Global Advanced Research Journal of Medicine and Medical Science (ISSN: 2315-5159) Vol. 3(10) pp. 298-307, October 2014
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Full Length Research Paper

Copper (I)-Nicotinate Complex Promoted the Synthesis of Aflatoxin M1 and Q1 More Efficiently than Butylated hydroxytoluene in Afaltoxicosed Rats

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Accepted 07 October, 2014

In a previous work we have reported that synthetic copper (I)-nicotinate complex efficiently prevented induced nephrotoxicity by Aflatoxin B1 (AFB1) specifically by promoting phase II detoxificating glutathione-S-transferase activity. This work has been conducted to evaluate the antitoxic effect of the complex on the metabolism of AFB1. Forty five healthy male Wister albino rats were intoxicated (20 µg/kg B.W.) day after day for five weeks. The individually collected urine was assayed for AFB1, AFM1 and AFQ1 contents by HPLC technique. One third of them were co-treated by butylated hdroxytoluene (BHT) (0.05 g/kg B.W.), the second was co-treated by the copper complex (400 µg/kg B.W.) while the last was only intoxicated untreated group. Significant increase in the less toxic AFM1 and AFQ1 metabolites was recorded by any of the co-treating agents. The output of the least toxic AFQ1 fraction was significantly increased by the copper complex as regard to the BHT, (P <0.05). Conclusively, AFM1 and AFQ1 fractions in the urine that were promoted by the two tested agents may be related to their potentiating activity upon the phase I detoxificating CYP 1A2 and 3A4 enzyme families respectively. The significantly increased detoxified AFQ1 metabolite may be preferentially promoted by the copper (I)-nicotinate complex. This beneficial detoxificating effect of the copper complex has been obviously confirmed by observed great reduction in the immunohistochemical detection of vascular endothelial growth factor that was highly initiated by aflatoxicosis. Hence, such a complex could be saftly accepted as a highly potent antitoxic agent against aflatoxicosis

Keywords: Butylated hydroxytoluene, Copper(I)-nicotinate complex, Aflatoxin Q1, Aflatoxicosis, Chemoprotectant, Antioxidant

INTRODUCTION

Aflatoxin B1 is the most toxic and carcinogenic compound among the known mycotoxins. Aflatoxin B1 has been shown to be immunosuppressant, mutagenic, teratogenic and hepatocarcinogenic in experimental animals (Mohammed and Metwally 2009; Creppy 2002; Etzel 2002). Biotransformation in toxicology plays an important defense mechanism in the elimination of the

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toxic xenobiotics and body wastes in which they are converted into less harmful and polar substances that can easily be excreted by the kidney (Monosson 2012; Vondracek et al., 2001; Eaton et al., 1995). After the AFB1 has been ingested, it is bio-activated by cytochrome P-450 to a genotoxic exo 8,9-epoxide (AFBO), a DNA reactive metabolite that forms N^7-guanine adducts and it is also able to bind with proteins (Cupid et al., 2004; Nassar et al, 1982) that ultimately can cause cancer, a risk factor for malnutrition in infants (Hatem et al., 2005; Nassar et al., 1985) as well as other oxidized metabolites with less harmful effect in vivo, including the hydroxylated forms, AFM1, AFQ and AFP1 in the phase I metabolism. Major CYP450 enzymes involved in the oxidation of AFB1 are CYP1A2, CYP3A4 and CYP3A5. As AFM1, AFP1 and AFQ1 have significantly lower toxic and less carcinogenic potentials than AFB1; their formation is considered to be detoxification metabolites (Eaton et al., 1995; Hsieh et al., 1984). Aflatoxin M1 is a hydroxylated metabolite of aflatoxin B1 originated from animal and human metabolism after the ingestion of AFB1, which may be presented in body fluids such as blood, urine, dairy milk, and colostrums (Omar 2013; Monosson 2012; Groopman, 1994; Hafez et al., 1985). Aflatoxin Q1 is one of the major AFB1 assayed metabolite, being 18 times less toxic than the parent molecule AFB1 in the chicken embryo simultaneously is not mutagenic, with or without microsomal activation. Furthermore, AFQ1 has been considered to be a major detoxification metabolite of AFB1, in primates (Hsieh et al., 1974).

Studies over the few past decades have demonstrated that the biotransformation of AFB1 is an essential component of its hepatocarcinogenicity, and differences in biotransformation contribute significantly to species and interindividual differences in susceptibility to AFB1 (Eaton et al., 2001), for example; there are several common polymorphisms in CYP3A5 that result in little or no expression of functional protein, and thus most people do not express a functional phenotype of CYP3A5 (Thompson et al., 2006). In rats, CYP2C11 and CYP3A2 have been reported to catalyze the activation step in phase I metabolism (Buetler et al., 1996; Shimada et al., 1987). CYP-mediated oxidation can also yield several hydroxylated metabolites AFM1, AFQ1 and AFP1 (Eaton and Gallagher 1994). In addition it was indicated that CYP1A, CYP2B and CYP3A are involved in the formation of these metabolites which considered as detoxification products (Gross-Steinmeyer and Eaton 2012; Manson et al., 1997; Buetler et al., 1996; Eaton and Gallagher 1994). The formation of AFM1 and AFQ1 probably counterbalances the increase of AFB1-epoxide. Moreover the metabolism of AFB1 to epoxide is mainly attributed to CYP2C11 and CYP3A2 in rats (Buetler et al., 1996; Shimada et al., 1987) while formation of AFQ1 can be attributed to CYP2B and CYP 3A families (Manson et al., 1997; Stresser et al., 1994).

One of the major factors determining the protective activity of chemopreventive agents against AFB1 hepatocarcinogenesis is their ability to increase the biotransformation of AFB1 into non-toxic products. Primary detoxification of AFB1 could be achieved by the increase in formation of AFM1 and AFQ1 according to Manson et al., (1997). Several compounds have been shown to possess chemoprotective properties against AFB1 in rodent models. Such protection is often mediated by biochemical modulation of either phase I or phase II enzymes or an increase in nucleophilic trapping of activated AFB1 intermediates (Kensler et al., 1994). Compounds such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) (Ch'ih et al., 1989), polyhalogentaed biphenyl (Shepherd et al., 1984), Pheonobarbital, β-naphthoflavone (Gurtoo et al., 1985) and bicyclol (Hong and Yan 2002) all directly inhibit AFB1 carcinogenesis in rats. Any of these synthetic agents induces the cytochrome P-450 isoenzymes that accelerate Phase I metabolism of AFB1 to hydroxylated products which are considerably less genotoxic than either AFB1 or its 8,9-epoxide metabolite (Wong and Hsieh 1976; Gurtoo et al., 1975). Presumably, these inductions serve to alter the balance between metabolic activation and detoxification of AFB1. Butylated hydroxytoluene was found to be effective for chromosomal aberrations in human lymphocyte cultures (Jaffe 1984) and induce mutagenic effects at high doses as well (Watt et al., 1975; Oliver 1967).

In a previous work, copper (I)-nicotinate complex showed statistically more significant antioxidant activity than the already accepted antioxidant BHT against induced aflatoxicosis in rats (Shatat et al., 2013). In the same area we have recently deduced (Nassar et al., 2014) that such a complex exhibits a highly protective activity against induced nephrotoxicity by AFB1 via potentiating the phase II detoxifying glutathione-S-transferase. The chelating complex which is composed of the organic residue of vitamin niacin chelated with the daily required copper, the metal that exhibits several biological actives especially cellular enzymes (Salama et al., 2007; El-Saadani 2004; EL-Saadani et al., 1993; Musa et al., 1987; Sorenson 1982). The measured competitive prophylactic action of copper (I)-nicotinate complex was apparent as more efficient against aflatoxicosis on lowering the oxidative stress than the well known BHT (Shatat et al., 2013). In this submitted work, the metabolic pathway of detoxification mechanisms under chronic intoxication by AFB1 was focused on the detoxification metabolites AFM1 and AFQ1 in the excreted urine.

In addition, the induced hepatotoxicity by AFB1 like other factor profoundly associated with vascular endothelial growth factor (VEGF) expression for initiating angiogenesis in experimental animals (Tsuchihashi et al., 2006; Taniguchi et al., 2001; Rosmorduc et.al., 1999; Shweiki et al., 1992). Accordingly determination of the

expressed VEGF in nephrotoxicosed tissue could be immunohistochemically assayed to clarify and compare the predicted detoxificating effect of each of BHT and copper nicotinate complex co-treating agents.

MATERIALS AND METHODS

Chemicals

Aflatoxin B1, AFM1 and AFQ1 authentic samples were purchased from Sigma Chemical Co. (St. Louis, MO, USA), 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), β- gulucuronidase was purchased from Sigma Aldrich, methanol (99.9% HPLC Fluka), acetonitrile (99.9% HPLC Sigma-Aldrich), nicotinic acid, copper chloride 5H$_2$O, ascorbic acid, sodium bicarbonate, orthophosphoric acid, chloroform and isopropanol all were purchased from El Nasr Pharmaceutical Chemicals Co., Egypt.

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.) were accommodated in appropriate normal conditions, housed individually in metabolic cages each to be intoxicated (20 µg/kg B.W.) day after day for five weeks and to collect the excreted urine. They were equally grouped; therapeutically co-treated group with BHT (0.05 g/kg B.W.) according to Williams et al., (1996), therapeutically co-treated group with copper complex (400 µg/kg B.W.) according to (Shatat et al., 2013) and aflatoxicosis group (20 µg/kg B.W.) without any prophylacting agent as a control in addition to five rats were taken as healthy reference immunohistochemical examination for the kidney tissue. The tested collected urine for each group was performed after one, two, three, four and five weeks of starting of exposure to dosage of AFB1.

Extraction of AFB1 and its metabolites in the excreted urine

The urine samples taken from each rat were volumetrically determined where one ml was incubated with 100,000 unites of β- gulucuronidase enzyme and then aflatoxins were extracted by the method of Coronel et al., (2011).

HPLC analysis for AB1 and AFM1 was performed by UFLC Shimadzu type, RF-20A prominence fluorescence detector and CTO-20A prominence column oven. Flow rate 1.2 ml/min, Pressure 1,600 psi, Column-phenomenex C18 (250 x 4.6 mm), 5 µm from Waters corporation (USA), Oven temp- 40 ˚C, Run time- 17 mins. Mobile phase was water/methanol/4M HNO_3/KBr (60/40/350ul/119mg), excitation wavelength-365 nm, emission wavelength- 455 nm and Kobra cell (R-BiopharmRhone ltd)- settings at 100 uA.

Derivatization of AFQ1

AFQ1 was derivatized by trifluoroacetic acid according to (AOAC 2000).

HPLC analysis for AFQ1 was performed by Waters HPLC Model 1525, a Watres 486 Ultraviolet Detector, Watres Spherisorb C18 (250 x 4.6 mm), 5 µm (USA). An isocratic system with water: methanol: acetonitrile 240:120:40 (Deabes et al., 2012). The separation was performed at ambient temperature at a flow rate of 1.5 mL/min. The injection volume was 20 µl for both standard solutions and the extracted samples. The ultraviolet detector was operated at wavelength of 360 nm.

Immunohistochemistry

The nephrotic VEGF content has been performed precisely by the recent published technique of Mahmut et al., (2014).

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 $p<0.05$.

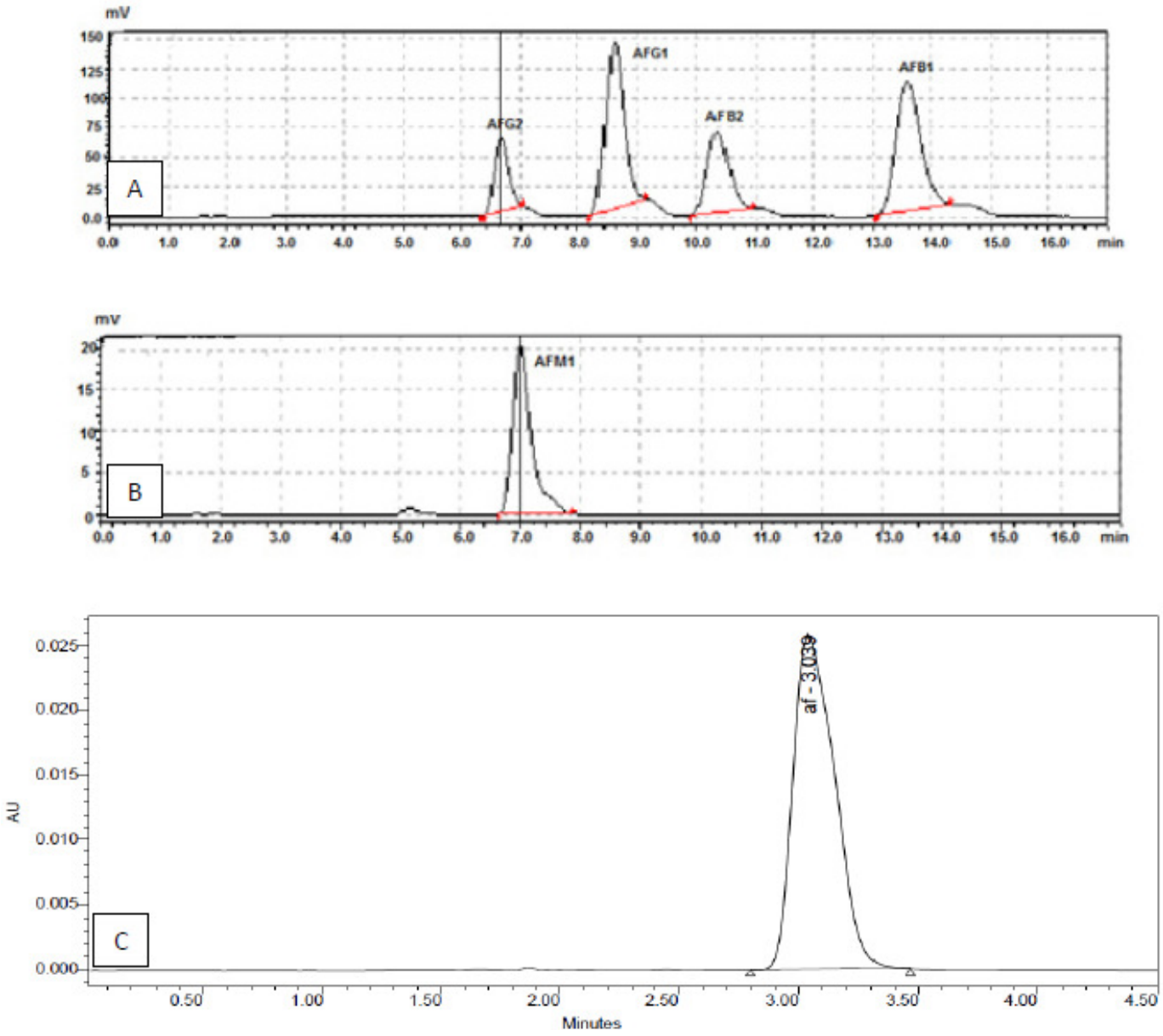

Figure 1 A, B, C. HPLC chromatogram of AFB1, AFM1 and AFQ1 standards.

RESULTS

Figure 1A, B and C shows the chromatogram of AFB1, AFM1 and AFQ1 standards. The concentration of the excreted parent AFB1 in urine as free or gulucuronid form was extracted and assayed by HPLC during the five weeks of exposure as chronic intoxication is presented in table 1, Figure 2. The significant reduction in the excreted AFB1 by treatment of BHT and Copper (I)-nicotinate complex was significantly observed specifically the effect of the copper chelating complex. The major excreted metabolite AFM1 was apparently increasing by time as well as by the effect of BHT and copper (I)-nicotinate complex as it's presented in table 2, Figure 3. The more excretable metabolite AFQ1 was increasing by time of exposure as well as by co-treating BHT and Copper (I)-nicotinate complex as it's presented in table 3, Figure 4.

The immunohistochemical investigation showed that the accumulated VEGF protein appeared as dense brown stained spots in the Bowman's capsule and renal tubules of aflatoxicoed cases. The co-treated cases with BHT showed characteristic findings of such brown spots but those with copper complex showed rare recognized brown spots in situ. While there was no reaction at all sliced of normal untoxicated cases Figure 5 A,B,C and D.

Table 1. Concentration of AFB1 (µg/ml) in excreted urine of aflatoxicosed rats, co-treated with BHT and copper complex rats.

	Aflatoxicosis	AFB1 + BHT	AFB1 + Cu -Complex
W1	1.907± 0.44	1.812± 0.43 ^{NS}	1.621± 0.29 ^{NS}
W2	3.231± 0.25	2.139± 0.29 *	1.974± 0.5**
W3	4.021± 0.24	2.840± 0.5**	2.415± 0.39**
W4	5.853± 0.44	2.319± 0.37***	2.211± 0.56***
W5	7.481± 0.21	1.026± 0.15***	0.916± 0.3***

Values are expressed as the mean ± SE for (15) animals in each group. *p<0.05; **p<0.01; ***p<0.001 represent significant differences as compared to aflatoxicosed animals

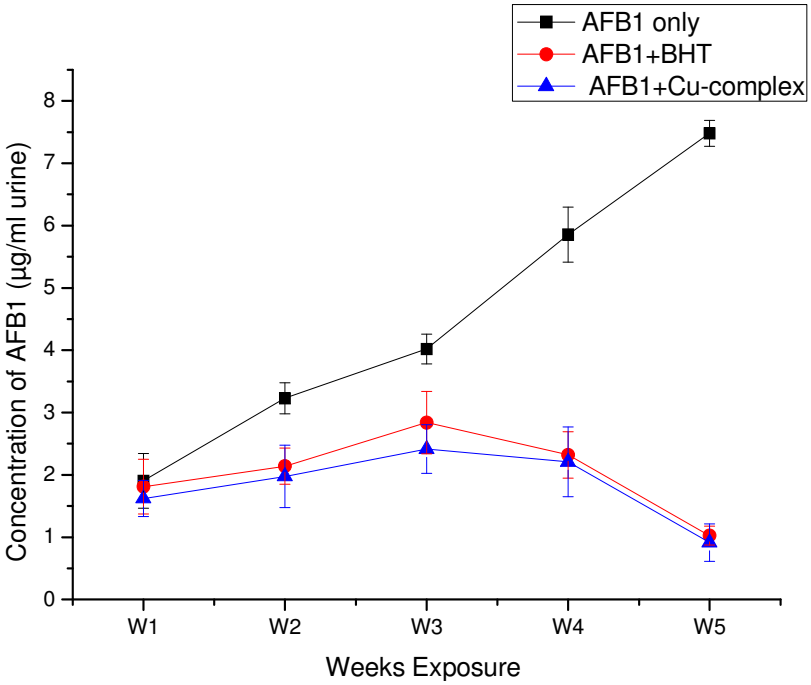

Figure 2. Concentration of AFB1 (µg/ml) in excreted urine of aflatoxicosed rats, co-treated with BHT and copper complex.

Table 2. Concentration of AFM1 (µg/ml) in excreted urine of aflatoxicosed rats, co-treated with BHT and copper complex rats.

	Aflatoxicosis	AFB1 + BHT	AFB1 + Cu -Complex
W1	ND	0.095± 0.04*	0.234± 0.13*
W2	0.092± 0.05	0.292± 0.06*	0.415± 0.19**
W3	0.183± 0.08	0.533± 0.19**	0.971± 0.34***
W4	0.537± 0.21	1.495± 0.26***	1.935± 0.27***
W5	0.951± 0.25	2.991± 0.15***	3.591± 0.26***

Values are expressed as the mean ± SE for (15) animals in each group. *p<0.05; **p<0.01; ***p<0.001 represent significant differences as compared to aflatoxicosed animals.

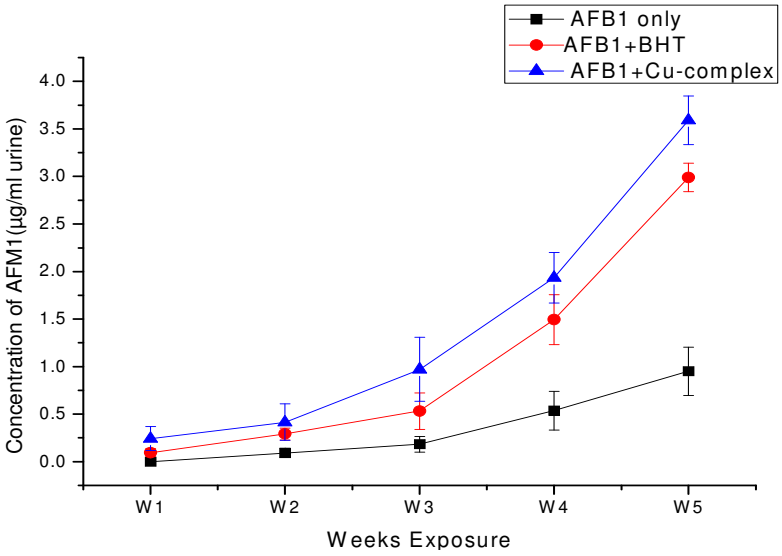

Figure 3. Concentration of AFM|1 (µg/ml) in excreted urine of aflatoxicosed rats, co-treated with BHT and copper complex.

Table 3. Concentration of AFQ1 (µg/ml) in excreted urine of aflatoxicosed rats, co-treated with BHT and copper complex rats.

	Aflatoxicosis	AFB1 + BHT	AFB1 + Cu -Complex
W1	ND	ND	0.053± 0.03 ^{NS}
W2	0.054± 0.06	0.083± 0.01 ^{NS}	0.133± 0.06*
W3	0.093± 0.03	0.191± 0.09*	0.253± 0.02**
W4	0.151± 0.04	0.354± 0.06***	0.445± 0.07***
W5	0.294± 0.05	0.513± 0.04***	0.693± 0.16***

Values are expressed as the mean ± SE for (15) animals in each group. *p<0.05; **p<0.01; ***p<0.001 represent significant differences as compared to aflatoxicosed animals

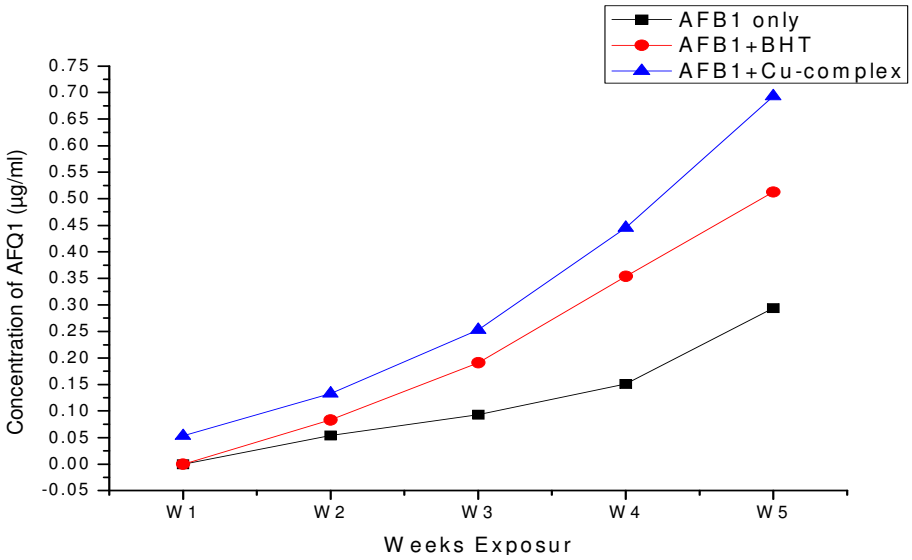

Figure 4. Concentration of AFQ1 (µg/ml) in excreted urine of aflatoxicosed rats, co-treated with BHT and copper complex.

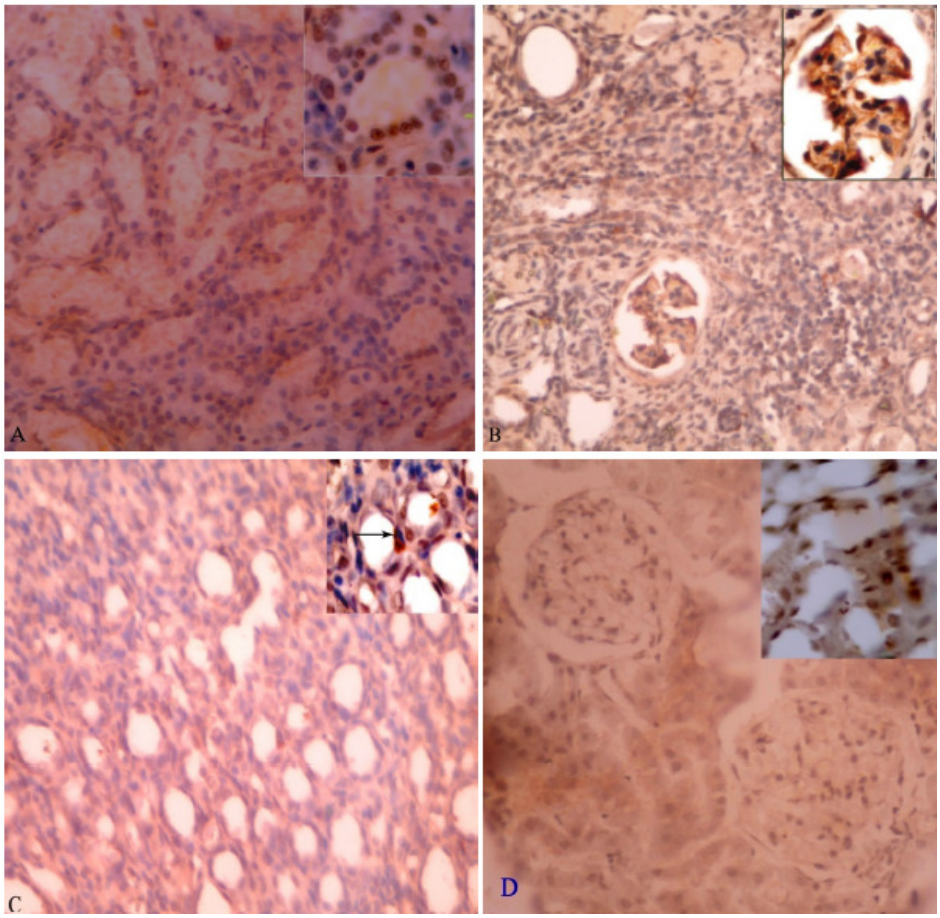

Figure 5. VEGF expression in renal tubules from the control groups (A). Cytoplasmic VEGF immunolabeling in treated by aflatoxin B1 (B). VEGF staining shown after BHT (C) as well as copper complex co-treated animals (D) were significantly decreased compared that observed in treated by Aflatoxin B1. Scale (VEGF x 400).

DISCUSSION

The detected increase in the excreted AFB1 in urine during the time course of chronic exposure for five weeks is consistent with reported data of others who postulated that approximately 50% of the oral dose of AFB1 is quickly absorbed in the small intestine and enter the liver to be concentrated there for oxidation while the kidney concentrates small portion, the free AFB1 and its water soluble metabolites can be found in the systemic blood to be excreted out in urine and feces (Coulombe et. al., 1985; Wogan et. al., 1967). The significant reduction in such excreted AFB1 by either co-treating BHT or Copper (I)-nicotinate complex could be attributed to the predicted promoting effect of both of them on the oxidative metabolic mechanisms that was detected by the corresponding increase in the urine output of AFM1 and AFQ1. This AFQ1 is more highly excretable by about 18 times than any other metabolite (Hsieh et al., 1974). Since, the treatment with BHT in vivo is well known to protect against AFB1-induced cytotoxicity and to reduce the covalent binding of AFB1 in primary culture of adult rat hepatocytes that was based on effects which alter AFB1 mechanisms by induction of several antioxidative and conjugating enzymes to play significant role in the activation and detoxification of AFB1 (Salocks et al., 1981). It seems that, the tested copper nicotinate chelating complex is more significantly efficient for promoting the metabolic pathways towards production of AFM1 and AFQ1 than the effect of BHT. Wild and Tuner in (2002) and Eaton et al., in (2001) have postulated that the available experimental evidence showed that excretion of AFB1 in urine and feces after exposure to AFB1 is followed by evolved AFP1 glucuronides regulated by undetected CYP family(s) but the AFQ1 glucuronides could be initiated by CYP 3A4 (3A5) while the hydrophilic AFM1 evolved by the active CYP 1A2.

Rationally, the more efficient detoxifying agents against aflatoxicosis whatever prophylactic or treating compounds is the more promoting effectors for directing the metabolic pathway towards further production of hydrophilic detoxified AFM1 as well as the more

excretable one AFQ1. The appreciated detoxifying agent for several decades was that of BHT (Ch'ih et al., 1989), herein it seems that the copper chelating complex somewhat more efficient in this virtue. Regarding the aim of this submitted work for getting up the most detoxifying agent especially that it is composed of daily required bio-elements (copper 3mg/day and nicotinic acid 15mg/day) for the human being that we have concerned in this research. It fortunately seems to be more efficient than BHT as detoxifying agent against aflatoxicosis. Wang et al., (1998) stated that, the AFB1 metabolizing enzymes depend on affinity as well as on the expression levels of human liver where CYP 3A4 is the predominant CYP450. Moreover, they postulated that the human hepatic CYP 3A5 expression is polymorphic with a proportion of individuals showing no expression in particular 40% of African-Americans do not express this enzyme. Sequentially, Hustert et al., and Kuehl et al., in 2001 identified polymorphisms in the promoter region of CYP 3A5 that alternative splicing and a truncated protein.

The Obvious observed metabolic pathway was directed towards formation of AFM1 and AFQ1 to be more accelerated by about 3 times that of the chronically untreated aflatoxicosed animals by induction of the tested copper (I)-nicotinate complex (Fukayama and Hsieh 1984). The metabolites that regulated by the CYP families 1A2, 3A4 and 3A5 that may similarly exhibit the character of the brain tryptophan hydroxylase which is not generally saturated with substrate; consequently the concentration of tryptophan in the brain influences the synthesis of 5-hydroxytryptamin (Goodman & Gilman 's 2008). Accordingly chronic exposure to aflatoxicosis resulted in further increase of such metabolites that were observed as products of parabolic function mechanism. The significant increase in these metabolites was reported by Halladay et al., (1980); Kawano et al., (1980) who stated that exposure to BHT increases the synthesis and activity of many microsomal and cytosolic enzymes including cytochrome P-450- dependent monooxygenases. Similarly it could be concluded that the more highly effective copper chelating complex promoted the endogenous biosynthesis of the cytosolic CYP 450 families responsible for detoxification of AFB1 into the excretable AFM1 specifically metabolite AFQ1.

The observed increased VEGF in the renal tubules and glomeruli resulted as signs of nephrotoxicity similar to those observed by intoxicated injured liver (Taniguchi et al., 2001). Noticeably, the VEGF is a transducer that conveys signals between endothelial and epithelial/mesenchymal cells and activates angiogenesis (Shweiki et al., 1992). The reduced accumulated in VEGF by BHT co-treated cases is similar to those reported by (Mahmut et al., 2014) by using a polyphenolic flavonoid antioxidant against injured hepatotoxicity. The authors interpreted such a reduction due to the antioxidant effect through reduces the in vivo the VEGF protein expression. Hence, the dramatic reduction by co-treated by copper complex could be accepted as a result of VEGF mRNA reduction or further positive effect in specific gen silences.

CONCLUSION

These biochemical findings enhance the beneficial idea for using this copper (I)-nicotinate complex as a potent food additive with most efficient detoxificating against probable aflatoxicosis of human being and animals.

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