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Assessment of the Ameliorative Effect of *Bacillus subtilis* against the Toxicity Induced by Aflatoxin B_1 in Rats

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Abstract

Aflatoxin B_1 (AFB_1) is the most toxic and common among the major types of aflatoxins. AFB_1 is hepatoxic and has been implicated in an increase risk of hepatocellular carcinoma, also the effect of AFs on the brain chemistry have been documented. Probiotics have health benefits and provide of powerful benefits for the body and brain. The present study was designed to reveal efficiency of *Bacillus subtilis* NS4182-01 against the hematological, biochemical and histopathological alterations induced by AFB_1 in rats. Both of AFB_1 and *B. subtilis* were studied on the experimental animals (rats) that were divided into 6 groups as the following: Group 1 (control) received distilled water orally. Groups 2&3 (*B. subtilis* treated groups) received *B. subtilis* orally at a dose of 0.25 and 0.50 ml, respectively. Group 4 (AFB_1 treated group) orally treated with AFB_1 at a dose of 2 mg/kg b.w. Groups 5&6 (AFB_1 + *B. subtilis* group) orally treated with AFB_1 and then treated with *B. subtilis* at two studied doses respectively. It was noticed that the hematological measurements declined and the most biochemical measurements elevated significantly ($P \leq 0.05$) in AFB_1 treated group. *B. subtilis* restored all studied measurements towards the normal values. Moreover, the native electrophoretic protein patterns showed that the physiological alterations occurred in the native protein and lipid moiety in addition to calcium moiety of native protein patterns as a result of AFB_1 treatment were represented by hiding one or more of normal protein types with existence of abnormal ones. Therefore, the similarity index (SI%) and genetic distance (GD%) values were altered with protein (SI=40.00%; GD=60.00%), lipid moiety (SI=50.00%; GD=50.00%) and calcium moiety of native protein patterns (SI=75.00%; GD=25.00%) in AFB_1 treated group. Although the treatment with *B. subtilis* at a dose of 0.25 ml showed ameliorative effect but could not restore the physiological state of the patterns to normalcy. While *B. subtilis* at a dose of 0.50 ml restored integrity of these native protein patterns by restoring the absent types with hiding the abnormal ones. Therefore, this group became physiologically similar to control group (SI=100.00%; GD=0.00%). These findings were supported by results of the histopathological examination in the most target organs (liver, kidney and brain) that were affected by AFB_1 and the *B. subtilis* restored their histopathological integrity to normal structure and maintained architecture of these organs.

Keywords: Aflatoxin B_1, *Bacillus subtilis*, Toxicity, Liver, Kidney, Brain, Electrophoresis, Rats.

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1. Introduction

World is facing a drastic problem regarding many of the diseases induced by food/feed stuff contamination that can be caused at any stage of production and storage due to as a result of unsuitable storage of food and foodstuff **[1]**. Fungi belong to the basic source of food contamination due to their ability to produce mycotoxins that are considered as toxic metabolites **[2]**. Aflatoxins (AFs) are the most common secondary fungal (toxic) metabolites that synthesized particularly by *Aspergillus parasiticus* and *Aspergillus flavus* that have a wide host ranges **[3-5]**. They mostly contaminate grains and nuts in tropical regions during pre or post-harvest conditions. Moreover, they contaminate not only food stuffs but also milk, eggs and edible tissues when farm animals consume contaminated feed **[6]**. Aflatoxin B_1 (AFB_1) in addition to three structurally similar compounds namely aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and Aflatoxin G_2 (AFG_2) belong to the naturally occurring AFs that able to contaminate food significantly. AFB_1 is the most hazardous and toxic mycotoxins for humans and livestock. It exhibits hepatotoxic, teratogenic, immunosuppressive, cytotoxic, genotoxic and carcinogenic effects on both human and animals **[7-12]**. The liver is its key target organ **[13-16]**.

After entering the body, AFB_1 is ingested and absorbed inside the body by intestine then carried to the liver **[17]**. In the liver, AFB_1 is first metabolized and biotransformed mainly by the microsomal cytochrome P-450 enzyme (CYP450) to a variety of highly reactive intermediates such as AFB_1 epoxide and hydroxylated metabolites (AFM1, AFP1, AFQ1, AFB 2α and aflatoxicol) **[18]**. These intermediates cause ultimate hepatotoxicity due to their affinity for binding the nucleic acids to form adducts affecting regulation of the functional gene expression to block transcription and translation **[16]**. Carcinogenicity and mutagenicity induced by AFB_1 due to accumulating the reactive oxygen species (ROS) that are considered as precursors of hydroxyl radicals. These reactive species have the affinity to interact with DNA leading to mutations in addition to a reactive epoxide formation at the 8, 9- position of the terminal furan ring and its subsequent covalent binding to nucleic acid **[19,20]**. The metabolized AFB_1 binds to proteins in the liver causing acute toxicity called "aflatoxicosis". Consequently, it leads to acute liver cirrhosis and liver damage, as well as tumors development or other genetic effects **[21]**. The exposure to high AFB_1 doses through diet over a short period that leads to hepatotoxicity is called "acute aflatoxicosis" while the exposure to low AFB_1 doses through diet over a long period that leads to hepatocellular carcinoma is called "chronic aflatoxicosis" **[22]**. It was demonstrated that incidence of human liver cancer occurred highly as a result of the exposure to AFB_1 in many countries. As compared to chronic aflatoxicosis, the acute aflatoxicosis is less common but it occurs occasionally **[23]**.

In the previous literature, it was reported that there are numerous strategies (physical and chemical) for AFs elimination or inactivation **[24]**. Nevertheless, these methods have some limitations such as losses in the nutritional value of the product, organoleptic qualities and the palatability of feeds, as well as high cost of the equipment required to implement these techniques in addition to the undesirable health effects, while none of these strategies is able or sufficient to fulfill the necessary safety, efficacy and cost requirements completely **[25]**. Recent developments in the field of nanotechnology have been made in the use of silver nanoparticles (Ag-NPs) as a novel therapeutic agent as an anti-inflammatory, antibacterial, antifungal, antiviral and anti-cancer agent **[26-28]**. The researchers have focused on utilizing microorganisms and / or the enzymatic preparations for the biological detoxification of mycotoxins due to their simplicity and affordability **[29]**. Although some microbes (including fungal and bacterial isolates) such as *Armillariella tabescens* **[30]**, *Rhodococcus erythropolis* **[25]** and *Myxococcus fulvus* **[31]** have been reported to possess various abilities regarding the degradation of AFs, most of these microorganisms are not officially allowed to be applied to foods or feeds under the US Food and Drug Administration (FDA) and the Association of American Feed Control Officials (AAFCO). Bacteria have more application for AFB_1 remediation and this attributed to their ability to eliminate AFB_1 producing non pigments as well as within shorter time **[32]**. Probiotics are the microorganisms that confer a health benefit on the host in addition to their vital role in maintaining well-being when they administered in

sufficient quantities **[33]**. The components in surface of probiotic bacteria have the affinity to bind with AFB_1. Therefore, they can be intervened to reduce the toxicity induced by AFB_1 **[34]**.

Bacillus subtilis is a gram-positive bacteria distributed widely in the environment. It is isolated and cultured easily and it is able to produce various biologically active compounds that have received much attention as a biological agent **[35]**. Among the hundred known Bacillus spp., (*B. subtilis*) have been approached as probiotics for human consumption **[36,37]**. A strain of *B. subtilis* ANSB060 that screened from fish gut is characterized by its nutritional and pharmaceutical benefits **[38]**. It is considered as appropriate due to its strong ability for AFB_1 detoxification **[39-41]**. In 2017, **Zhang *et al.* [42]** postulated that this bacterial strain increased activity of the antioxidant enzymes and reduced the AFB_1 concentration in the liver from 0.12 to 0.06 µg/kg. Combination of two strains of *B. subtilis* (ANSB060 and ANSB01G) reduced amount of the bacteria needed to neutralize AFB_1 **[43]**. Objective of the present study was designed to investigate efficiency of *B. subtilis* NS4182-01 against the hematological, biochemical and histopathological alterations induced by AFB_1 in rats.

2. Materials and Methods

2.1. Preparation of Bacterial Strains

Spores of Bacilli strain (*Bacillus subtilis* NS4182-01) were cultured at 37 °C on Trypticase soy broth (TSB) medium for preparing broth with a concentration to 10^{12} bacteria / ml. The viable bacteria were counted by both flow cytometry (FCM) and traditional plate counting methods. Bacterial counts were expressed as colony-forming units (CFU) per ml media **[44]**.

2.2. Animals and Treatments

Healthy thirty six (36) *Sprague Dawley* rats (adult males) with age 2 months old were obtained from the Animal House Colony, National Research Centre, Giza, Egypt. They were divided into 6 groups and housed in cage (filter-top) under controlled normal environmental and nutritional conditions. Rats within different groups were daily treated for 17 days as the following:

Group 1 (Control group): rats were fed with normal diet and received tap water without any treatment. Group 2 (*B. subtilis* 10^{12} bacteria /ml (0.25 ml) treated group): rats were fed with normal diet associated with administration of aqueous *B. subtilis* 10^{12} bacteria /ml distilled water orally at a dose of 0.25 ml by stomach tube. Group 3 (*B. subtilis* (0.5 ml) treated group): rats were fed with normal diet associated with administration of *B.subtilis* 10^{12} bacteria orally at a dose of 0.5 ml by stomach tube. Group 4 (AFB_1 treated group): rats were fed with normal diet associated with orally treatment of AFB_1 at a dose of 2 mg/kg b.w. **[45]**. Group 5 (AFB_1 + *B. subtilis* 10^{12} bacteria / ml (0.25 ml) treated group): rats were fed with normal diet associated with treatment of AFB_1 and administration of *B. subtilis* at a dose of 0.25 ml. Group 6 (AFB_1 + *B. subtilis* 10^{12} bacteria / ml (0.5 ml) treated group): rats were fed with normal diet associated with treatment of AFB_1 and administration of *B. subtilis* 10^{12} bacteria at a dose of 0.5 ml.

2.3. Collection of Blood Samples and Tissues

Rats were anaesthetized at end of the experimental period. For the hematological measurements, the heparinized blood samples were obtained from retro orbital plexus. The blood samples were allowed to clot then centrifuged for 15 min at 4000 rpm and the clear serum were collected for biochemical measurements. Rats were sacrificed by decapitation then liver, kidneys and brain tissues were autopsied and fixed immediately in formal saline (10%) for histopathological examination.

2.4. Hematological Measurements

All hematological measurements including hemoglobin (HB), red blood cells (RBCs), hematocrit (HCT), corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDW), mean platelet volume (MPV) and platelet count (PLT) and white blood cells (WBCs) in addition to their differential blood cells (lymphocytes, monocytes and granulocytes) were quantified in heparinized blood samples using an automatic blood analyzer (ABX Micros 60 manufactured by HORIBA ABX SAS).

2.5. Biochemical Measurements

Activities of liver enzymes (alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH)) and levels of lipid measurements (total

cholesterol (TC) and triglycerides (T.Gs)) were measured in addition to levels of renal functions (urea, creatinine, total protein and albumin) in sera samples by conventional colorimetric methods using spectrophotometer using commercially available kits (Spectrum Diagnostics Egyptian Company for Biotechnology).

2.6. Markers of Oxidative stress

The total antioxidant capacity (TAC), total protein carbonyl content (TPC) and the lipid peroxidation products that were used as markers of the oxidative stress, were spectrophotometrically measured. They were expressed as mM/L **[46]**, nmol of reactive carbonyl compounds per mg protein of tissue **[47]** and nmol/L **[48]**, respectively.

2.7. Statistical Analysis

Data were analyzed statistically using the Statistical Package for Social Sciences (SPSS for windows, version 11.0) by one-way analysis of variance (one-way ANOVA) test followed by Post-Hoc "Bonferroni test". All results were compiled in Tables and illustrated in Figures as mean ± standard error (SE). The differences between the groups were considered statistically significant at a "P" value of less than 0.05.

2.8. Electrophoretic Assays

The samples were prepared by pooling equal volumes of the individual serum samples for each group and used as one pooled sample. In the pooled samples, concentration of total protein was quantified to ensure that the samples loaded in all wells with equal protein concentration. After electrophoretic run, the native proteins were detected by staining the gel in commassie brilliant blue **[49,50]**. The electrophoretically separated proteins appeared as blue bands. The electrophoretic lipid moiety of native protein pattern was detected by staining the gel with Sudan Black B (SBB) and appeared as black bands **[51]**. Electrophoretic calcium moiety of native protein pattern was detected based on the method described by **Abd Elhalim *et al.* [52]** and **Abulyazid *et al.* [53]** using staining solution of alizarin Red 'S' prepared by the method suggested by **Zacharia and Kakati [54]**. The stained calcium moieties appeared as yellow bands.

2.9. Data Analysis

The relative mobility (Rf), band percent (B%) and relative band quantity (Qty%) of the electrophoretically separated bands were determined in addition to illustrating the dendogram by the Quantity One software (Version 4.6.2). The similarity index (SI%) and genetic distance (GD%) molecular weights (Mwts) were calculated by the method suggested by **Nei and Li [55]**.

2.10. Histopathological Examination

The tissues specimens (liver, kidneys and brain) that were collected from all rats and fixed in formal saline (10%) washed with tap water, dehydrated and cleared then embedded in blocks of paraffin wax. The tissue was prepared as sections with thickness 4-5 micron then stained with Haematoxylin and Eosin according to the method suggested by **Suvarna *et al.* [56]** to be examined by light microscope (Olympus BX50, Tokyo, Japan). Histopathological damages were scored and ranged from 0-3. Values of the damage score might be 0 (no damage), 1 (mild), 2 (moderate) or 3 (severe damage), while the grading was determined by the percentage that might be <30% for mild changes, <30% - 50% for moderate changes and >50% for the severe changes **[57]**.

3. Results and Discussion

Hematological Measurements

Hematopoietic system reflects the alterations occurred in animal or human body as a result of the exposure to chemicals, drugs and toxic agents. Therefore, it is considered as body mirror **[58]**. As depicted in **Table 1**, the *B. subtilis* caused no significant changes in all hematological measurements when it was administrated alone at both doses (0.25 and 0.5 ml). As compared to control group, AFB_1 caused significant ($P \leq 0.05$) decline in the hematological measurements that include RBCs, HB, HCT, MCV, RDW, MPV, PLT and WBCs in addition to its differential count (lymph, mono and gran.%). This was in agreement with **Husain *et al.* [59]** who suggested that the decline in the hematological measurements attributed to occurrence of hyperplasia in the bone marrow in addition to dissolution of the red blood cells that consequently leads to induction of the lytic anemia and hence decreasing HB. Furthermore, **Umar *et al.* [60]**

reported that AFB_1 induced macrocytic hypochromic anemia that was confirmed by reduction in RBCs count, HB concentration, MCH and MCHC. This anemia might be attributed to the hemopoietic cellular defects and the hemolytic anemia that occurred by AFB_1 through decreasing the circulating mature erythrocytes and lysis of erythrocytes. Treatment with *B. subtilis* at a dose of 0.25 ml increased all the measurements significantly ($P \leq 0.05$) as compared to AFB_1 treated group but it could not restore them to normal values. *B. subtilis* at a dose of 0.5 ml restored these measurements to normalcy. It elevated their levels significantly ($P \leq 0.05$) as compared to the group treated with AFB_1 alone and that treated with *B. subtilis* at a dose of 0.25 ml. This might be attributed to ability of *B. subtilis* to stimulate the immune system in addition to enhancing the systemic as well as intestinal and respiratory mucosal immune responses **[61]**.

Biochemical Measurements

Liver is considered the primary target organ for the AFB_1 that is accumulated predominantly then metabolized in that tissue after absorption **[62]**. Activity of ALT is considered a gold standard for measuring liver injury **[63]**. It exists in cytoplasm and mitochondria. Therefore, its level increases due to accumulation of the lipids that lead to changes in membrane permeability and hence these enzymes leak out into the cytosol. While ALP is localized within the cell membrane of tissues **[64]**. It was postulated that activities of ALT, AST and ALP were recognized in serum as sensitive serological indicators in hepatic tissues impairment and level of total protein TP used as indicator of protein synthesis **[65]**. Data compiled in **Table 2** showed that no significant alterations were noticed in all biochemical functions as a result of administration of *B. subtilis* alone at both doses. AFB_1 caused a significant ($P \leq 0.05$) elevation in activities of the liver enzymes (ALT, AST, ALP and LDH). This was in agreement with **Eftekhari *et al.* [66]** who revealed that the liver enzymes might increase due to the liver injury that induced by and occurred in association to the hepatobiliary action and tends towards cholestatic induction. In addition, it altered the kidney functions by increasing urea and creatinine levels significantly ($P \leq 0.05$) associated with lowering total protein and albumin levels as compared to control group. This was in accordance with **Husain *et al.* [59]** who suggested that alteration in the biochemical measurements attributed to the cellular damage that caused by AFB_1. The immune responses were initiated through production of antimicrobial peptides and globulins that act as inhibitory factors for the defense against the pathogens and other foreign materials. Therefore, the decline in concentration of the total protein leads to lowering efficiency of the immune system **[67]**. Lipids are the macromolecules that exhibit key roles in metabolic pathways. The TC, T.Gs and phospholipids are the most common and significantly clinical and physiological lipids. These lipids are transported as lipoproteins in blood stream. These lipoproteins are made up of a hydrophobic core surrounded by a hydrophilic layer **[68]**. The dyslipidemia that was characterized by hypertriglyceridemia occurred as a result of disturbances in the homeostasis of these lipids and lipoproteins **[69,70]**. During the current study, it was noticed that AFB_1 caused a significant ($P \leq 0.05$) elevation in levels of the lipid measurements (TC and T.Gs). This was in accordance with **El-Nekeety *et al.* [4]** who postulated that AFB_1 caused alterations in plasma and liver lipid levels and the doses at which these effects occur and the mechanisms underlying these alterations need further exploration. *B. subtilis* at a dose of 0.25 ml showed ameliorative effect by decreasing levels of the liver enzymes, lipid and protein measurements in addition to lowering levels of urea and creatinine as compared to AFB_1 treated group but it could not restore them to normalcy. *B. subtilis* at a dose of 0.5 ml restored all the measurements to normal values. It decreased levels of the liver enzymes, lipid and protein measurements in addition to lowering levels of urea and creatinine significantly ($P \leq 0.05$) as compared to the group treated with AFB_1 alone and that treated with *B. subtilis* at a dose of 0.25 ml. Administration of *B. subtilis* alone showed no negative impact on the biochemical functions while its administration in association with AFB_1 showed beneficial effect and decreased the biochemical alterations and this was in accordance with **Ma *et al.* [40]** who suggested that *B. subtilis* was found to counteract the biochemical changes induced by AFB_1 in a dose-related manner, and highest dose provided maximum protection. Moreover, **Gao *et al.* [39]** added that the beneficial effect of *B. subtilis* might be attributed to its ability to biodegrade AFB_1 directly without inducing undesirable byproducts. Therefore, the application

of *B. subtilis* in feedstuffs may exhibit a more bright and valuable future.

Oxidative stress is defined as a disturbance in the balance between antioxidants (enzymatic & nonenzymatic) and the prooxidants that lead to potential damage. This imbalance might occur due to the decrease of endogenous antioxidants, low intake of dietary antioxidants, and/or increased formation of the reactive species. Either of both circumstances occurring together or separately eventually lead to adverse modifications in biomolecules and multitude of downstream consequences **[71]**. Oxidative stress plays an important role in aflatoxicosis. It might occur due to direct effect of AFB_1 themselves or by their metabolites. Metabolizing AFB_1 increases the production of ROS and lipid peroxides, resulting in cell damage **[72]**. The TAC, LPO and TPC levels were used as indicator of the oxidative stress. As presented in **Fig. 1**, it was found that AFB_1 caused significant ($P \leq 0.05$) elevation in levels of these measurements. This might be attributed to ability of AFB_1 to induce generation of the ROS that cause oxidative stress and the genetic toxicity of AFB_1 is partly attributed to the accumulation of ROS such as O_2^-, $^{\cdot}OH$, and H_2O_2 radical during the metabolic processing of AFB_1 by CYP450 in the liver. These reactive species attack the cellular (soluble) compounds as well as membranes, eventually leading to impairment of cell functioning and cytolysis **[73]**. The *B. subtilis* at a dose of 0.25 ml ameliorated the antioxidant status by increasing the TAC associated with decreasing levels of the LPO and TPC significantly ($P \leq 0.05$) as compared to AFB_1 treated group but it could not restore them to normal levels. As regard to the *B. subtilis* at a dose of 0.5 ml, it restored these measurements to normalcy. It elevated the TAC associated with decreasing levels of the LPO and TPC significantly ($P \leq 0.05$) as compared to the group treated with AFB_1 alone and that treated with *B. subtilis* at a dose of 0.25 ml. This was supported by **Fan *et al.* [74]** who suggested that administration of *B. subtilis* was able to counteract the oxidative stress and this protective and antioxidant effect is primarily based on absorption or the binding to the bacterial cells.

For separation, identification and quantification of different proteins in addition to analyzing stoichiometry of their specific subunits were carried out by electrophoresis **[75]**. Disappearance of normal bands with existence of one or more abnormal ones belongs to the mutations that occur at qualitative level. There is inversely correlation between SI and genetic variation. The low SI values between control and all the treated groups indicate differences in number and arrangement of electrophoretically separated bands **[76,77]**. Otherwise, the alterations that were detected by altering quantities of normal bands belong to the quantitative mutations. The SI is only correlated to the qualitative alterations **[78]**. Proteins that are considered as the key players inside the cells, are susceptible to be oxidized depending on relative content of oxidation-sensitive amino acid residues **[79,80]**.

As illustrated in **Fig. 2**, it was found that native electrophoretic protein pattern was represented in sera of control rats by 5 types identified at Rfs 0.13, 0.36, 0.74, 0.81 and 0.92 (B% 19.04, 20.08, 20.67, 20.24 and 19.97 ; Qty% 6.39, 8.91, 7.55, 4.79 and 6.50, respectively). Two common bands were identified at Rfs 0.74 and 0.92 (B% 20.67 and 19.97 ; Qty% 7.55 and 6.50, respectively). Three characteristic bands were identified in AFB_1 treated group at Rfs 0.26, 0.46 and 0.64 (B% 20.05, 20.23 and 20.14 ; Qty% 6.15, 6.79 and 7.34, respectively). *B. subtilis* alone at both doses (0.25 and 0.5 ml) caused no physiological variations as compared to control group. AFB_1 caused alterations in the protein pattern represented by hiding 3 normal types with existence of 3 abnormal (characteristic) bands. Therefore, the SI% value (SI=40.00%) decreased with increasing GD% value (GD=60.00%) in AFB_1 treated group. The treatment with *B. subtilis* at both doses (0.25 and 0.50 ml) restored the normal physiological state of the protein pattern through hiding the abnormal types with re-appearance of the normal ones at Rfs 0.14, 0.36 and 0.81 (B% 19.18, 19.62 and 20.66 ; Qty% 6.83, 9.13 and 4.09, respectively) at a dose of 0.25 ml of *B. subtilis* and identified at Rfs 0.14, 0.35 and 0.81 (B% 18.50, 20.03 and 20.93 ; Qty% 6.15, 7.83 and 4.30, respectively) at a dose of 0.50 ml of *B. subtilis*. Therefore, the protein pattern in the groups treated with *B. subtilis* became completely similar to that in control group (SI=100.00%; GD=0.00%).

As revealed in **Fig. 3**, it was noticed that electrophoretic lipid moiety of native protein pattern was represented in sera of control rats by 4 types identified at Rfs 0.10, 0.29, 0.45 and 0.94 (B% 15.41,

16.50, 29.28 and 38.81 ; Qty% 4.46, 4.99, 12.33 and 23.49, respectively). Two common bands were identified at Rfs 0.29 and 0.94 (B% 16.50 and 38.81 ; Qty% 4.99 and 23.49, respectively). One characteristic band was identified in AFB_1 treated group at Rf0.77 (B% 23.37 and Qty% 9.79). The *B. subtilis* alone at both doses (0.25 and 0.5 ml) caused no physiological variations when compared to control group. AFB_1 caused alterations in lipid moiety of native protein pattern represented by hiding 2 normal types with existence of 2 abnormal bands identified at Rfs 0.56 and 0.77 (B% 19.49 and 23.37 ; Qty% 8.73 and 9.79, respectively). Therefore, the SI% value (SI=50.00%) decreased with increasing GD% value (GD=50.00%) in AFB_1 treated group. The treatment with *B. subtilis* at a dose of 0.25 ml restored the normal bands physiological state of the protein pattern through re-appearance of the normal bands at Rfs 0.09 and 0.46 (B% 16.02 and 19.67 ; Qty% 5.55 and 12.38, respectively) with hiding one of the two abnormal ones. Therefore, the SI% value (SI=88.89%) increased with decreasing GD% value (GD=11.11%) in the AFB_1 + *B. subtilis* (0.25 ml) treated group. The treatment with *B. subtilis* at dose of 0.50 ml restored the normal physiological state of the lipid moiety of native protein pattern through hiding the abnormal types with re-appearance of the normal ones at Rfs 0.08 and 0.46 (B% 17.52 and 26.00 ; Qty% 8.00 and 12.96, respectively). Therefore, the lipid moiety of native protein pattern in the AFB_1 + *B. subtilis* (0.50 ml) treated group became completely similar to that in control group (SI=100.00% ; GD=0.00%).

As presented in **Fig. 4**, it was found that electrophoretic calcium moiety of native protein pattern was represented in sera of control rats by 5 types identified at Rfs 0.19, 0.64, 0.77, 0.84 and 0.95 (B% 15.99, 14.47, 26.57, 21.99 and 20.99 ; Qty% 14.12, 10.65, 16.62, 13.76 and 16.99, respectively). Three common bands were identified at Rfs 0.77, 0.84 and 0.95 (B% 26.57, 21.99 and 20.99; Qty% 16.62, 13.76 and 16.99, respectively). No characteristic bands were identified. The *B. subtilis* alone at both doses (0.25 and 0.5 ml) caused no physiological changes when compared to control group. AFB_1 caused alterations in calcium moiety of native protein pattern represented by hiding 2 normal types without existence of abnormal ones. Therefore, the SI% value (SI=75.00%) decreased with increasing GD% value (GD=25.00%) in AFB_1 treated group. The treatment with *B. subtilis* at both doses (0.25 and 0.50 ml) restored the normal physiological state of the calcium moiety of native protein pattern through re-appearance of the normal bands at Rfs 0.19 and 0.64 (B% 17.65 and 17.76; Qty% 10.09 and 10.15, respectively) at a dose of 0.25 ml of *B. subtilis* and identified at Rfs0.19 and 0.63 (B% 15.67 and 15.10 ; Qty% 12.46 and 12.98, respectively) at a dose of 0.50 ml of *B. subtilis*. Therefore, the protein pattern in the groups treated with *B. subtilis* became completely similar to that in control group (SI=100.00%; GD=0.00%).

During the current study, it was found that AFB_1 caused alterations in the native proteins and their lipid and calcium moieties that were electrophoretically detected. This was supported by **Swenson *et al.* [81]** who documented that AFB_1 is oxidized into several products by CYP450 subfamilies and specific isoforms of enzymes. AFB_1 epoxide is the mutagenic and others are considered as detoxification products. The putative AFB_1 epoxide is the most accepted active electrophilic AFB_1 form. It exhibits high affinity to attack nucleophilic nitrogen, oxygen and sulfur heteroatoms in cellular constituents. The alteration in the protein pattern might be attributed to covalently binding of the reactive AFB1-8,9-epoxide that obtained by the CYP450-mediated oxidation during the primary (Phase 1) bioactivation pathway of AFB_1 to cellular macromolecules (e.g., protein and/or DNA). Moreover, the electrophoretic alterations might refer to protein oxidation, thiols depletion, lipid peroxidation and DNA damage that caused by reactive species induced by AFB_1 **[82]**. In addition, **Peng *et al.* [83]** reported that these alterations might be attributed to capacity of AFB_1 to generate ROS that promote oxidation of amino acid residue side chains, formation of protein-protein cross-linkages and oxidation of the protein backbone leading to protein fragmentation. The modified forms of proteins accumulate in living tissues due to the inhibitory effect of AFB_1 on some (serine) proteolytic enzymes responsible for the degradation of damaged proteins and / or due to its inhibitory effect on the 20S proteasome that is the proteolytic machinery responsible for removing oxidized proteins and this consequently leads to elevating protein carbonyl content. The alterations in calcium moiety of native protein might be attributed to reduction of protein synthesis that affected certain metal ions. The

inhibitory effect of AFB_1 on protein synthesis altered composition of serum protein resulting in suppression of the production of nonspecific humoral substances important to native defense **[84]**. The treatment with *B. subtilis* showed ameliorative effect againt the electrophoretic alterations induced by AFB_1 and this was in accordance with **Fan *et al.* [74]** who suggested that supplementation of *B. subtilis* exhibit antagonistic effect to against the oxidative damage induced by AFB_1 in a dose-related manner and this was supported by our findings obtained during this study and this might refer to biodegradation of activity of *B. subtilis* on the AFB_1 that decreased by 56% and 73%, indicating the detoxification effect of *B. subtilis* on AFB_1.

Histopathological Examination

Microscopic examination of liver in control rats showed hepatic lobule with normal histological structure **(Fig. 5a)**. Meanwhile, liver sections of rats from groups 2 & 3 exhibited no histopathological alterations **(Fig. 5e & f)**. However, liver of aflatoxicated rats (group 4) showed histopathological changes demonstrated as vacuolar degeneration of hepatocytes, oedema and inflammatory cells infiltration **(Fig. 5b)** in the portal triad as well as focal hepatocellular necrosis associated with inflammatory cells infiltration **(Fig. 5c)** and hepatocellular apoptosis **(Table 3 & Fig. 5d)**. This was in agreement with **Gursoy *et al.* [85]** and **El-Nekeety *et al.* [82]** who suggested that the histopathological abnormalities in liver tissues might be attributed to the oxidative damage occurred as a result of AFB_1 that stimulate overproduction of ROS and this is considered as one of the underlining mechanisms for AFB_1-induced tissue injury. **Sun *et al.* [86]** added that AFB_1 induced liver cell injury due to elevation of nitric oxide significantly. Moreover, these alterations might refer to the lipid peroxidation that was accompanied in the liver with lowering the total antioxidant capacity in rats. Also, the exposure to AFB_1 produced a marked oxidative impact as evidenced by a significant increase in product of the peroxidation reactions in both of liver and kidneys of rats. These alterations might be attributable to the direct effects of AFB_1 or by their metabolites and/or by the ROS that were generated during formation of these metabolites. Also, mechanism of the free radical damage includes ROS-induced peroxidation of polyunsaturated fatty acids in lipid bilayer of the cell membrane that consequently causes disruption of the cell membrane leading to further oxidation of membrane proteins and lipids and hence leads to DNA damage **[87]**. On the other hand, sections from group 5 revealed no changes except slight vacuolar degeneration of some hepatocytes **(Fig. 5g)**. Furthermore, the histological structure of hepatic parenchyma was restored in liver from group 6 and in which the examined sections revealed binucleation of some hepatocytes **(Fig. 5h)**. This might be attributed to effect of this bacterial strain in alleviating the liver damage that induced by AFB_1 in rats via their ability to bind AFB_1 in the gastrointestinal tract **[88]**.

Microscopically, kidneys of control normal rats showed normal histological structure of renal tissue (normal glomeruli and renal tubules) (Fig. 3a). Meanwhile, kidneys of rats from group 2 & 3 manifested no histopathological changes and appeared histologically normal **(Fig. 6c & d)**. Adversely, kidneys of aflatoxicated rats revealed cytoplasmic vacuolization of renal tubular epithelium, thickening of the parietal layer of Bowman's capsule, perivascular and periglomerularoedema **(Table 3 & Fig. 6b)**. This was in accordance with **Marquez *et al.* [89]** who postulated that these alterations were associated with a significant increase in formation of conjugated diene in addition to the products of the peroxidation reactions in rat tissues. **Gesing and Karbownik-Lewinska [90]** added that 4-Hydroxynonenal (4-HNE) is a major lipid peroxidation (electrophilic) by-product that was caused by oxidative stress as a result of effect of AFB_1 in the most target organs in rats and it interacts with DNA forming exocyclic guanine products that increase in rats during tissue carcinogenesis. On the other hand, kidneys from group 5 showed mild damage and the examined sections showed slight cytoplasmic vacuolization of epithelial lining some renal tubules **(Fig. 6e)**. Moreover, kidneys from group 6 restored the histological architecture of renal tissue **(Fig. 6f)**. This was in accordance with **Nada *et al.*, [91]** who showed co-administration of probiotic bacteria reduced the toxic effect induced by AFB_1 significantly and this might refer to elevating level the antioxidants especially level of the reduced glutatione (GSH). Therefore, liver and kidney structures were preserved as demonstrated by histopathological examination.

TABLE 1. Effect of *B. subtilis* against Aflatoxin B_1 (AFB_1) induced toxicity on different hematological measurements in rats

		C	*B. subtilis*		AFB_1	AFB_1 + *B. subtilis*	
			0.25 ml	0.5 ml		0.25 ml	0.5 ml
Formed Elements	**RBCs (10^6/ul)**	5.59 ± 0.01	5.56 ± 0.01	5.57 ± 0.00	2.51 ± 0.01[a]	4.32 ± 0.08[ab]	5.57 ± 0.01[bc]
	HB (g/ dl)	12.72 ± 0.01	12.69 ± 0.01	12.69 ± 0.05	8.17 ± 0.02[a]	10.39 ± 0.01[ab]	12.70 ± 0.01[bc]
	HCT (%)	32.77 ± 0.04	32.71 ± 0.02	32.70 ± 0.02	20.11 ± 0.03[a]	25.49 ± 0.01[ab]	32.69 ± 0.01[bc]
	MCV (um^3)	55.29 ± 0.02	55.30 ± 0.03	55.30 ± 0.02	32.05 ± 0.05[a]	40.83 ± 0.02[ab]	55.28 ± 0.01[bc]
	MCH (pg)	21.38 ± 0.02	21.38 ± 0.03	21.38 ± 0.01	16.21 ± 0.01[a]	18.20 ± 0.01[ab]	21.37 ± 0.00[bc]
	MCHC (g/ dl)	36.38 ± 0.05	36.38 ± 0.04	36.39 ± 0.04	24.20 ± 0.01[a]	28.74 ± 0.01[ab]	36.40 ± 0.03[bc]
	RDW (%)	14.02 ± 0.08	14.08 ± 0.07	14.06 ± 0.07	9.35 ± 0.03[a]	12.41 ± 0.01[ab]	14.03 ± 0.06[bc]
	MPV (um^3)	8.16 ± 0.01	8.16 ± 0.00	8.17 ± 0.01	5.37 ± 0.01[a]	6.87 ± 0.01[ab]	8.15 ± 0.00[bc]
	PLT (10^3/ul)	445.83 ± 0.50	445.20 ± 0.61	445.00 ± 0.45	288.00 ± 1.05[a]	350.00 ± 0.81[ab]	446.00 ± 0.63[bc]
	WBCs (10^3/ul)	7.39 ± 0.01	7.38 ± 0.00	7.40 ± 0.02	4.71 ± 0.03[a]	5.20 ± 0.01[ab]	7.39 ± 0.01[bc]
Differential Count	**Lymp.** (10^3/ul)	5.54 ± 0.01	5.53 ± 0.00	5.55 ± 0.01	3.54 ± 0.02[a]	3.90 ± 0.00[ab]	5.54 ± 0.01[bc]
	Mono. (10^3/ul)	0.73 ± 0.00	0.73 ± 0.00	0.73 ± 0.00	0.47 ± 0.00[a]	0.51 ± 0.00[ab]	0.73 ± 0.00[bc]
	Gran. (10^3/ul)	0.66 ± 0.00	0.66 ± 0.00	0.67 ± 0.00	0.42 ± 0.00[a]	0.47 ± 0.00[ab]	0.67 ± 0.00[bc]

Values were expressed as mean ± standard error, **a:** significant difference from control group, **b:** significant difference from AFB_1 treated group, **c:** significant difference from AFB_1 treated group and treated with *B. subtilis* (0.25 ml) at $P \leq 0.05$.

TABLE 2. Effect of *B. subtilis* against Aflatoxin B_1 (AFB_1) induced toxicity on different biochemical measurements in rats

		C	*B. subtilis*		AFB_1	AFB_1 + *B. subtilis*	
			0.25 ml	0.5 ml		0.25 ml	0.5 ml
Liver Enzymes	ALT (U/L)	42.28 ± 0.01	42.28 ± 0.01	42.28 ± 0.01	63.08 ± 0.42[a]	55.13 ± 0.06[ab]	42.28 ± 0.01[bc]
	AST (U/L)	98.18 ± 0.09	98.19 ± 0.09	98.18 ± 0.08	123.18 ± 0.18[a]	110.34 ± 0.01[ab]	98.22 ± 0.09[bc]
	ALP (U/L)	25.88 ± 0.08	25.91 ± 0.08	25.89 ± 0.07	45.89 ± 0.07[a]	35.52 ± 0.04[ab]	25.92 ± 0.09[bc]
	LDH (U/L)	995.88 ± 0.08	995.91 ± 0.08	995.89 ± 0.07	1227.69 ± 0.63[a]	1125.24 ± 0.10[ab]	995.92 ± 0.09[bc]
Lipid Profile	TC (mg/dl)	73.55 ± 0.16	73.59 ± 0.15	73.59 ± 0.15	94.15 ± 0.32[a]	84.98 ± 0.06[ab]	73.39 ± 0.13[bc]
	T.Gs (mg/dl)	67.07 ± 0.05	67.12 ± 0.05	67.12 ± 0.04	87.07 ± 0.05[a]	77.18 ± 0.01[ab]	67.12 ± 0.03[bc]
kidney functions	Urea (mg/dl)	48.70 ± 0.01	48.69 ± 0.01	48.69 ± 0.01	68.69 ± 0.01[a]	58.61 ± 0.03[ab]	48.72 ± 0.00[bc]
	Creatinine (mg/dl)	0.91 ± 0.01	0.90 ± 0.00	0.90 ± 0.00	1.90 ± 0.00[a]	1.29 ± 0.01[ab]	0.91 ± 0.00[bc]
	Total Protein (g/dl)	8.25 ± 0.01	8.27 ± 0.02	8.26 ± 0.01	4.25 ± 0.01[a]	6.26 ± 0.01[ab]	8.24 ± 0.00[bc]
	Albumin (g/dl)	3.73 ± 0.01	3.73 ± 0.00	3.73 ± 0.00	1.73 ± 0.01[a]	2.55 ± 0.01[ab]	3.73 ± 0.00[bc]

Values were expressed as mean ± standard error, **a:** significant difference from control group, **b:** significant difference from AFB_1 treated group, **c:** significant difference from AFB_1 treated group and treated with *B. subtilis* (0.25 ml) at $P \leq 0.05$.

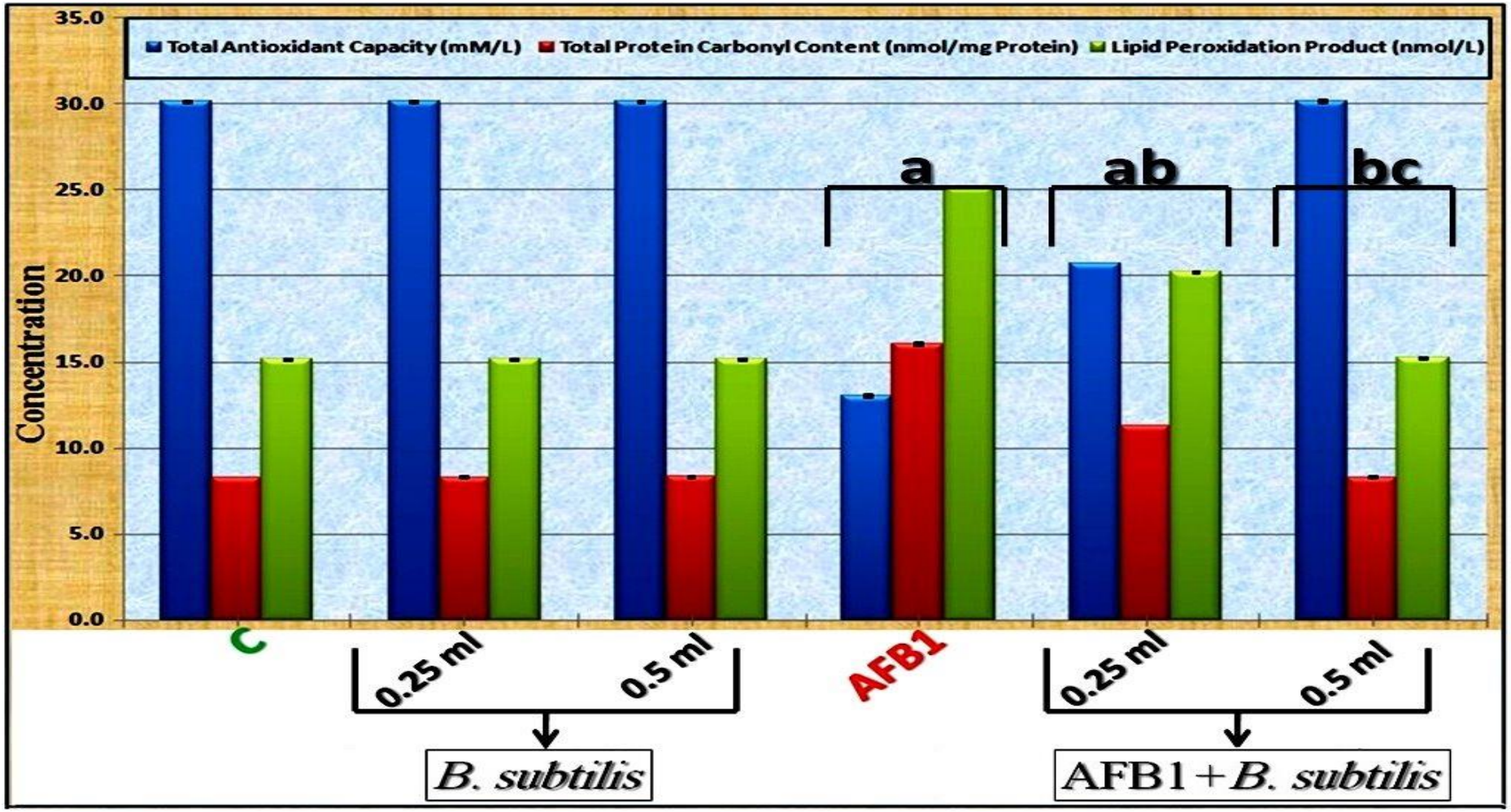
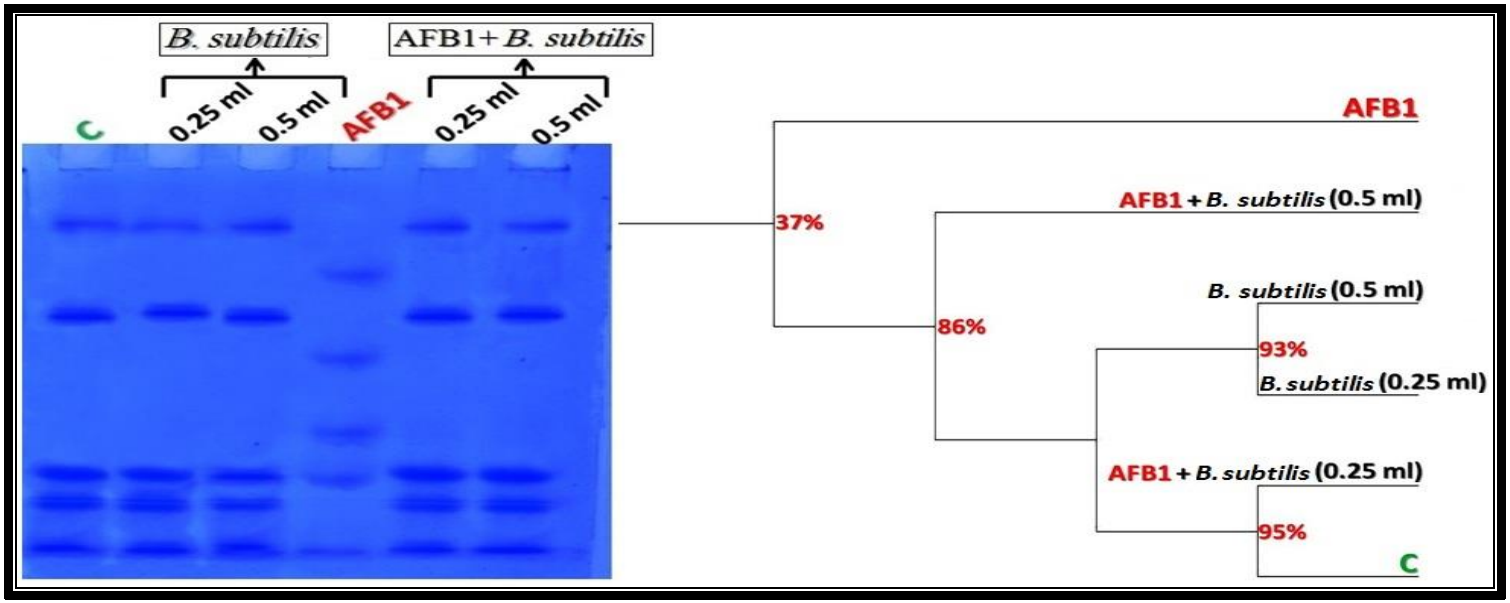


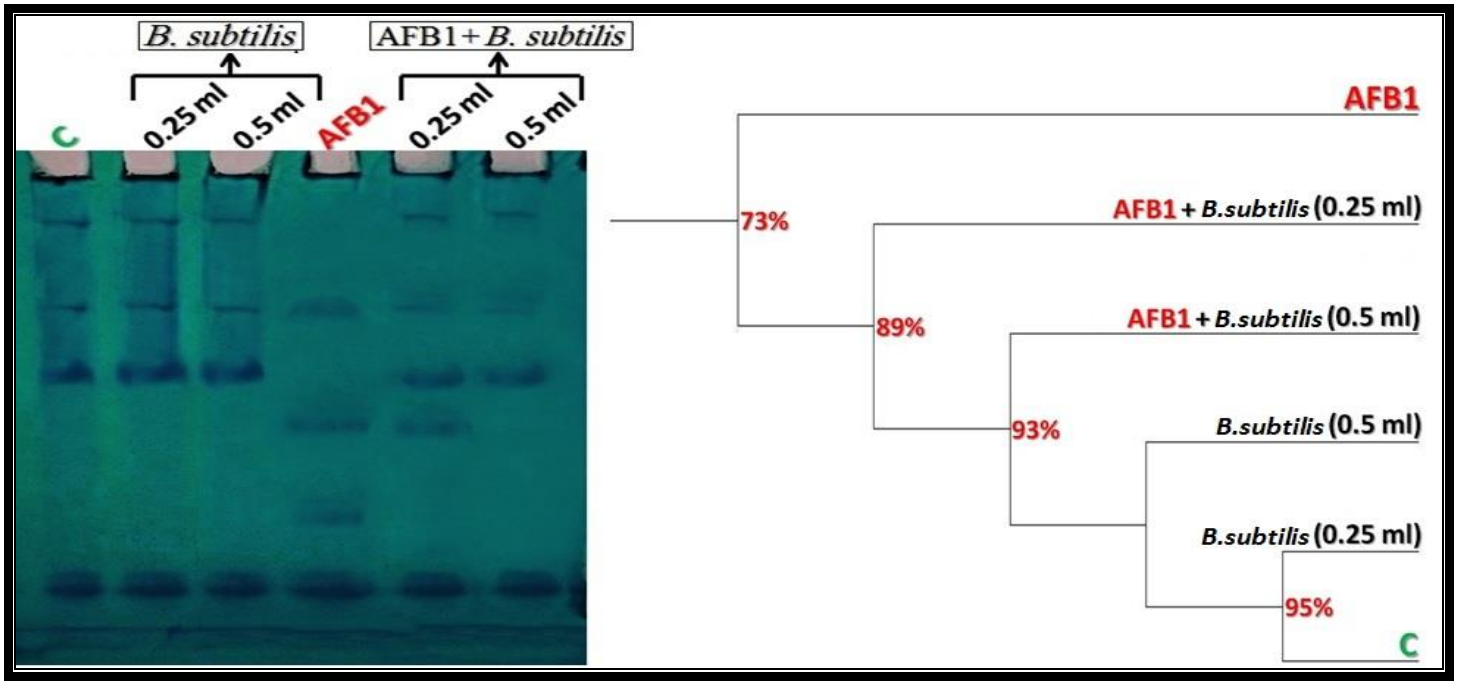
Fig. 1. Effect of *B. subtilis B. subtilis* against Aflatoxin B_1 (AFB_1) induced toxicity on major markers of oxidative stress in rats. Values were expressed as mean ± standard error, **a:** significant difference from control group, **b:** significant difference from AFB_1 treated group, **c:** significant difference from AFB_1 treated group and treated with *B. subtilis* (0.25 ml) at $P \leq 0.05$.



Control			*B. subtilis*						AFB_1			AFB_1 + *B. subtilis*					
			0.25 ml			0.5 ml						0.25 ml			0.5 ml		
Rf	**B%**	**Qty%**	**Rf**	**B%**	**Qty%**	**Rf**	**B%**	**Qty%**	**Rf**	**B%**	**Qty%**	**Rf**	**B%**	**Qty%**	**Rf**	**B%**	**Qty %**
0.13	19.04	6.39	0.14	18.72	6.08	0.14	19.51	7.60	-	-	-	0.14	19.18	6.83	0.14	18.50	6.15
-	-	-	-	-	-	-	-	-	0.26	20.05	6.15	-	-	-	-	-	-
0.36	20.08	8.91	0.35	20.56	7.68	0.36	20.07	8.79	-	-	-	0.36	19.62	9.13	0.35	20.03	7.83
-	-	-	-	-	-	-	-	-	0.46	20.23	6.79	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	0.64	20.14	7.34	-	-	-	-	-	-
0.74	20.67	7.55	0.74	20.46	6.24	0.74	19.99	7.79	0.75	19.95	7.27	0.74	20.71	8.4	0.74	20.78	8.12
0.81	20.24	4.79	0.81	19.83	5.85	0.81	19.73	8.26	-	-	-	0.81	20.66	4.09	0.81	20.93	4.30
0.92	19.97	6.50	0.92	20.44	8.04	0.91	20.7	7.46	0.92	19.63	6.4	0.92	19.83	8.24	0.92	19.76	9.85

Rf: Relative Mobility, **B%:** Percent of Band Intensity, **Qty%:** Percent of Band Quantity.

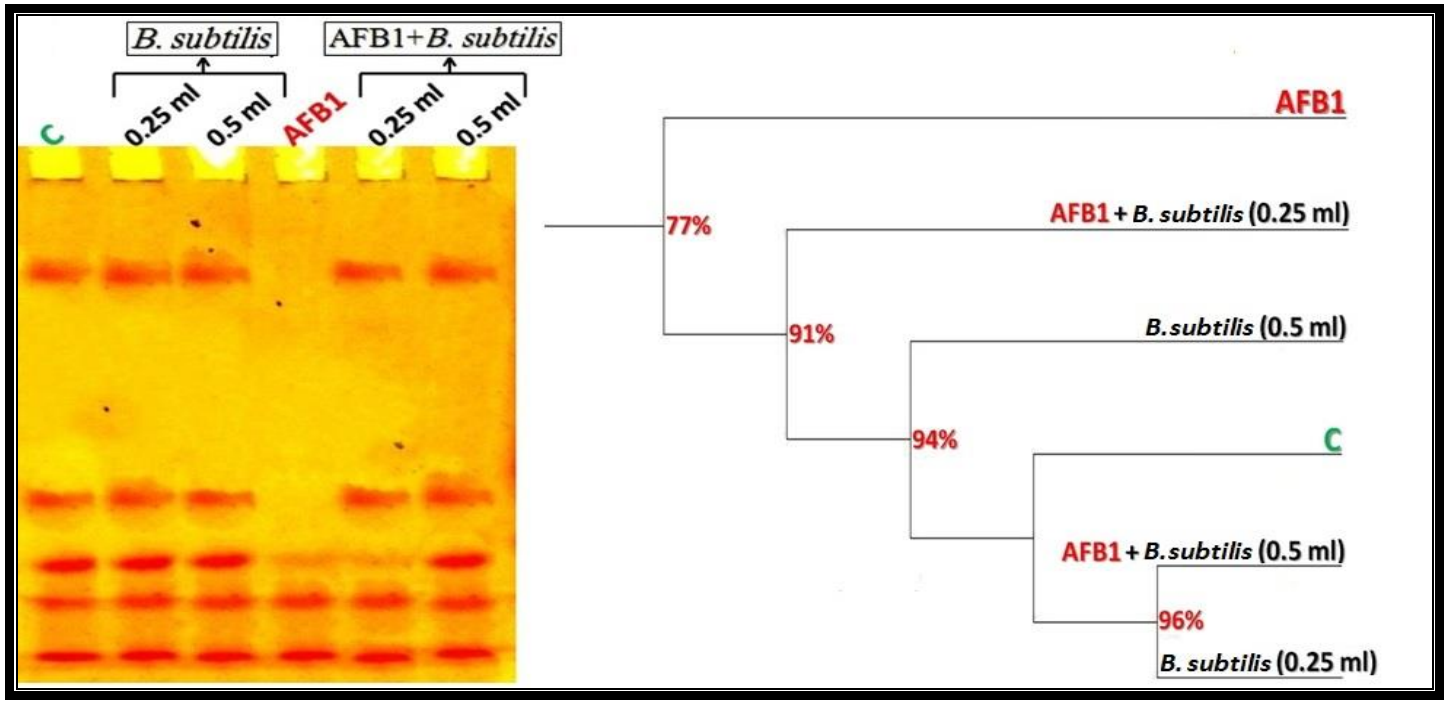
Fig. 2. Native electrophoretic protein pattern showing physiological effect of *B. subtilis* against the toxicity induced by Aflatoxin B_1 (AFB_1) on number and arrangement of the native bands in addition to similarity percents of this pattern in sera of rats.



Control			*B. subtilis*						AFB_1			AFB_1 + *B. subtilis*					
			0.25 ml			0.5 ml						0.25 ml			0.5 ml		
Rf	**B%**	**Qty%**	**Rf**	**B%**	**Qty%**	**Rf**	**B%**	**Qty%**	**Rf**	**B%**	**Qty%**	**Rf**	**B%**	**Qty%**	**Rf**	**B%**	**Qty %**
0.10	15.41	4.46	0.10	14.17	4.12	0.10	15.51	6.00	-	-	-	0.09	16.02	5.55	0.08	17.52	8.00
0.29	16.50	4.99	0.29	21.96	7.40	0.30	16.82	5.97	0.30	17.15	8.42	0.30	14.84	4.90	0.30	14.18	2.91
0.45	29.28	12.33	0.44	25.86	14.26	0.44	28.89	19.57	-	-	-	0.46	19.67	12.38	0.46	26.00	12.96
-	-	-	-	-	-	-	-	-	0.56	19.49	8.73	0.57	15.89	8.25	-	-	-
-	-	-	-	-	-	-	-	-	0.77	23.37	9.79	-	-	-	-	-	-
0.94	38.81	23.49	0.94	38.01	34.34	0.93	38.78	26.90	0.94	40.00	31.78	0.94	33.58	31.17	0.93	42.30	29.14

Rf: Relative Mobility, **B%:** Percent of Band Intensity, **Qty%:** Percent of Band Quantity.

Fig. 3. Native electrophoretic lipid moiety of protein pattern showing physiological effect of *B. subtilis* against the toxicity induced by Aflatoxin B_1 (AFB_1) on number and arrangement of the native bands in addition to similarity percents of this pattern in sera of rats.



Control			*B. subtilis*						AFB_1			AFB_1 + *B. subtilis*					
			0.25 ml			0.5 ml						0.25 ml			0.5 ml		
Rf	**B%**	**Qty%**	**Rf**	**B%**	**Qty%**	**Rf**	**B%**	**Qty%**	**Rf**	**B%**	**Qty%**	**Rf**	**B%**	**Qty%**	**Rf**	**B%**	**Qty %**
0.19	15.99	14.12	0.20	16.85	11.69	0.19	15.27	11.71	-	-	-	0.19	17.65	10.09	0.19	15.67	12.46
0.64	14.47	10.65	0.64	14.55	13.82	0.64	15.72	9.76	-	-	-	0.64	17.76	10.15	0.63	15.10	12.98
0.77	26.57	16.62	0.77	23.99	15.33	0.76	25.66	14.99	0.76	20.29	8.66	0.762	9.91	5.83	0.76	24.80	12.47
0.84	21.99	13.76	0.84	22.42	13.09	0.85	21.94	15.62	0.84	37.62	20.34	0.84	28.13	14.19	0.85	23.95	10.49
0.95	20.99	16.99	0.95	22.19	16.20	0.95	21.41	18.76	0.95	42.08	22.75	0.95	26.55	17.86	0.94	20.48	13.96

Rf: Relative Mobility, **B%:** Percent of Band Intensity, **Qty%:** Percent of Band Quantity.

Fig. 4. Native electrophoretic calcium moiety of protein pattern showing physiological effect of *B. subtilis* against the toxicity induced by Aflatoxin B_1 (AFB_1) on number and arrangement of the native bands in addition to similarity percents of this pattern in sera of rats.

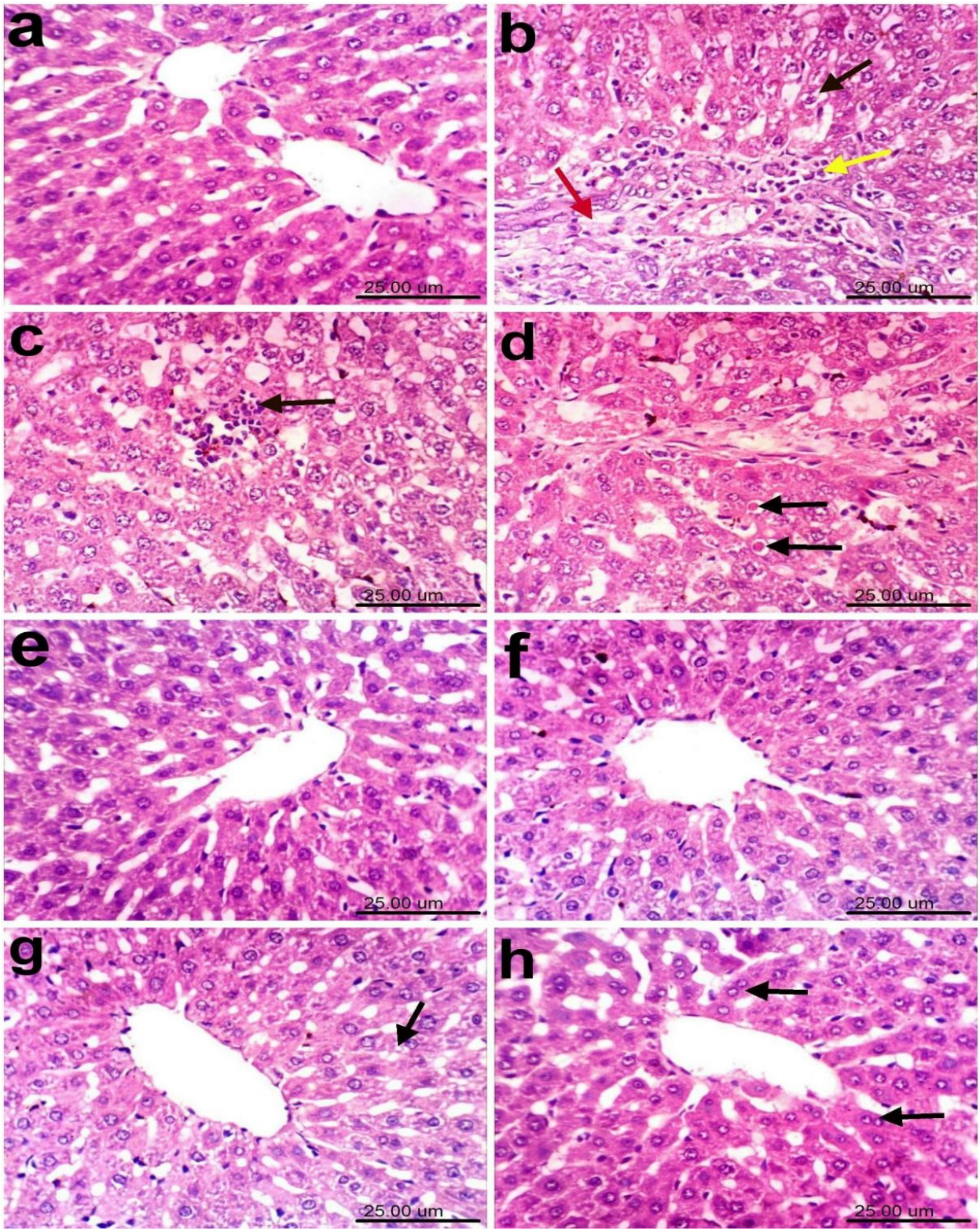


Fig. 5. Photomicrograph of histological H&E stained liver tissue of rats: a) group 1 (control) showing normal histological structure of hepatic lobule, b) group 4 (AFB_1 treated group) showing vacuolar degeneration of hepatocytes (black arrow), oedema (red arrow) and inflammatory cells infiltration (yellow arrow) in the portal triad, c) group 4 showing focal hepatocellular necrosis associated with inflammatory cells infiltration (arrow), d) group 4 showing hepatocellular apoptosis (arrows), e) & f) groups 2 (*B. subtilis* (0.25 ml) treated group) & 3 (*B. subtilis* (0.5 ml) treated group), respectively showing no histopathological alterations, e) group 5 (AFB_1 + *B. subtilis* (0.25 ml) treated group) showing slight vacuolar degeneration of some hepatocytes (arrow), f) group 6 (AFB_1 + *B. subtilis* (0.5 ml) treated group) showing binucleation of some hepatocytes (arrow) **(scale bar 25um, X400)**.

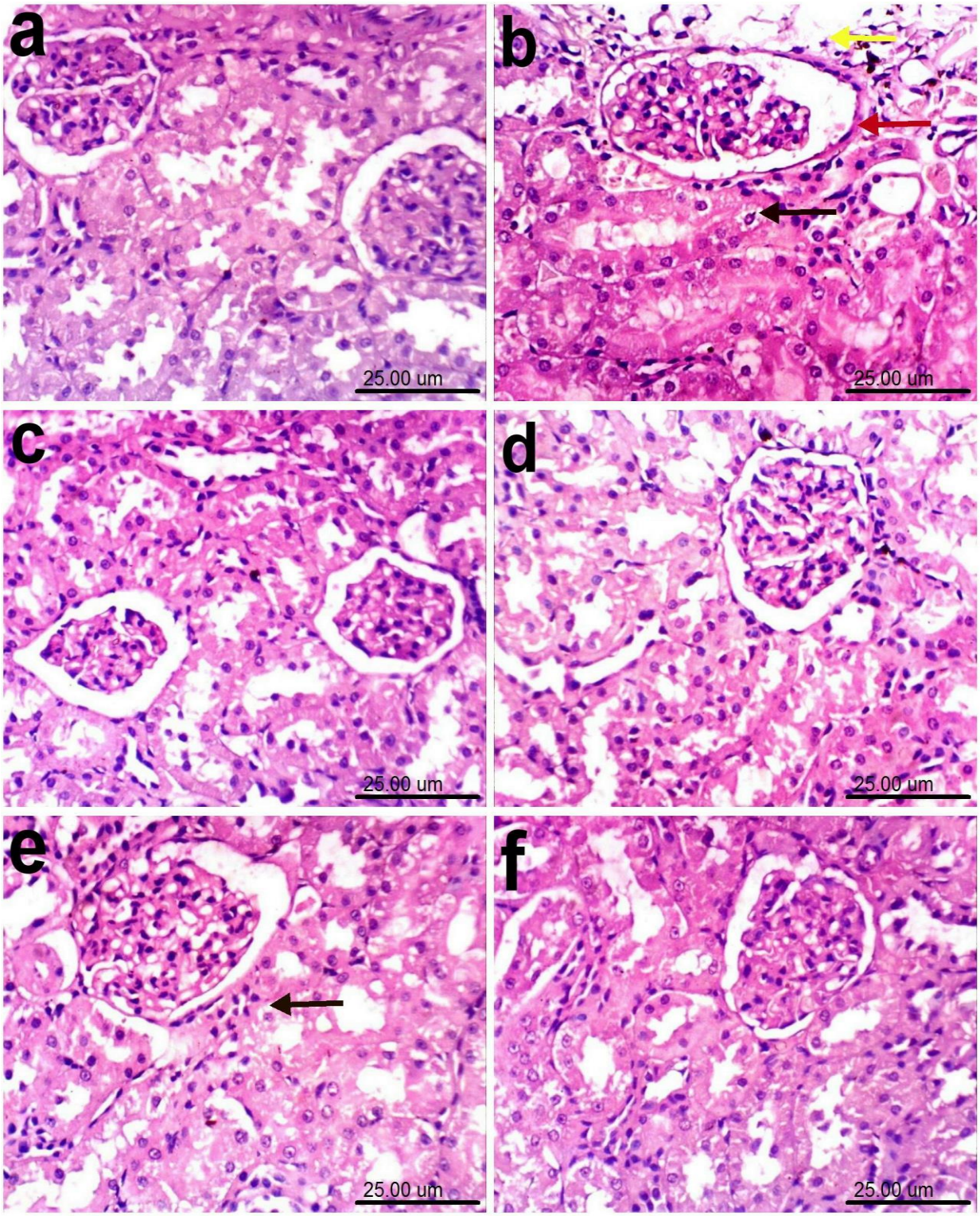


Fig. 6. Photomicrograph of histological H&E stained renal tissue of rats: a) group 1 (control) showing normal histological architecture of renal tissue (normal glomerulus and normal renal tubules), b) group 4 (AFB_1 treated group) showing cytoplasmic vacuolization of renal tubular epithelium (black arrow), thickening of the parietal layer of Bowman's capsule (red arrow) and periglomerularoedema (yellow arrow), c) & d) groups 2 (*B. subtilis* (0.25 ml) treated group) & 3 (*B. subtilis* (0.5 ml) treated group), respectively showing no histopathological changes, e) group 5 (AFB_1 + *B. subtilis* (0.25 ml) treated group) showing slight cytoplasmic vacuolization of epithelial lining some renal tubules (arrow), f) group 6 (AFB_1 + *B. subtilis* (0.5 ml) treated group) showing restored the histological architecture of renal tissue **(scale bar 25um, X400)**.

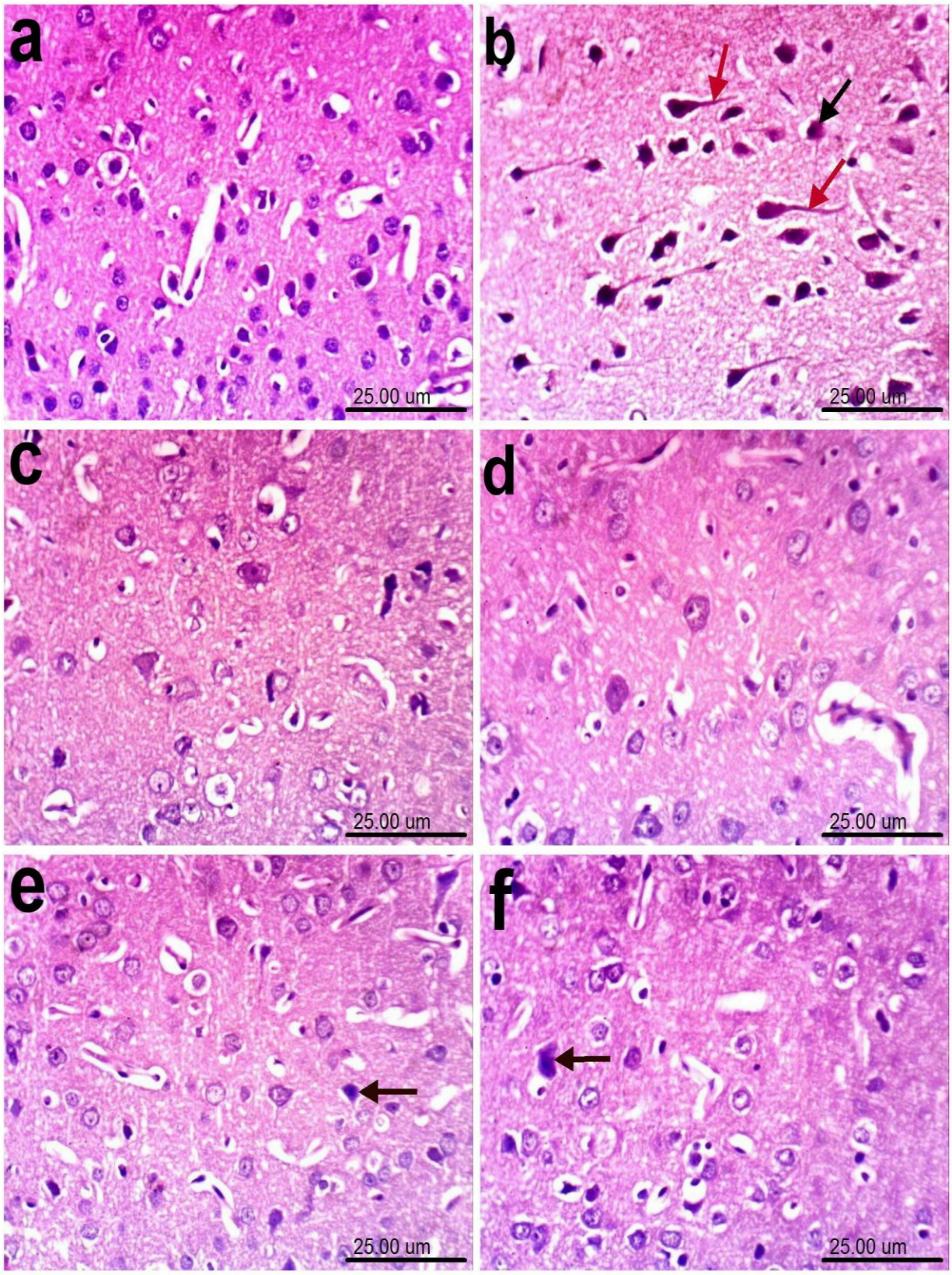


Fig. 7. Photomicrograph of histological H&E stained brain sections of rats: a) group 1 (control) showing no histopathological changes, b) group 4 (AFB_1 treated group) showing shrunken, atrophy and necrosis of neurons (black arrow) and neurofibrillary tangles (red arrows), c) & d) groups 2 (*B. subtilis* (0.25 ml) treated group) & 3 (*B. subtilis* (0.5 ml) treated group), respectively showing normal histological architecture of the brain tissue, e) & f) group 5 (AFB_1 + *B. subtilis* (0.25 ml) treated group) & 6 group 5 (AFB_1 + *B. subtilis* (0.5 ml) treated group), respectively showing necrosis of some neurons (arrows) **(scale bar 25um, X400)**.

TABLE 3. The histopathological score showing effect of *B. subtilis* against the lesions induced by Aflatoxin B_1 (AFB_1) in different tissues

Organs	Lesions	C	*B. subtilis*		AFB_1	AFB_1 + *B. subtilis*	
			0.25 ml	0.5 ml		0.25 ml	0.5 ml
Liver	Vacuolar degeneration of hepatocytes	0	0	0	3	1	0
	Hepatocellular necrosis	0	0	0	2	1	0
	Hepatocellular apoptosis	0	0	0	2	0	0
	Oedma in the portal triad	0	0	0	2	0	0
	portal inflammatory cells infiltration	0	0	0	1	0	0
Kidney	Vacuolization of renal tubular epithelium	0	0	0	3	1	0
	thickening of the parietal layer of Bowman's capsule	0	0	0	2	0	0
	periglomerularoedema	0	0	0	2	0	0
Brain	Shrunken, atrophy& necrosis of neurons	0	0	0	3	1	1
	Neurofibrillary tangles	0	0	0	2	0	0
	Cellular oedema	0	0	0	3	0	0

Brain tissue of control rats revealed normal histological architecture **(Fig.7a)**. No histopathological alterations were recorded in brain tissues from groups 2 & 3 **(Fig. 7c & d)**. In contrast, brain of rats from group 4 exhibited neuropathological alterations described as shrunken, atrophy and necrosis of neurons associated with appearance of neurofibrillary tangles and cellular oedema **(Table 3 & Fig. 7b)**. This was supported by **Naaz *et al.* [92]** who showed that the histopathological alterations were noticed as a result of the exposure to AFB_1 not only in the liver but also in the kidney and brain. They reported that polyunsaturated lipids are considered as the most important constituents in endoplasmic reticulum and mitochondria in addition to the cell membranes. Thus, they are essential for cells and disruption in their structural properties could have consequences for the cellular function. Lipid peroxidation is the most important factor that induced by oxidative stress and responsible for functional and structural alterations of the cell membrane. The mycotoxins stimulate the lipid peroxidation directly through increasing the ROS formation or sensitivity of the tissue to the peroxidation that is compromised antioxidant defence **[93]**. Meanwhile, brain of rats from groups 5 & 6 showed regression of the histopathological lesions and the examined sections only demonstrated necrosis of some neurons **(Fig. 7e & f)**. This was in accordance with **Fan *et al.* [41]** and supported by **Fan *et al.* [74]** who suggested that the beneficial effect of *B. subtilis* against the histopathological alterations induced by AFB_1 might be attributed to the linear decrease in recovery of AFB_1 from duodenal contents. These findings implied that *B. subtilis* was able to detoxify AFB_1 in the gastrointestinal tracts by reducing amount of AFB1 that absorbed into the body and this consequently prevented the detrimental AFB_1 effects to the animals and environment.

4. Conclusion

The study concluded that *B. subtilis* restored all hematological and biochemical measurements when administrated at a dose of 0.50 ml in association with AFB_1 treatment towards the normal values. AFB_1 caused physiological alterations detected electrophoretically in the native protein and lipid moiety in addition to calcium moiety of native protein patterns and represented by hiding normal protein types with existence of abnormal ones. Therefore, the SI% and GD% values were altered with protein (SI=40.00%; GD=60.00%), lipid moiety (SI=50.00%; GD=50.00%) and calcium moiety of native protein patterns (SI=75.00%; GD=25.00%) in AFB_1 treated group. *B. subtilis* at a dose of 0.50 ml restored integrity of these native protein patterns by restoring the absent types with hiding the abnormal ones. Therefore, this group became physiologically similar to control group (SI=100.00%; GD=0.00%). These findings were supported by the severe lesions that were noticed histopathologically in the most target organs (liver, kidney and brain) AFB_1 treated group and *B. subtilis* restored their normal architecture. *B. subtilis* exhibited antioxidant activity enables it to be used as promising product as probiotics to eliminate the unbalance in oxidative stress and to moderate the health hazards induced as a result of the exposure to AFB_1 direct or indirect.

5. Conflict of Interests

The authors who are responsible for the theoretical and practical parts of the manuscript declare that there are no conflicts of interests financially or non-financially.

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