

## Full Paper

### **Ameliorative effects of bradykinin potentiating factor, butylated hydroxy toluene and oltipraz on biochemical and histological structure of lymphoid organs in aflatoxicated female rats**

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#### **Abstract:**

Aflatoxins (AFs) are chemically secondary metabolites produced by *Aspergillus*, *Penicillium* and *Fusarium* genera. It has adverse effects on humans and animals health due to inhibition of macromolecule synthesis. The current research investigate the pathological and biochemical changes in lymph follicles of female rats exposed to aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) for one month and the efficacy of isolated bradykinin potentiating factor (BPF) from cobra snake venom, butylated hydroxy toluene (BHT) and oltipraz (OPZ) to ameliorate those changes. Aflatoxicosis cause significant increase of lipid peroxidation (LPO) and nitric oxide (NO) in addition to significant decrease in the level of total thiols, glutathione (GSH) and the activities of glutathione peroxidase (GPx) and glutathione S-transferase (GST). Moreover, AFB<sub>1</sub> caused

histopathological changes in lymph follicles represented by depletion of the lymphoid cells. Treatment of aflatoxicosed rats with BPF, BHT or OPZ resulting in amelioration of the oxidative stress markers and improvement in the histological structure of lymph follicles represented by increase of lymphoid cell population with presence of mast cells and collagen bundle. In conclusion BPF, BHT or OPZ ameliorate the aflatoxicosis with priority for the BPF.

**Key Words:** lymph follicles, aflatoxin B<sub>1</sub>, oxidative stress, butylated hydroxy toluene, bradykinin potentiating factor, oltipraz.

### 1- Introduction:

Aflatoxins (AFs) are chemically secondary metabolites produced by *Aspergillus*, *Penicillium* and *Fusarium* genera which causes immunosuppression, genotoxicity, carcinogenic and teratogenic effects on humans and animals health due to inhibition of macromolecule synthesis [1,2]. Also, AFs deplete the cell populations of the lymph organs and decreases the concentrations of immunoglobulins and phagocytic activity [3,4]. Several kinds of animal venom from spiders, snakes, cone snails, scorpions contain short polypeptide such as bradykinin-potentiating peptides and natriuretic peptides [5,6]. The Egyptian cobra (*Naja haje haje*) contains a number of low molecular weight basic polypeptides has low toxicity and distinct immunochemical properties [7]. Moreover, BPF extracted from *scorpion* venom *Buthus occitanus* have antioxidants, anti-inflammatory, immunomodulatory effects that improve cellular growth of the uterus

and ovarian follicle in mice and healing of guinea skin burned [8-11]. Also, BPF generates thymus and spleen cellularity promoted vascular permeability and mitogenesis in irradiated guinea pigs [12-14].

Oltipraz (OPZ) [4-methyl-5-(2-pyrazinyl)-1,2-dithiole-3-thione] as a cancer chemopreventive drug due to enhancement of the antioxidant response element binding activity of NFE<sub>2</sub> related factor-2 and the induction of the glutathione S-transferase (GST) A<sub>2</sub> gene in cirrhotic liver [15,16]. High doses OPZ may increase the production of its oxidized metabolites via extensive metabolism and made changes in target gene transactivation [17].

Butylated hydroxy toluene (BHT) is an antioxidant phenolic acid which used as additive in polymers such as foamed plastic, food grade plastics, natural and synthetic rubbers [18]. BHT protect against oxidative stress, cancer, HIV and cardiovascular diseases [19,20].

From the for mentioned the current research was planned to investigate the pathological and biochemical changes in lymph follicles of female rats exposed to AFB<sub>1</sub> for one month and the efficacy of isolated BPF from the cobra snake venom, the BHT and OPZ to ameliorate those changes.

## **2- Materials and methods:**

### **2.1- Chemicals used:**

Butylated hydroxy toluene (W218405), oltipraz (O9389), thiobarbutric acid (T5500), naphthylethylene diamine dihydrochloride (N9125), 5,5- dithiobis-2-nitrobenzoic acid (D8130), sulphanilamide (S9251) and coomassie brilliant, blue R stain (B0149) were purchased from Sigma-Aldrich Company (St. Louis, MO), USA.

### **2.2- Extraction, purification and identification of AFB<sub>1</sub>:**

AFB<sub>1</sub> was extracted from harvested media of growing *Aspergillus flavus* according to the method of Booth [21], purified by TLC method of El- Kady and Moubasher [22].

### **2.3- Extraction, purification and identification of BPF:**

The collected amount of the Egyptian cobra snake crude venom was dialyzed according to the method of Abdel-Raheim *et al.* [11]. Isolation and purification of

BPF from the dialyzed performed by the chemical method of Ferreira [23]. Isolated BPF was identified by its hypotensive effect on arterial blood pressure of rabbit by oscillograph in the Central Lab of Zoology Department, Faculty of Science, Assiut University. The amino acid content of the isolated BPF was analyzed by amino acid analyzer SW (Hydrolisate Separation Method) at Desert Research Center, Ministry of Agriculture and Land Reclamation, Cairo, Egypt.

### **2.4- Experimental animals:**

In this study, 120 female Wistar albino rats were used and obtained from the Animal House of the Faculty of Medicine, Assiut University, Assiut, Egypt with mean body weight of 130±10 gm. Rats were housed in cages, kept at room temperature with normal 12h light/12h dark cycle and supplemented with standard commercial pellets for feeding, water, *ad libitum*. All of the animal procedures were performed in accordance with the guidelines for the care and use of experimental animals established by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the protocol of the National Institutes of Health (NIH) [24].

#### 2.4.1- Design of the experiment:

Rats were divided randomly into 6 groups of 20 rats each as follows:

**G I:** Reference normal group.

**G II<sub>0</sub>:** Intoxicated group treated with AFB<sub>1</sub> for one month.

**G II<sub>1</sub>:** Self- recovery for the same period after intoxication.

**G II<sub>2</sub>:** Treated with BPF for the same period after intoxication.

**G II<sub>3</sub>:** Treated with BHT for the same period after intoxication.

**G II<sub>4</sub>:** Treated with OPZ for the same period after intoxication.

#### 2.4.2- Administration protocol of intoxicating solutions and the other treating agents:

The animals of GII<sub>0</sub> were individually intoxicated by oral administration three times weekly (500µg, AFB<sub>1</sub> suspended in milk/kg b.wt) for one month according to Raisuddin *et al.* [25]. GII<sub>1</sub> animals after intoxication were left without treatment for self-recovery. GII<sub>2</sub> intoxicated animals were treated by intraperitoneally three doses weekly for one month (1µg BPF in PBS, pH 7.4/ kg b.wt) according to Omar and Meki [26]. GII<sub>3</sub> intoxicated animals were orally treated by 0.5mg BHT in PBS, pH 7.4/ kg b.wt three times weekly for one month according to Hocman [27]. Similarly to GII<sub>3</sub>, the animals of GII<sub>4</sub> were orally administrated by OPZ (2mg in PBS,

pH 7.4)/ kg b.wt) according to Dimitrov *et al.* [28].

#### 2.4.3- Collection and preparation of the samples for biochemical determination and histopathological examinations:

At the end time of the experiment, blood samples were collected from the heart under an aesthesia by ether and sacrificed by cervical dislocation. At time of scarifying, the blood was collected in tubes and centrifuged after blood clotting at 4,000 rpm for 10 min to separate serum. Spleen, thymus and axillary lymph node (ALN) was quickly removed, washed with saline solution and small slices were fixed in glutaraldehyde for electron microscopic examinations and the remnant was imbedded in liquid nitrogen and kept frozen at -80 for biochemical studies. 10% w/v homogenate of ALN in 0.1M phosphate buffer (pH 7.4) was prepared then the homogenate was preserved at -20<sup>0</sup>C for the subsequent biochemical indices.

#### 2.5- Biochemical determinations:

Total protein content in the supernatant of ALN tissue homogenates was performed according to the method of Lowry *et al.* [29]. Alanine aminotransferase (ALT) activity in serum was determined by ALT-Liquizyme (4+1) E.C.2.6.1.2 kit which purchased from Egyptian Company for

Biotechnology. Nitric oxide (NO) was determined by Gries reagent according to Ding [30]. Lipid peroxidation as MDA was evaluated according to the method of Wills [31]. Total thiols content was assayed according to the method of Ellman [32]. Glutathione (GSH) was estimated according to the method of Beutler *et al.* [33]. The activity of GSH-peroxidase (GPx) and transeferase (GST) were assayed according to Habig *et al.* [34].

### 2.7- Histopathological features:

Semi-thin section and transmission electron microscope observations were done in Electron Microscope Unit (E.M.U) of Assiut University according to Bozzola and Russell [35].

### 2.8- Statistical analysis:

Statistical differences of parameters are presented as Mean  $\pm$  SEM, statistical significance was determined using the one way ANOVA test in Graph Pad Prism 5.03 at  $P < 0.05$  where <sup>a</sup> is a significant of aflatoxicosed GII<sub>0</sub> with respect to reference normal GI, <sup>b</sup> is a significant of treated groups GII<sub>1, 2, 3, 4</sub> with respect to reference normal GI and <sup>c</sup> is a significant of treated groups GII<sub>1, 2, 3, 4</sub> with respect to aflatoxicosed GII<sub>0</sub>.

## 3-Results

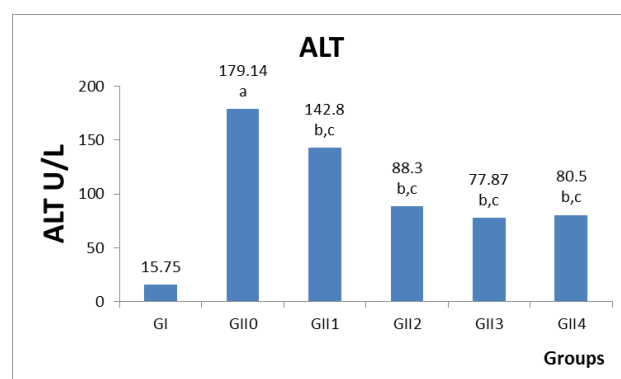
### 3.1- Identification and characterization of AFB<sub>1</sub> and isolated BPF from Cobra snake venom:

The results were identified and data not shown in the current paper (submitted and under revision in the journal of applied animal research (JAAR-2020-0109)) [36].

### 3.2- Biochemical indices:

#### 3.2.1- ALT activity (U/L) in serum of experimental groups

ALT in serum was significantly increased in the intoxicated rats (GII<sub>0</sub>) in comparison to the normal (GI) and still elevated in the self-recovery group (GII<sub>1</sub>). However, in treated rats with BPF (GII<sub>2</sub>), BHT (GII<sub>3</sub>) and OPZ (GII<sub>4</sub>), the levels were decreased but still higher than normal (GI) as shown in Fig (1).



**Fig (1):** ALT activity in serum of experimental groups.

Data presented as M  $\pm$  SEM, <sup>a</sup> is a significant of aflatoxicosed (GII<sub>0</sub>, GII<sub>00</sub>) with respect to reference normal GI, <sup>b</sup> is a significant of treated groups GII<sub>1, 2, 3, 4</sub> with respect to reference normal GI and <sup>c</sup> is a significant of treated groups GII<sub>1, 2, 3, 4</sub> with respect to aflatoxicosed GII<sub>0</sub>.

### 3.2.2- Oxidative stress markers in ALN

#### tissue

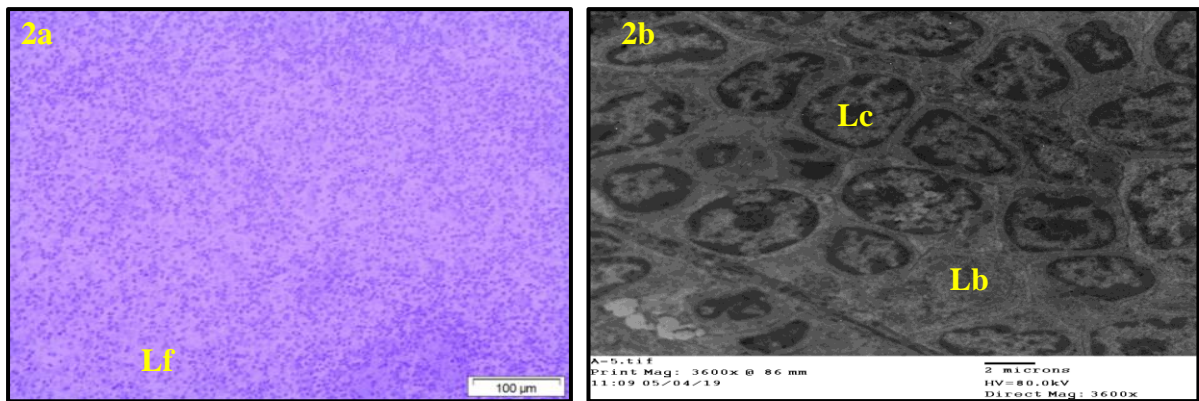
LPO as MDA, NO in ALN tissue were significantly increased in rats that treated with AFB<sub>1</sub> for one month (GII<sub>0</sub>) in comparison to the control group (GI) and still elevated in the recovery group (GII<sub>1</sub>), however, in the groups co-treated with BPF (GII<sub>2</sub>), BHT (GII<sub>3</sub>) and OPZ (GII<sub>4</sub>) was similar to the control one. Total thiols and GSH content were significantly reduced in the ALN tissue of rats that treated with AFB<sub>1</sub> for one month (GII<sub>0</sub>) and the recovery (GII<sub>1</sub>) group in comparison with control one (GI), however, in groups of rats that co-treated with BPF (GII<sub>2</sub>), BHT (GII<sub>3</sub>) and OPZ (GII<sub>4</sub>) were increased but still lower than control group. The enzymatic activities of GPx and GST were significantly depleted in the ALN tissue of rats that treated with AFB<sub>1</sub> for one month (GII<sub>0</sub>) and the recovery (GII<sub>1</sub>) group in comparison with control one (GI), however, in groups of rats that co-treated with BPF (GII<sub>2</sub>), BHT (GII<sub>3</sub>) and OPZ (GII<sub>4</sub>) were increased but still lower than control group as shown in Table (1).

**Table (1):** Oxidative stress markers in ALN tissue of the tested groups (Mean± SEM)

| Groups Parameters              | GI                | GII <sub>0</sub>               | GII <sub>1</sub>                 | GII <sub>2</sub>                 | GII <sub>3</sub>                 | GII <sub>4</sub>                 |
|--------------------------------|-------------------|--------------------------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
| MDA (nmol/ mg protein)         | 3.41<br>±<br>0.03 | 4.68<br>±<br>0.04 <sup>a</sup> | 4.67<br>±<br>0.06 <sup>b,c</sup> | 3.84<br>±<br>0.06 <sup>b,c</sup> | 3.75<br>±<br>0.09 <sup>b,c</sup> | 3.85<br>±<br>0.07 <sup>b,c</sup> |
| NO (nmol/ mg protein)          | 31.6<br>±<br>0.65 | 47.8<br>±<br>0.53 <sup>a</sup> | 44.7<br>±<br>1.08 <sup>b,c</sup> | 36.4<br>±<br>1.52 <sup>b,c</sup> | 34.9<br>±<br>1.06 <sup>b,c</sup> | 35.8<br>±<br>1.05 <sup>b,c</sup> |
| Total thiols (nmol/mg protein) | 30.8<br>±<br>0.03 | 23.4<br>±<br>0.04 <sup>a</sup> | 24.8<br>±<br>0.06 <sup>b,c</sup> | 26.3<br>±<br>0.54 <sup>b,c</sup> | 27.2<br>±<br>0.61 <sup>b,c</sup> | 26.9<br>±<br>0.52 <sup>b,c</sup> |
| GSH (nmol/ mg protein)         | 14.2<br>±<br>0.50 | 12.7<br>±<br>0.29 <sup>a</sup> | 12.9<br>±<br>0.38 <sup>b,c</sup> | 13.1<br>±<br>0.35 <sup>b,c</sup> | 13.7<br>±<br>0.41 <sup>b,c</sup> | 13.5<br>±<br>0.23 <sup>b,c</sup> |
| GPx (nmol/min/ mg protein)     | 22.2<br>±<br>1.22 | 12.8<br>±<br>1.07 <sup>a</sup> | 14.6<br>±<br>2.20 <sup>b,c</sup> | 16.4<br>±<br>1.35 <sup>b,c</sup> | 17.4<br>±<br>2.79 <sup>b,c</sup> | 19.8<br>±<br>1.81 <sup>b,c</sup> |
| GST (nmol/ mg protein)         | 1.35<br>±<br>0.05 | 0.59<br>±<br>0.16 <sup>a</sup> | 0.65<br>±<br>0.05 <sup>b,c</sup> | 0.89<br>±<br>0.05 <sup>b,c</sup> | 0.86<br>±<br>0.07 <sup>b,c</sup> | 1.06<br>±<br>0.09 <sup>b,c</sup> |

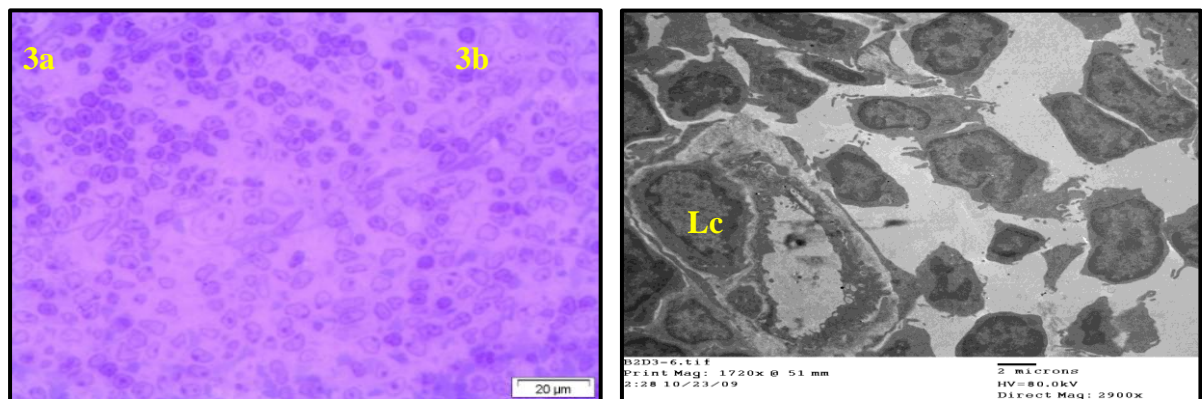
Lipid peroxidation as malondialdehyde (MDA), Nitric oxide (NO) and Glutathione (GSH) and the activities of glutathione peroxidase (GPx) and transeferase (GST).<sup>a</sup> is a significant difference between aflatoxicosed and control group, <sup>b</sup> is a significant difference between treated groups and control group and <sup>c</sup> is a significant difference between treated groups and aflatoxicosed group.

### 3.3- Histopathological examinations:



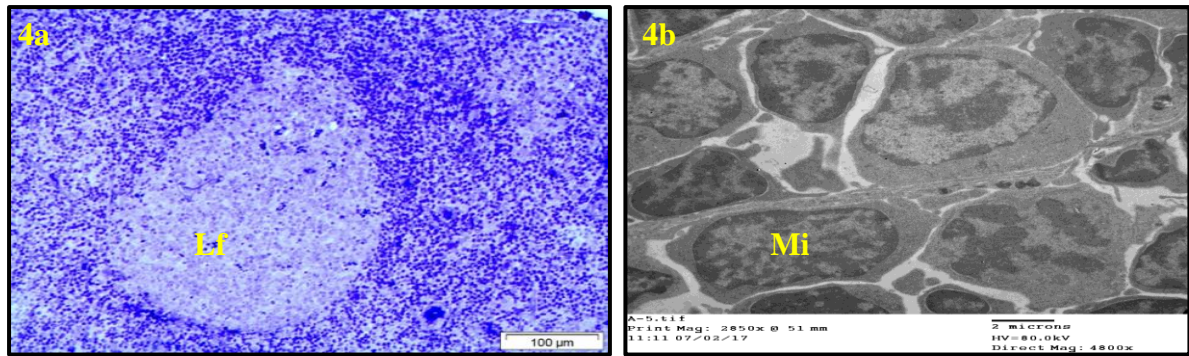
**Fig 2a:** Light micrograph of semi thin section of lymph follicles of normal rats (GI) showing normal population of lymphoid cells of lymph follicles (Lf). Toluidine blue T.B. stain.

**Fig 2b:** T.E. micrograph of the lymphoid follicle of normal rats (GI) showing lymphoid tissue formed by lymphoblasts (Lb) and lymphocytes (Lc).



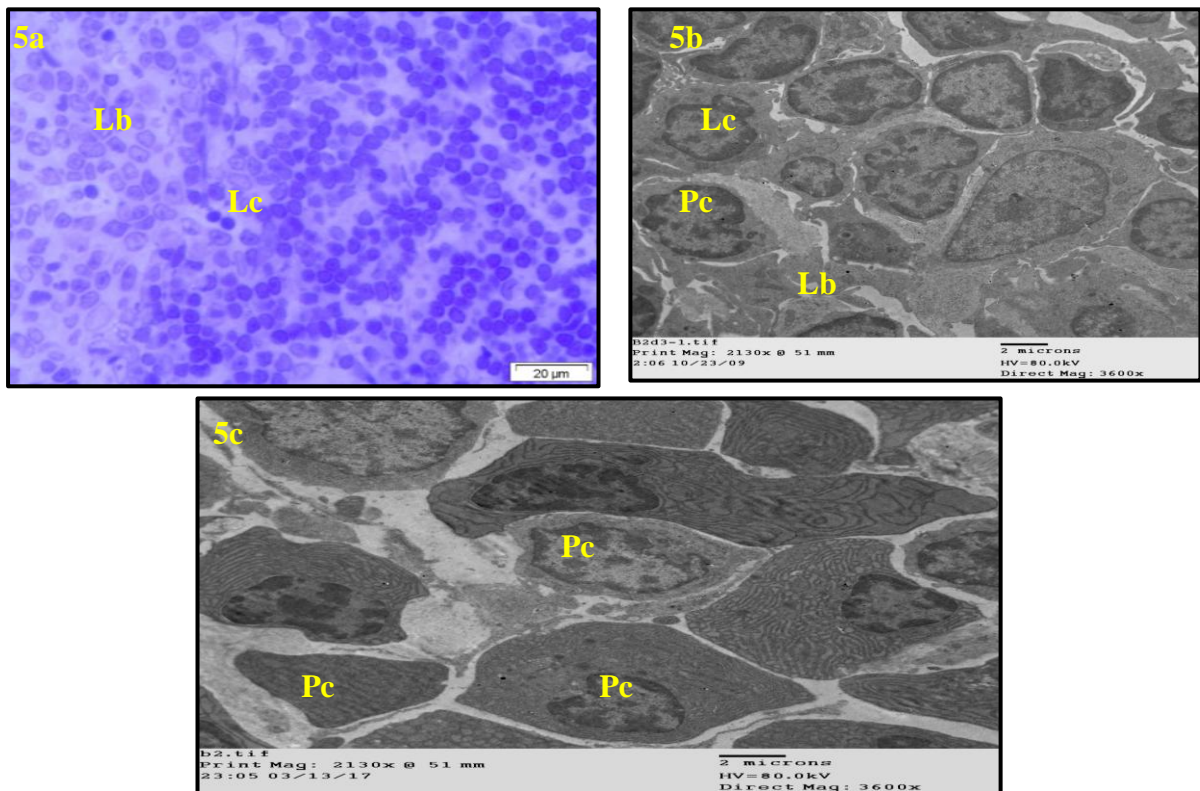
**Fig 3a:** Light micrograph of lymph follicles of rats treated with AFB<sub>1</sub> for one month (GII<sub>0</sub>) showing marked depletion of the lymphoid cells in the lymph follicles. T.B. Stain

**Fig 3b:** T.E. micrograph of lymph follicles of rats treated with AFB<sub>1</sub> for one month (GII<sub>0</sub>) showing marked depletion with prominent decreases of the lymphoid cells (Lc).



**Fig 4a:** Light micrograph of lymph follicles of rats left for self-recovery for one month (GII<sub>1</sub>) showing proliferation of lymphoid cells in the lymph follicle (Lf).T.B. stain.

**Fig 4b:** T.E. micrograph of lymph follicle of rats left for self-recovery for one month (GII<sub>1</sub>) showing lymphoblast in state of mitosis (Mi).

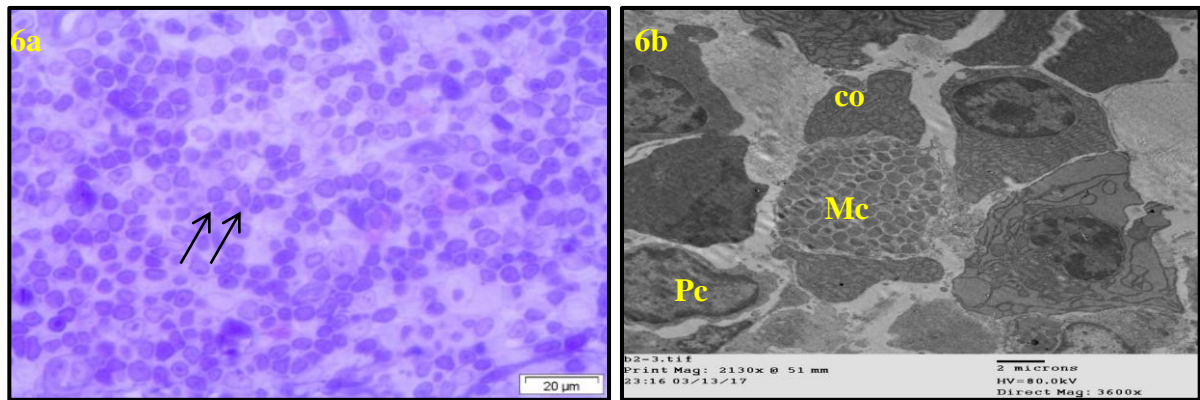


**Fig 5a:** Light micrograph of lymph follicle of rats treated with AFB<sub>1</sub> for one month then treated with BPF for one month (GII<sub>2</sub>) showing presence of large number of lymphocyte (Lc) and lymphoblast's (Lb). T.B. stain.

**Fig 5b:** T.E. micrograph of lymphoid tissue of rats treated with AFB<sub>1</sub> for one month then treated with BPF for one month (GII<sub>2</sub>) showing presence of large number of lymphocyte (Lc) and lymphoblast's (Lb) as well as plasma cells (Pc).

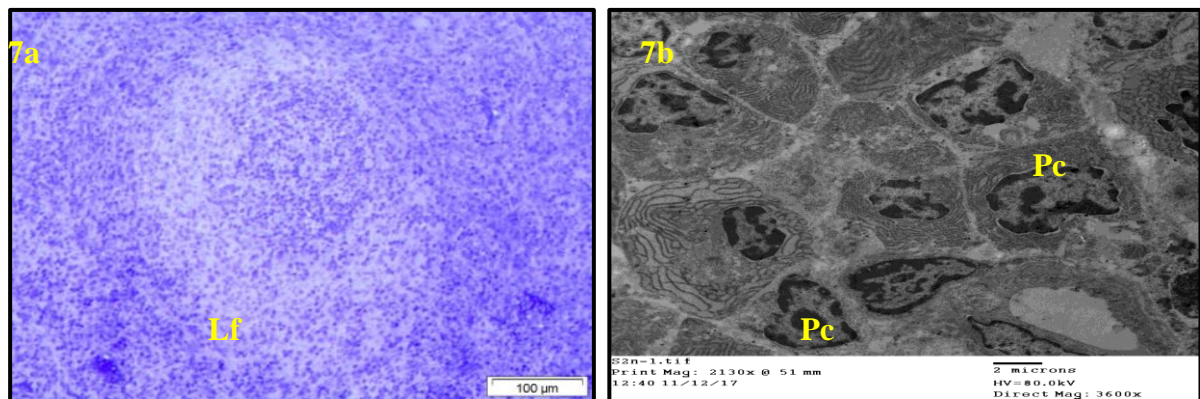
**Fig 5c:** T.E. micrograph of lymph follicles of rats treated with AFB<sub>1</sub> for one month then treated with BPF for one month (GII<sub>2</sub>) showing presence of large number of plasma cells (Pc).





**Fig 6a:** Light micrograph of lymph follicles of rats treated with AFB<sub>1</sub> then treated with BHT (GII<sub>3</sub>) for one month showing increase lymphoid cell population with presence of mast cells (arrow). T.B.stain.

**Fig 6b:** T.E. micrograph of lymphoid tissue of rats treated with AFB<sub>1</sub> then treated with BHT (GII<sub>3</sub>) for one month showing presence of plasma cells (Pc), mast cell (Mc) and collagen bundle (co).



**Fig 7a:** Light micrograph of lymph follicles of rats treated with AFB<sub>1</sub> then treated with OPZ (GII<sub>4</sub>) for one month showing lymph follicle (Lf) of normal morphological appearance. T.B.stain.

**Fig 7b:** T.E. micrograph of lymphoid tissue of rats treated with AFB<sub>1</sub> then treated with OPZ (GII<sub>4</sub>) for one month showing presence of numerous plasma cells (Pc).

#### 4- Discussion

ALT is commonly measured liver function test and used as a way of screening for liver damage. ALT activity was significantly increased in AFB<sub>1</sub> intoxicated rats (GII<sub>0</sub>) in comparison to control one (GI) and still elevated in the self-recovery

(GII<sub>1</sub>), however, in treated rats with BPF (GII<sub>2</sub>), BHT (GII<sub>3</sub>) and OPZ (GII<sub>4</sub>), the levels were elevated to those of normal (GI) as in Fig (1). These results agreed with who reported that these changes represent the initial protective response of the mitochondria against the aflatoxin toxicity and the increased activity of these enzymes provided evidence for their

release from damaged hepatocytes [37]. In the present study, the reduction of serum ALT activity after treatment with BPF, BHT and OPZ suggested healing of hepatic parenchymal cells and regeneration of hepatocytes [38-40]. In the current study, the authors found a significant increase in the levels of LPO as MDA, NO in aflatoxicosed rats and a significant decrease in the levels of total thiols, GSH and the activities of GPx and GST. AFs transformed *in vivo* into active metabolites which inhibits RNA and protein synthesis leading to change in cell cycles, impaired proliferation, differentiation of immune cells and promotes inflammatory responses by regulating cytokines expression [41, 42]. AFs affected cellular and humoral immune reactions and caused immunosuppression [43]. As well as AFs depresses the T-cell dependent functions of splenic lymphocytes and affects the lymphoid follicles result in depleting the lymphocytes [44]. In the present study, lymph follicles of intoxicated rats with AFB<sub>1</sub> showed marked depletion with prominent decreases of the lymphoid cells, moreover, lymph follicles of self-recovery rats showed lymphoblast in state of mitosis. All the previous changes by AFB<sub>1</sub> may return to elevation of LPO which is considered a key event in cellular damage [45]. Treatment of aflatoxicosed rats with

BPF improved the oxidative stress parameters and the histological changes such as presence of large number of lymphocyte and lymphoblast's as well as plasma cells.

In this aspect, BPF stimulated the release of prostaglandin and cytokines that important in reconstituting the hematopoietic organs and increases immunoglobulin production [26, 46]. Moreover, the authors found increase in hepatic IL-1 $\beta$  gene expression (data not shown) which is known enhance the synthesis of prostaglandin E<sub>2</sub> via the induction of phospholipase A<sub>2</sub> and cyclo-oxygenase activities by potentiation the response to BK [47]. In the present study, treatment of aflatoxicosed rats with BHT improved the oxidative stress parameters and the histological changes in lymph follicles such as increase lymphoid cell population with presence of mast cells, plasma cells and collagen bundle could be return to the increased of GST activity which is important for the detoxification process [48,49]. Moreover, BHT as antioxidants transfer immunoprotective effects during aflatoxicosis that increase immune sensitivity and/or the activation of NK cells which related to the alleviation of lymphedema [50,51]. In the current study, treatment of aflatoxicosed rats with OPZ enriched the oxidative stress parameters

and the histological changes such as presence of lymphoid cells with numerous plasma cells and showed normal morphological appearance. In this aspect, Velayutham *et al.* [52] suggested that OPZ enhanced phase 2 enzyme induction via Nrf2 activation and inhibit CYP<sub>1A2</sub> and CYP<sub>3A4</sub>, the principal enzymes involved in AFB<sub>1</sub> detoxification, especially GSTs. OPZ have anti-inflammatory effects that provoke the induction of phase II detoxification enzymes and enhancer of wound healing and fibroblast proliferation [53].

From the previous discussion we conclude that AFB<sub>1</sub> induced biochemical and pathological changes in lymphoid tissue and any of the BPF, BHT and OPZ have the capacity to ameliorate those biochemical and pathological changes with the priority for the BPF.

No conflict between the authors; the authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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