: G_0 and (G_I, G_{II} and G_{III}). [a] P< 0.05 comparison between G_{II} and G_{III}). N.S= non significant

DISCUSSION

The observed results in this study generally denote that chronically ingested AFB1 led to characteristic features of kidney intoxication in exposed rats. Such signs could be attributed to a decrease in the activity of antioxidative biological factors in renal tissue and uncontrol chain reactions of overproduction of free radicals (Katarzyna et al., 2013; Shatat et al., 2013). Hence, many believe that both the hepatotoxicity and renal toxicity by AFB1 are due to the storm of generated active metabolites especially AFB1-8,9-epoxide (Autrup et al., 1996) that rapidly produced by at least five members of CYP450 families that many reach its maximum six to twelve hours post dosing in rats (Chou et al., 1997). Indeed, the significant reduction of oxidative stress markers resulted by co-treating of inducing aflatoxicosis in rats with the copper nicotinate complex than that of the BHT in verified has been reported in a previous work (Shatat et al., 2013). In this work the light microscopic examination and the electron transmission one further confirms such findings by illustrating that glomerular and tubular structure seem morphologically normal even the exposure to aflatoxicosis was processing by time.

Noticeably, niacin like other natural antioxidant food additive against biotransformation of AFB1 into harmful metabolites in the kidney (Sharma et al., 2011), the niacin which is present as a chelating compound with the copper in the nicotinate complex could be accepted as more biologically active than the parent niacin molecule according to Sorenson (1989) who stated that the copper complexes usually exhibit significantly stronger activity than their parent compounds without copper. According to Prochaska and Talaly (1988), the chemoprotectors

against body intoxication which induce both enzymes of phase I and phase II as biofunctional inducers, this chelating complex was proven to more efficient than BHT for initiation specific phase I CYP 450 families for production of AFM1 and AFQ1 metabolites (Halladay et al., 1980; Kawano et al., 1980). Some workers have reported that some unite of GST isoforms exhibit antioxidant as well as detoxificating power towards epoxided AFB1 (Hayes et al., 1998). Concomitantly, the results of this work detect that such a complex is still more efficient for enhancing GST activity. Since, one of the important detoxification pathways the activated AFB1-epoxide is the formation of GSH conjugate (Degen and Neumann 1978, 1981), Fukayama and Hsieh (1984) reported that BHT increased the activity of phase II enzymes. Basically, it could be concluded that the copper nicotinate complex which is similarly acting as bifunctional inducer to that of BHT but significantly more efficient and more safely used as food additives biologically active agent against aflatoxicosis.

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