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Expression of Biotransformation and Antioxidant Genes in the Liver of Albino Mice after Exposure to Aflatoxin B1 and an Antioxidant Sourced from Turmeric (*Curcuma longa*)

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Abstract

The present investigation aims to determine the effects of aflatoxin B1 (AFB1) on biotransformation and antioxidant genes and the protective effects of curcumin, present in turmeric (*Curcuma longa*) powder (TMP). Specifically, the study included four groups of albino mice were fed for 30 days on diet Group I: Control, Group II: animals fed on the conventional basal diet supplemented with 0.5% food grade TMP that supplied 74 mg/kg total curcuminoids. Group III contained animals reared on conventional basal diet supplemented with 1.0 ppm AFB1 supplied by ground aflatoxin culture material (760 ppm AFB1). Finally, Group IV comprised of albino mice fed with basal diet supplemented with 1.0 ppm AFB1 and 0.5% TMP that supplied 74 mg/kg of the body weight. After treatment, a number of physical parameters were assessed (gain in body weight, average quantity of feed intake and relative liver weight) and it was found that the subjects fed on diet containing curcumin and aflatoxin B1experienced ameliorative effect on the impact of aflatoxin B1 and performed better on growth and liver weight parameters. RNA extracted from the mice liver successfully was subjected to quantitative real time PCR analysis (Q PCR) and the results revealed no high significant difference in the expression of *CAT* gene between studied groups with probability value ≥0.005. However, at the other hand a decreased in expression statically of *SOD, GPx, GST, EH* genes was observed while there was an increased Synchronous and consistent expression of *CYP1A1* and *CYP2H1* genes display in the studied groups in the current study.

Keywords: Aflatoxin B1, *Curcuma longa*, Gene expression, Antioxidant.

1. Introduction

Fungi contaminate the food in a variety of ways including food spoilage and toxicity depending on their biological make-up and the eco-physiological conditions (Ahmadib *et al*., 2011). Some fungi are toxigenic, synthesising one or more mycotoxins (Ahmed *et al*., 2013), having impact on humans and certain animals when ingested in large quantities. Amongst these, Aflatoxins (AF), especially Aflatoxin B1 (AFB1), are wide spread in distribution, potent contaminants of foodof humans and animals, and result in food security issues and mortality. (Kumar *et al*., 2017). These toxins travel in the food chain and causing wide spread impact in a variety of organisms apparently not under the direct influence of the fungus (Moosavy *et al*., 2013). Recognizing the significance of food security and these toxins rendering the food unsuitable for human consumption has led to a large number of studies (Moosavy *et al*., 2013) on various aspects of the toxicity and remedies thereof. When consumed by Albino mice, AFB1 causes invariably cell damage, production of free radical, and lipid peroxidation in different organs (Dheeb,2013; Nogueira *et al*., 2015). Protective effects against damage due to oxidative are normally redressed by antioxidants that obstruct the free radicals like reactive oxygen species (ROS) reactions. Various studies have been reported the healing effect of aflatoxins through use of medicinal plants (Abdulmajeed, 2011) and the curative role of curcuminoid pigments extracted from roots and rhizomes of turmeric (*Curcuma longa*) (WH, 2009; Bayram *et al*., 2008) is one such example. The present study was therefore initiated to investigate and evaluate this curative role of curcumin and its influence on the expression of biotransformation and anti-oxidant function genes, using Quantitative real time PCR.

2. Materials and Methods

2.1. Animals and Experimental Design

A total 40 male Albino mice (age 7weeks) obtained from the National Centre of Research and Drugs

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Monitoring in Baghdad were employed in the study. The mice were allowed to adapt for two weeks in the Biotechnology Research Centre at Al Nahrain University before the commencement of the study. The animals were reared separately in clean, disinfected and pathogen-free facility fed on commercially available assorted pellets and tap water *ad libitum* (Jun *et al*., 2006). Experiment design "A completely randomized design" was adopted with ten replicates of eight Albino mice assigned to each of four dietary treatments: Group I: Control, Group II: animals fed on the conventional basal diet provided with TMP 0.5% food grade that supplied total curcuminoids74 mg/kg. Group III contained animals reared on conventional basal diet supplemented with 1.0 ppm AFB1 supplied by ground aflatoxin culture material (760 ppm AFB1). Finally, Group IV comprised of albino rats fed with basal diet supplemented with 1.0 ppm AFB1 and 0.5% TMP that supplied 74 mg/kg of the body weight. During the 30 days' dietary treatment, mortality of the animals was recorded as and when noticed and mice were inspected daily for any health related anomalies. At the end of the treatment, mice were sacrificed, the liver was weighed and the tissue collected, immediately frozen in liquid nitrogen, and stored in a freezer at-80 °C until RNA extraction in order to use it in molecular analysis and determination expression of biotransformation and antioxidant genes. Dietary AFB1 concentrations and the content of all the diets were confirmed by the ELISA technique performed using an ELISA kit (Wacoo *et al*., 2017). All treatments on present experiments were approved based on The Committee Ethics of Baghdad University ID: 2398.

2.2. Extraction of Curcuminoid and Determination of the Concentration

The potent curcuminoid was extracted from naturally grown, commercially available Turmeric. A sample of (10) g powdered root and rhizomes was extracted using hexane (50 mL) as a solvent. Re-extracted done to Hexane extracted powder (I gm) with methanol (20 ml) for 2 hours, aliquot centrifuged at 13,000 rpm for 5 min. The supernatant (1 mL) was removed, diluted methanol (4 mL) and total curcuminoid content (curcumin, bis-dimethoxy curcumin and dimethoxy curcumin) was ascertained by High Performance Liquid Chromotography following Gowda *et al*. (2009). To achieve highest possible degree of precision, Sampler and Column (Hitachi Model L–7200 autosampler, 250 x 4.6 mm HyperSil reverse phase C18 column) were employed. Following Dheeb (2015), Hitachi D-7000 data acquisition interface and Concert Chrome software were used to gather data at a detection wavelength of 425 nm. The mobile phase was a mixture of methanol, acetonitrile and acetic acid (1:11:10) with a flow rate of 1mL/min. The curcumin standards were established following Gowda *et al*. (2009). The total curcuminoid content of TMP was determined by adding up the concentrations of the three curcuminoids: curcumin, bis-dimethoxy curcumin and dimethoxy curcumin (Buchau *et al*., 2007) .

2.3. Gene Expression Study of Biotransformation and Antioxidants

2.3.1. RNA Isolation and cDNA Synthesis

An RNA extraction kit (Promega) was used for extraction of Total RNS following the manufacturer's instructions. Although not considered mandatory step, more complete DNA removal was carried out by treating isolated RNA with RNase-free DNase I (Biobasic, Canada) for 20 min at 37°C. Quantus Florometer was used to detect the concentration of extracted RNA in order to detect the goodness of samples for downstream applications. For 1 µL of RNA, 199 µL of diluted Quanty Flour Dye was mixed. After 5min incubation at room temperature, RNA concentration values were detected.

The DNase I was inactivated at 65°C for 10 min. The integrity of the RNA was ascertained by gel electrophoresis (1.5% agarose gel containing 0.5% (v/v) ethidium bromide) following Rassin *et al* (2015). First-strand cDNA was synthesised from 500 ng of total RNA using a Reverse Transcription system (Bioneer, Korea) with an oligo-dT_{15} primer. The reaction solution was used as a template for reverse transcriptase polymerase chain reaction (RT-PCR) .

2.3.2. Amplification of Biotransformation and Antioxidant Genes

Target gene and housekeeping B-actin (reference gene) cDNA were amplified using biotransformation and antioxidant primers in order to measure the expression patterns of genes involved in antioxidant function. Primers were as follows:

Catalase (CAT) Forward 5′GGGGAGCTGTTTACT GCAAG-3′ and a reverse primer5′TTCCATTGGCTATG GCATT-3′, product size 139bp; super oxide dismutase (SOD, GPx, GST biotransformation genes EH, CYP 2H1, CYP 1A1,), Forward5′- AGGGGGTCATCCACTTCC-3′ and a reverse primer 5′ CCCATTTGTGTTGTCTCCAA-3′, product size 122 bp; glutathione peroxidase (GPx), Forward 5′- TTGTAAACATCAGGGGCAAA-3′and a reverse primer 5′ TGGGCCAAGATCTTTCTGTAA-3′, product size 140 bp; glutathione S-transferase (GST) Forward GCCTGACTTCAGTCCTTGGT-3′ and a reverse primer 5′ 5′- CCACCGaATTGACTCCATCT -3′, product size 131 bp.

The following primers were used for biotransformation genes: epoxide hydrolase (EH) Forward5′-AAAGGGACAGAAGCCTGACA -3′ and a reverse primer 5′ CCTCCAGTGGCTCAGTGAAT-3′,product size 128 bp; cytochrome P450'sCYP2H1 Forward 5′-ATCCCCATCATTGGAAATGT-3′ and a reverse primer 5′ TCGTAGCCATACAGCACCAC -3′,product size 137 bp; cytochrome CYP 1A1Forward 5′CACTTTCTGCCTGCTCCTG-3′ and a reverse primer 5′ GGTCCTTCCTCAGCTCCAG -3′, product size 125 bp polymerase chain reaction (PCR) was initiated by employing cDNA template on a Lab net Thermocycler (USA) at the following conditions : (95°C for 5 min and 40 cycles at 95°C for 1 min, 60°Cfor 45 s and 72°C for 1 min). Primers were designed according to Livak *et al*., (2017)using the Primer3 program with an annealing temperature of 60 °C (Al-Tekreeti *et al*., 2017).

2.4. Gene Expression Analysis Study through SYBR Green Real-Time RT-PCR

The gene expression of biotransformation and antioxidant was evaluated through use of SYBR real-time RT-PCR using Exicycler real time PCR (Bioneer, Korea) following Al-Mashhadani (2014). Quantitation of relative expression was determined by the following equation (Yarru, 2008):

Gene expression (Quantity) $=10^{[(CT-b)/slop]}$

Gene for Glyceraldehyde Phosphate Dehydrogenase (GAPDH) was used as the endogenous control gene in the qRT-PCR experiments.

2.5. Quantitative Real Time PCR (qRT–PCR)

The expression levels of antioxidant genes CAT, SOD, GPx, GST, biotransformation genes EH, CYP 2H1, and CYP 1A1, were estimated by One Step qRT-PCR. To confirm the expression of target gene, quantitative real time one step qRT-PCR sybr Green assay was used. Primers sequences for each gene were prepared. The mRNA levels of endogenous control gene GAPD H were amplified and used to normalize the mRNA levels of the up genes , reaction volume and Thermal Cycler Programming summarized in Table 1 and 2.

Table 1. Reaction volume and components of RT qPCR

Components	Conc.	Volume(µL)\Reaction
GoTaqqPCR master mix	2X	10
RT mix	10µM	0.4
Forward Primer	10µM	2
Revers Primer	1-2ng	2
RNA	-	4
RNase-free water		1.6
Total per reaction		20

Table 2. Thermal Cycler Programming

Steps	˚C	min:sec	Cycles
cDNA Synthesis	37	15min	1
Initial Denaturation	95	5 min	1
Denaturation	95	30 sec	40
Annealing	60	30 sec	
Extension	72	30 sec	
Melt	65-90		1

2.6. Statistical Analysis

Data were analysed using the model procedures of SAS. The differences of values of the investigated parameters among different groups of the subjects (mean ± standard error) were assessed by analysis of variance using SAS version 7.5, (difference $p<=0.05$ and 0.001) (Choi *et al*.,2010).

3. Results

3.1. Influence of Dosage of AFB1on Total Body Weight and Some Properties of Mice Organs

Data for the animals belonging to control and experimental groups regarding average food intake, gain in weight, and liver weight (% BW) after 30 days of experimentation are presented in Table 3. The data reflects that the mice treated with TMP alone (Group II) and the control (Group I) had similar pattern of weight gain and feed intake while the mice in Group III(receiving dose of AFB1) had significantly lower feed intake and corresponding weight gain. For animals receiving 0.5% TMP (74 mg/kg curcuminoids) together with AFB1 (Group IV), feed intake and body weight both increased, suggesting a protective action of the curcuminoids present in TMP. The results of the present investigation are in conformity with some previous studies investigating the effects of AFB1 (Choi *et al.*, 2008, Livak *et al.*, 2008),and other chemicals (Yarru, 2008, Livak *et al.*, 2008).The condition of the mice exposed to AFB1 together with TMP (Group IV) and the ones receiving dose of TMP alone (Group II) are a reflection of curcumin present in turmeric works as an antioxidant through inhibition of the biotransformation of AFB1 to aflatoxicol in the liver (Yarru, 2008, Livak *et al.*, 2008), and may also have antimutagenic and anticarcinogenic effects (Yarru, 2008, Cleveland *et al.*,2009). In another study (Hismiogullari, 2014; Livak *et al.*, 2008), where mice were fed on different concentrations of turmeric powder over a period of49 days, the animals experienced a positive effect on liver enzymes that directly or indirectly reflect a healthier liver status.

Table 3. Average feed intake, weight gain, feed efficiency and relative liver weight in mice during the study.

Treatment	Av. Feed intake (gm/mice)	Av. Body weight gain (gm/ mice)	Wt of liver (% BW)
I(Control)	1047.3±0.22 a	33.5± 0.12a	1.74 ±0.44b
II(animals fed on the conventional basal diet supplemented with 0.5% food grade TMP that supplied 74 mg/kg total curcuminoids)	857.8±0.75b	26.0 ±0.34c	1.37±0.19a
III(animals reared on conventional basal diet supplemented with 1.0 ppm AFB1 supplied by ground aflatoxin culture material (760 ppm AFB1)	905.5±0.73b	27.6 ±0.32b	1.09± 0.09ab
IV (animals feed basal diet supplemented with 1.0 ppm AFB1 and 0.5% TMP that supplied 74 mg/kg of the body weight)	1004.9±0.20a	29.9 ±0.18ab	1.016±0.007ab

*$p \leq 0.05$. *different letter means significant difference between the treatment

3.2. Quantitative Real Time PCR Results

The expression of genes responsible for antioxidant and biotransformation functions was ascertained using the quantitative real time PCR techniques show in Figure 1. The results of the present investigation showed similar pattern of gene expression among the four groups receiving different dietary treatments for the catalase (CAT).

However, super oxide dismutase (SOD), glutathione peroxidase (GPx), glutathione S-transferase(GST) and epoxide hydrolase (EH) genes exhibited a decreased activity in animals treated with 1 ppm AFB1 (Group III). These results may be interpreted as the effects of AFB1are neutralized by inclusion of curcumin in the diet (Group IV). Increased expression of CYP1A1 and CYP2H1 genes may also be attributed to curcumin's alleviation of the toxic effects of AFB1. The present results may be due to the presence of TMP stimulating the antioxidant system in the livers of albino mice in order to counteract the oxidative damage caused by AFB1 (Dwivedi, 2013, Cleveland *et al.*, 2009). The animals fed on diet containing AFB1exhibited an increased liver peroxide level and a decrease in SOD, GPx and CAT activities, and some studies have shown the supplementation of diets with the phenolic compounds of plants origin have reduced the free radical production and apoptosis in human hepatoma cells induced by AFB1(Helal *et al.*, 2012).

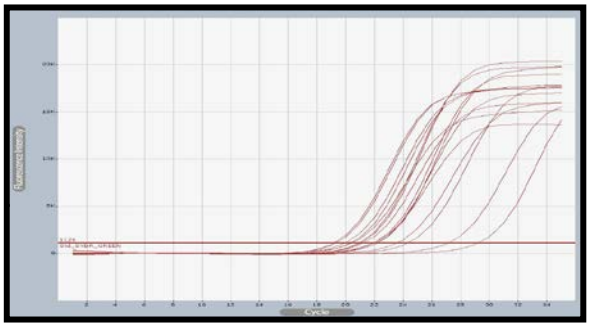

Figure 1, Quantitative Real Time PCR Curves of antioxidant genes CAT, SOD, GPx, GST, biotransformation genes, and GAPD Hwith threshold (177.8)

Some other studies have demonstrated the carbonyl functional group of curcuminoids to be responsible for its antimutagenic and anticarcinogenic effects (Buchau and Gallo, 2007), and curcumin also has inhibitory effects on superoxide anion generation and the biotransformation of Aflatoxin B1 to aflatoxicol in liver (Buchau and Gallo, 2007). Supplementation of turmeric is known to reduce the formation of adducts through modulation of cytochromeP450 function (Buchau and Gallo, 2007). Aflatoxin B1 is known to cause lipid peroxidation in liver (Buchau and Gallo, 2007), induces oxidative damage in the cell, and is also a potent carcinogen that forms DNA adducts (Buchau and Gallo, 2007). Feeding AFB1 to rats increased liver peroxide levels and was associated with a decrease in activities of superoxide dismutase, catalase, and glutathione peroxidase. Supplementation of root extracts of *Picrorhiza kurroa* and seeds of *Silybum marianum* ameliorated the effects of Aflatoxin B1, reduced peroxide levels, and returned antioxidant enzymes to control levels (Buchau and Gallo, 2007). Rosamarinic acid, a phenolic compound present in *Boraginaceae* species of plants reduced free radical production and apoptosis in human hepatoma cells induced by Aflatoxin B1(Buchau and Gallo,2007). The carbonyl functional group of curcuminoids was responsible for its antimutagenic and anticarcinogenic actions (Ahmeda *et al.*, 2014). Further, earlier reports suggest that curcumin has a strong inhibitory action on superoxide anion generation (Choi *et al.*, 2010) and biotransformation of Aflatoxin B1 to aflatoxicol in liver (Buchau and Gallo, 2007). Supplementation of turmeric is known to reduce the formation of adducts through modulation of cytochrome P450 function (Shivanoor *et al.*, 2016). The above findings demonstrated the possible mode of action of curcumin as an antioxidant and the results we obtained in the present study suggest that curcumin in TMP gave partial protection against aflatoxicosis. Current findings also suggest that curcumin may need to be supplemented at levels higher than 74mg/kg to achieve optimum protection against 1.0 ppm AFB1.

4. Discussion

In the present study, expression of gene for super oxide dismutase (SOD) was down-regulated in albino mice fed a diet containing AFB1 and this reduced expression of gene has been attributed to the superoxide anions accumulated within the mitochondria, thus leading to an oxidative stress and thereby hindering the cellular processes (Ahmeda *et al.*, 2014). Curcumin is known to boost the antioxidant activity especially through SOD (Thinarayanana *et al.*, 2017). The animals receiving dose of AFB1 together with curcumin had greater expression of SOD gene compared to those receiving AFB1 dose alone, an indication of ameliorative effects of curcumin in improving the antioxidant status in aflatoxin fed mice. Similarly, the expression of the GPx gene was decreased in mice fed on diet containing AFB1 (Group III) compared to the Control. Such a decrease in GPx expression may result in impaired conversion of hydrogen peroxide to water and thus maintaining hydroxyl radicals within hepatocytes (Buchau and Gallo, 2007). Decreased expression of GPx due to AFB1 was normalized in animals containing in group IV, receiving curcumin in the diet (Group III). The addition of curcumin seems to support the role of GPx in protecting against high cellular concentrations of hydrogen peroxide (Nouri *et al.*, 2015). Supplementation of curcumin, however, was not completely protective against the toxic effects of AFB1 since catalase levels remained low in these mice compared to controls. Like other genes, the expression of Glutathione S-transferase (GST) was decreased in mice exposed to AFB1 in their diet and potentially it was due to the ability of hepatic tissue to conjugate reactive metabolites1 . As a result, curcumin in the diet alleviated the negative effect of AFB1 on GST expression. Studies have established that the curcumin reduced iron-induced hepatic damage, AFB1 induced mutagenicity and hepatocarcinogenecity by inhibiting cytochrome P450 in the liver and also induction of antioxidant enzymes (Ahmeda *et al.*, 2014). The expression of Epoxide hydrolase (EH), GST and Cytochrome P450genes show that there was an increase in the expression of hepaticCYP1A1 and CYP2H1 genes in the albino mice fed on diet containing AFB1. The gene

CYP450 isoforms have previously been shown to be overexpressed, leading to hepatocellular injury and inducing death through chronic oxidative stress, excess ROS and transformation of aflatoxin B1 to toxic metabolite aflatoxin (Jesuthasan *et al.*, 2005). This toxic metabolite is produced from the oxidation of AFB1 by the CYP450 isoforms reactive intermediates, AFB1 -8,9-epoxide (AFBO), and aflatoxin M1 (aflatoxin M1) The fact that, in the present study, CYP450 isoform genes were up-regulated and epoxide hydrolase and GST genes were down-regulated in AFB1receiving group, compared to the control suggests that there is a greater chance of the formation of more toxic intermediate metabolites such as aflatoxin 8, 9 epoxide. Furthermore, down-regulation of these detoxification genes could reduce the ability of the mice to detoxify AFB1 which could lead to various toxicological effects. The present study highlights the antioxidant role of curcumin in different parameters investigated and it is linked to decrease CYP1A gene expression compared to mice fed on AFB1. It further suggests chemo protective action against the negative effects of aflatoxin B1 (Haas *et al.*, 2006) .

There are important interactions among the activities of several antioxidant enzymes and various ROS and cellular reactions, all of which could be responsible for some of the observations in the present study. The decrease in the expression and activity of SOD, GST and GPx observed in the present study are additive with respect to oxidative damage. On-enzymatic decomposition of hydrogen peroxide involving transition metals, such as iron, in a Fenton-type reaction can be more damaging to the cell than the production of the hydroxyl radical species (Giray *et al.*, 2008). Furthermore, increased levels of hydrogen peroxide within the cells reduce SOD activity (Mathuria *et al.*,2007), thereby increasing superoxide levels within the cell and reducing catalase activity. It is evident from the results of the present study that transcriptional activation ofCYP1A1 and CYP2H1 isoforms, in response to aflatoxin has the potential to increase oxidative stress. Also, these CYP 450 isoforms are involved in biotransformation of aflatoxin B1 to the highly toxic metabolite aflatoxin 8,9 epoxide (Buchau and Gallo,2007). CYP 450 isoforms oxidize Aflatoxin B1into two metabolites: the reactive intermediate, 71Aflatoxin B1 -8,9-epoxide (AFBO), and aflatoxin M1 (AFM1). Because of the importance of AFBO and AFM1 in the toxicity of AFB1, CYP450 isoforms play an important role in the well-known hypersensitivity of mice to Aflatoxin B1 (Magnoli *et al.*, 2011). Since genes coding for CYP 450 isoforms were up regulated and epoxide hydrolase and GST genes were downregulated in aflatoxin fed mice compared to controls in the present study, there is a greater chance for formation of more toxic intermediate metabolites such as aflatoxin 8, 9 epoxide. Furthermore, down-regulation of these detoxification genes could reduce the mice ability to detoxify Aflatoxin B1 which could lead to various toxicological effects. The inclusion of butylated hydroxyl toluene, an antioxidant in the diet has any chemo protective effects in Aflatoxin B1 fed mice (Osawa, 2007). "They observed decreased activity of hepatic microsomal CYP1A as well as conversion of Aflatoxin B1 to the putative toxic metabolite, AFB1-8,9-epoxide (AFBO), compared to controls (Matur *et al.*,2007). Similar to the above findings, the antioxidant, curcumin in the current study decreased CYP1A gene expression compared to mice fed Aflatoxin B1 suggesting chemo protective effects .

5. Conclusion

The above findings demonstrate the possible mode of action of curcumin as an antioxidant, and the results obtained in the present study suggests that curcumin present in TMP gave partial protection against aflatoxicosis. Results also suggest that curcumin may need to be supplemented at levels higher than 74 mg/kg in order to achieve optimum protection against 1.0 ppm AFB1. These results suggest that mice fed AFB1 had impaired antioxidant activities along with decreased growth, development, and detoxification mechanisms, making them susceptible to various other stressors. Furthermore, addition of TMP containing curcumin to the AFB1-contaminated diet partially protected mice against AFB1.

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