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# EXTRA CELLULAR ABILITY OF *BACILLUS SUBTILIS* AND *PSEUDOMONAS FLUORESCENS* TO DEGRADE AFLATOXIN B1 PRODUCED BY *ASPERGILLUS FLAVUS*

**Labeed A. Al-Saad*[1], Adnan I. Al-Badran[2], Sami A. Al-Jumayli[3]**

[1]University of Basrah, College of Agriculture, Dept. of Plant Protection.

[2]University of Basrah, College of sciences, Dept. of Biology.

[3]University of Karbala, College of Applied Medical Sciences.

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***Correspondence for Author**
**Dr. Labeed A. Al-Saad**
University of Basrah,
College of Agriculture,
Dept. of Plant Protection.

## ABSTRACT

This study was designed to evaluate the extra cellular ability of *Bacillus subtilis* isolates (BSS1, BSS2, BSS3, BSS4 and BSW) and *Pseudomonas fluorescens* isolates (PFMst and PFDL) to degrade aflatoxin B1 (AFB1) that produced by *Aspergillus flavus* AFL14. The extra-cellular degradation ability of bacterial isolates to AFB1 was evaluated quantitatively using HPTLC technique. *B. subtilis* isolates BSS4, BSW and BSS3 were exceeded the rest of bacterial isolates significantly (100, 100 and 99.55) % respectively, followed by *P. fluorescens* isolates PFMst and PFDL (97.805 and 97.396) % respectively, with no significant differences among individuals in the same group. The degradation residues administrated to rats to determine their effect on biosystems, the blood parameters showed a significant reduction in WBC, HB, RBC, and P.C.V while there was a significant increase in Urea, Glutamic pyrovate transaminase (GPT) and Glutamic oxaloacetic transaminase (GOT) in AF+DMSO treatment comparing with the negative and positive control which is may indicate that AFB1 caused immune-malfunction, hemolysis, kidney malfunction and liver stress. The biocontrol treatments showed a significant increase in WBC caused by BSS4+AF treatment, significant reduction in RBC caused by BSS4 and PFMst and significantly high Urea levels exceeded AF+DMSO treatment caused by PFMst and PFMst+AF treatments respectively. According to platelet count parameter, no significant differences were observes among all treatments and control treatments.

**KEYWORDS:** *Aspergillus flavus*, aflatoxin B1, extra-cellular, degradation, *Pseudomonas fluorescens, Bacillus subtilis*.

## INTRODUCTION

*Aspergillus flavus* is a worldwide fungus, highly distributed in soil, water and air and posses the ability to contaminate crops, processed foods and feedstuff (Vujanovic *et al*., 2001). A broad spectrum of human diseases were reported to be caused by *A.flavus* which included human tissue infections which involved skin, oral mucosa, or subcutaneous tissues and hypersensitivity reactions in addition to producing Aflatoxins, especially AFB1 which is the most toxic and potent hepatocarcinogenic natural compound ever characterized (Hedayati *et. al.*, 2007).

The control of fungal diseases was conventionally performed by chemical compounds which were mostly affected the environment and human and animal health in addition to economical costs as this type of control is usually expensive. However, since 1930 the scientists discovered interesting beneficial microbes with antifungal activities that could be employed in controlling plant fungal diseases (Cawoy *et al.*, 2011). Among those microorganisms, a nontoxigenic filamentous fungi like *A.flavus* and *A.parasiticus strains* (Rajani *et al*., 2012), *Tricoderma harzianum* (Al-Othman *et al*., 2013), yeasts like *Candida krusei, Saccharomyces cerevisiae* and *Pichia anomala* (Sari *et al.*, 2008; Rajani *et al.*, 2012) and bacteria like *Bacillus subtilis, Pseudomonas spp., Ralstonia spp.* and *Lactobacilli spp.* (Mejía-Teniente *et al*., 2011; Rajani *et al.*, 2012). *Bacillus subtilis* have some characteristics that enabled it to be highly recommended as a biological control agent as being recognized as non-pathogenic organism by the US Food and Drug Administration (USFDA), high tolerant to hard conditions like low nutrition and water supply, high temperature and unfavourable pH in addition to its ability to produce spores which is important benefit enabled ease of handling of this bacteria by converting to powder formulation (Cawoy *et al*., 2011). *B.sutilis* have the ability to inhibit spore germination and *A.flavus* growth in addition to its extracellular activity to degrade more than 50-78.39% of aflatoxin B1 with maximum activity at 50ºC and wide range of pH (5-8) (Farzaneha *et al.*, 2010; Thakaew and Niamsup, 2013; Watanakij, 2013). *P.fluorescens* is one of the important biocontrol agents found to inhibit *A.flavus* growth and AFB1 production and/or AFB1 content reduction either *in vitro* or *in vivo* (Luna-Romero *et al*, 2000; Reddy *et al.* 2010; Singh *et al.*, 2011; Mohale, 2013). *P.fluorescens* possessed some characteristics helped to considering it as effective biocontrol agent that included the ability

to grow and rapid colonization of the rhizosphere and spermosphere in soil environment, aggressive competitor with high ability to adapt environmental stress, the ability to produce wide spectrum of extra-cellular enzymes (cellulase, chitinase, amylase, protease, and gelatinase), antibiotics, siderphore, growth promoter substances and volatiles, furthermore, each strain is able to act against the same pathogene in multiple patho-system (Weller, 2007; Couillerot *et al.*, 2009; Soesanto *et al.*, 2011). The Public Health Agency of Canada classified *P.fluorescen*s as a risk group 1 which means that it can infect human in contact with contaminated medical devices, immunocompromised patients and people with outbreaks but it is unlikely infects general population or causes diseases to healthy research works (Canadian Dept. of Environment and Canadian Dept. of Health, 2013).

## MATERIALS AND METHODS

Microorganisms: *A.flavus* AFL14 was isolated from soil using dilution method on MEA (Malt Extract Agar: Oxoid Limited) then preliminary identified morphologically according to Klich (2002) , Molecular identification was made to confirm isolates identification according to . Rodriguez *et al.* (2012) using Internal Transcript spacer ITS1-ITS2 primers:

(For: 5'TCCGTAGGTGAACCTGCGG3',
Rev:5' GCTGCGTTCTTCATCGATGC 3')
and ITS3-ITS4 region (For: 5'GCATCGATGAAGAACGCAGC3',
Rev: 5'TCCTCCGCTTATTGATATGC3') (Bellemain *et al*., 2010)

*Bacillus subtilis* isolates BSS1, BSS2, BSS3, and BSS4 are obtained as a gift from Dr. Sami Al-Jumaily, Karbala Univ., College of Applied Medical Sciences; BSW is obtained as a gift from Dr. Wael Al-Waely, Basrah Univ., College of Agriculture, *Pseudomonas fluorescens* PFMst are obtained from Dr. Mohammed Amir; PFDL is obtained from Dr. Dehya Al-Waely, Basrah Univ., College of Agriculture, Plant Protection Dept.

All *Bacillus subtilis* isolates are confirmed morphologically and biochemically (Gram staining, shape, spore existence, oxidase test, catalase test, gelatin liquefaction, indol test, methyl red test, Voges-Proskauer test, citrate test, salt tolerance test and amylase test) whereas *Pseudomonas* isolates are confirmed by (Fluorescens under UV light, gram staining, shape, oxidase test, catalase test, gelatin liquefaction, indol test, methyl red test, Voges-Proskauer test) (Henayl, 2000; Barrow and Filtham, 2003; Forbes *et al.*, 2007).

**The extra-cellular ability of bacterial isolates to degrade AFB1**

Aflatoxin B1 was mixed with 10 ml of sterilized Nutrient broth medium at a concentration of 250 ng/ml. The medium was inoculated with 1 ml of ($1\times10^6$ CFU) of 24h age bacterial broth culture of *B. subtilis* isolates BSS1, BSS2, BSS3, BSS4, BSW and *P. fluorescens* isolates PFMst and PFDL. Treated tubes were incubated at 35ºC in shaking incubator for 48h (Zuo *et al*., 2012).

**High performance thin layer chromatography (HPTLC)**

AFB1 was extracted according to Al-Jumaily (1996) and the qualitative TLC was obtained according to Goldblatt (1969). Quantitative estimation was obtained using 6 captures for each TLC plate from different positions using a digital camera (CASIO EXILIM EX-Z80, Japan) to achieve precision, the images were analyzed with Image J 1.46r open source software (National Institute of Health, USA: http://imagej.nih.gov/ij) to record fluorescens intensities of the AFB1 spots. A standard curve was made using linear plotting of AFB1 standard spots fluorescens (under UV light λ=366 NM) values versus AFB1 concentration (Hoeltz *et al*., 2010; Hoeltz *et al*., 2012). The experiment was designed in one way CRD and the statistical analysis was carried out by SPSS® Ver. 16.0 software.

**Bioassay for AFB1 degradation residues**

Two bacterial isolates, *B. subtilis* BSS4 and *P. fluorescens* PFMst were chosen according to the HPTLC results that showed a high ability to degrade AFB1 (approximately 100%) and to determine the effect of AFB1 degradation residues *in vivo*. The bacterial broth cultures, after incubation for 48h with 250 ng/ml of AFB1 were preceded with equal amounts of chloroform then filtrated with Whatman No. 1 filter paper using Buchner funnel. The extracts were concentrated to dryness in water bath at 40ºC and the crystallized residues were re-dissolved with the physiological solution Dimethyl sulfoxid (DMSO) in addition to the standard positive treatment involved dissolving of AFB1 (250 ng/ml) in DMSO to be obtained in the oral injection of experimental animals. The bioassay is held using 28 albino rats, *Rattus rattus* of 8 weeks age. The animals were divided into 7 groups that were exposed to 7 different treatments. The treatments were (PFMst, PFMst+AFB1, BSS4, BSS4+AFB1, DMSO+AFB1, DMSO and non treated animals as a negative control), each treatment was obtained in 4 replicates. The treatments were performed by oral gavage of 4 doses equivalent to 250 ng/kg of body weight, with an interval of 2 days, after the last dose the animals left for one month then they were scarified through opining the abdominal alveolus by dissection after

anesthetization with Di-ethyl ether. Blood samples were collected by heart puncture technique and collected blood is divided into two groups, the first one placed in EDTA containing tubes for physiological tests that carried out using an Auto Hematology analyzer (ABX Micros ES60, France), whereas the second group is placed in EDTA free tubes for biochemical tests which is carried out using Reflotron®Plus devise (Roche Ltd., Germany) **(**Gagini *et al.*, 2010; El-Hady *et al.*, 2011; Al-Saadi, 2012**).** The experiment was designed in one way CRD and the statistical analysis is carried out by SPSS® ver. 16.0 software.

## RESULTS AND DISCUSSION

The morphological identification test approved the identity of the isolate AFL14 as *A.flavus* which is confirmed by molecular identification results which revealed that 100% alignment between ITS1-ITS2 PCR product sequence with the standard isolate (CBS 100558; ID:gb|KJ175473.1|) and 100% alignment between ITS3-ITS4 PCR product sequence with the standard isolate (M1204.653; ID:gb|KJ175474.1|) in NCBI gene bank.

### Bacterial isolate

The biochemical test results of *B. subtilis* and *P. fluorescens* isolates presented in tables (1 and 2) respectively were consistent with the same test results in Henayl, (2000), Barrow and Filtham (2003), Forbes *et al.* (2007) which confirmed the previous definition conducted in advance by the source donors.

**Table (1): Biochemical tests of *B. subtilis*.**

| Isolate | Gram stain | Shape | Spores | Oxidase | Catalase | Gelatine | IMVC | | | | NaCl 6.5% | Amelase |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Indol | MR | VP | Citrate | | |
| BSS1 | + | + | + | + | + | + | - | + | + | + | + | + |
| BSS2 | + | + | + | + | + | + | - | + | + | + | + | + |
| BSS3 | + | + | + | + | + | + | - | + | + | + | + | + |
| BSS4 | + | + | + | + | + | + | - | + | + | + | + | + |
| BSW | + | + | + | + | + | + | - | + | + | + | + | + |

**Table (2): Biochemical tests of *P. fluorescens*.**

| Isolate | fluoresces under UV | Gram stain | shape | Oxidase | Catalase | Gelatine | IMVC | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Indol | MR | VP | Citrate |
| PFMst | + | - | + | + | + | + | - | - | - | + |
| PFDL | + | - | + | + | + | + | - | - | - | + |


**Fig. 1: TLC plate of bacterial degradation of AFB1.**

The quantitative analysis of TLC plates captures (Figure 1 and 2) revealed the high ability of all bacterial isolates to degrade AFB1 in liquid medium. The degradation percentage ranged from (95.56-100%) with a significant differences among isolates. BSS4, BSW and BSS3 exceeded the rest of isolates significantly with no significant differences among them followed by PFMst and PFDL which surpassed BSS1 whereas BSS2 possessed the lowest degradation ability. The extracellular degradation of AFB1 is mostly attributed to the extracellular enzymatic activity of the bacteria which is usually depended on the level of the genes that controlling the production of those enzymes (Reddy *et al.*, 2010; Afsharmanesh *et al.*, 2014) adding to that, there are many bacterial species (*B.subtilis*, *Escherichia.coli* and *Lactococcus lactis*) posses a high ability to detoxify AFB1 in liquid medium by binding AFB1 to bacterial cell wall due to the intimacy between them which is lead to detoxify aflatoxin B1 (Al-Jumaily, 2014).

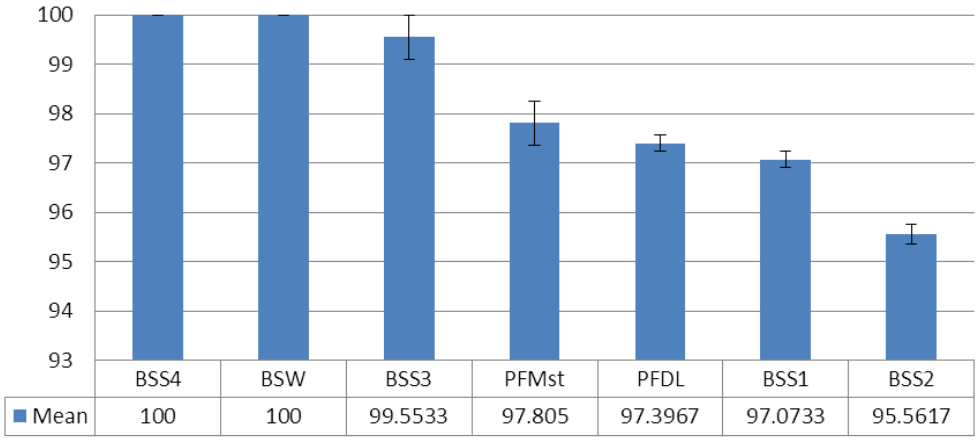


**Fig. 2: AFB1 degradation (%) by bacterial isolates. LSD=0.765 at P≤ 0.05**

**Bioassay for AFB1 degradation residues**

**Blood physiological measurements**

The results of the several physiological parameters of blood were considered to determine the impact of AFB1 and its degradation side products on biological systems (rat was our model). The parameters included:

**White blood cells (WBCs)**

The number of WBCs was influenced by the treatment derivatives that administered to the animals (Figure 3). AF+DMSO showed the lowest WBCs rate ($1.6\times10^9$/L) that was significantly differed from all other treatments, followed by (-) Control, PFMst, DMSO, PFMst+AF (7, 7.966, 9.6 and $10.35\times10^9$/L) respectively with no significant differences among them, in addition to significant increase in WBCs number in BSS4+AF ($14.375\times10^9$/L) treatment compared to AF+DMSO, (-) Control and PFMst (1.6, 7 and $7.966\times10^9$/L ) respectively and similar increase in BSS4($12.6\times10^9$/L) compared to (AF+DMSO and (-) Control) with no significant differences between BSS4+AF and BSS4. Generally, the results revealed that WBCs levels were not affected by *P.fluorescens* in opposite to *B.subtilis* and its AFB1 degradation derivatives which caused WBCs levels to be doubled. This may be an indication that the immune system had not been affected in addition to that the bacterium itself may have antigens stimulated immune activity toward it.

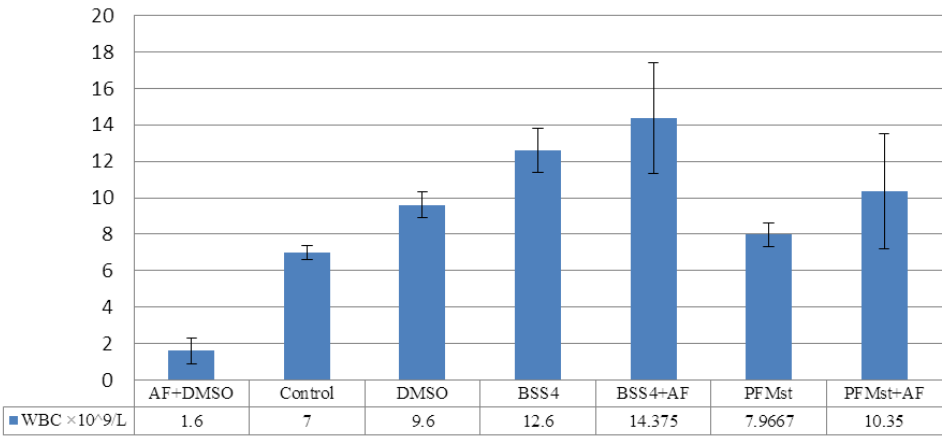


**Fig. 3: The effect of interaction of AFB1×biocontrol agents on WBCs levels. LSD=4.204 at P≤ 0.05**

The reduction in WBCs levels in AF+DMSO treatment could be returned to the deterioration of the immune system of the rat as AFB1 is well known as immunosuppressant (Mogilnaya *et al.*, 2010; Anokwuru *et al.*, 2011). The effect of AFB1 on the number of the WBCs found to be dose dependent due to stimulation or suppression of the immune system (Hinton *et al.*,

2003; Mogilnaya *et al.*, 2010; Anokwuru *et al.*, 2011). Our study corresponded with previous studies which reported a significant decrease in WBCs count due to AFB1 activity (El-Shewy and Ebrahem., 2004; Mogilnaya *et al.*, 2010; Omoya *et al.*, 2014).

**Hemoglobin (HB)**

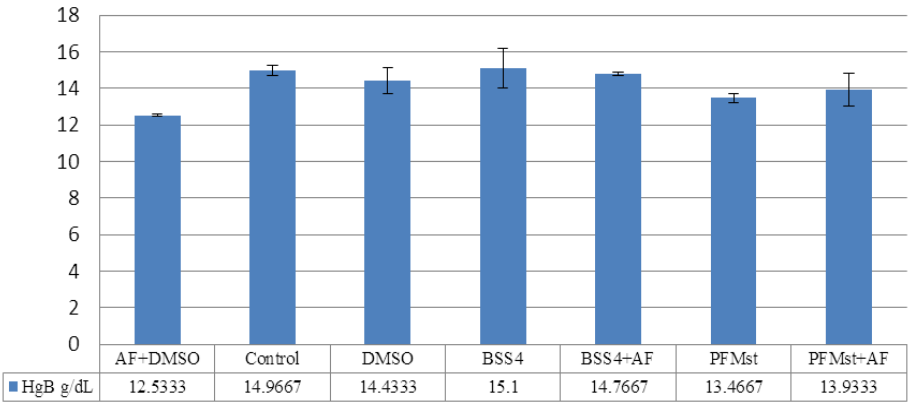


**Fig. 4: The effect of interaction of AFB1×biocontrol agents on HB levels. LSD=1.840 at P≤ 0.05**

AF+DMSO treatment displayed a significant reduction level (12.533 g/L) compared to DMSO, BSS4+AF, (-) Control and BSS4 (14.433, 14.766, 14.966 and 15.1g/L), respectively with no significant differences with PFMst and PFMst+AF treatments (13.466 and 13.933 g/L) respectively (Figure 4). All other treatments had no significant differences among them. The reduction of HB could be caused as a result of oxidative damage due to the activity of AFB 1 which is reported to increase auto-oxidation rate of oxyhemoglobin in addition to haemolysis (Abdel-Wahhab *et al.*, 2014) or due to the binding of AFB1 with the cell membrane proteins which is affect the energy supply (ATP) to the red blood cells, as well as the transfer of sugar in to the cells which is eventually lead to shorten the cell life and death (Cloherty *et al.*, 2001) . Kubena *et al.*(1997) found that secondary metabolic products of *A.flavus* (aflatyoxins: B1, B2, G1and G2) have the ability to bind with blood proteins that are responsible for the biosynthesis of red blood cells leading to the lake in cell numbers which is affect the balance of the blood (Hemostasis).

**Red blood cells (RBCs)**

A significant reduction in RBCs number was observed in AF+DMSO treatment (Figure 5) compared to (BSS4+AF, DMSO and (-) Control) respectively, those treatments, on the other hand did not show a significant differences among them. BSS4 and PFMst also recorded a significant reduction in RBCs level compared to ((-) Control and DMSO) treatments, respectively while BSS4 reduction rate was significantly lower than (BSS4+AF, DMSO, (-)

Control) respectively. No significant differences observed among AF+DMSO, PFMst and BSS4 which were ordered respectively. RBCs reduction could be resulted in blood haemolysis that may be caused due to the potential of AFB1 (Abdel-Wahhab *et al.*, 2014).

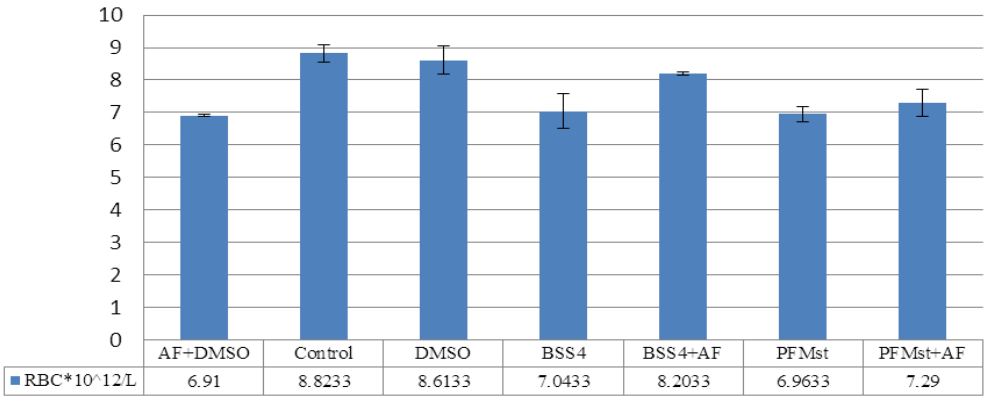


**Fig. 5: The effect of interaction of AFB1×biocontrol agents on RBCs levels. LSD=1.002 at P≤ 0.05**

**Hematocrit (HTC% or P.C.V.)**

Figure (6) displayed that P.C.V. rate of AF+DMSO treatment was lower than all other treatments with significant differences compared to (DMSO, (-) Control, BSS4 and BSS4+AF) respectively and not significantly differed than (PFMst+AF and PFMst), respectively. No significant differences were observed among all other treatments. The percentage of red blood cells reduction may be related to the impact of AFB1 which causes chronic blood haemolysis that leads to cause folate deficiency that impaired the DNA synthesis and nuclear development causing anaemia which reduces P.C.V. level (Golder , 2007; Mandal *et al.*, 2013; Abdel-Wahhab *et al.*, 2014).

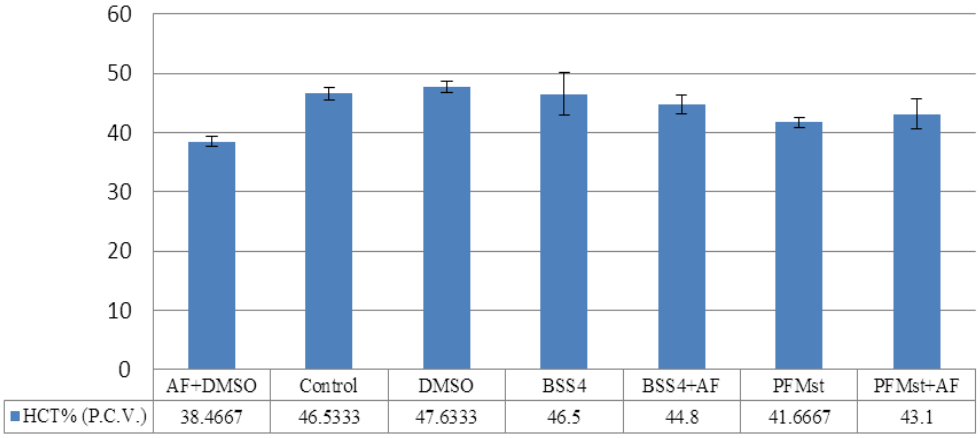


**Fig. 6: The effect of interaction of AFB1×biocontrol agents on P.C.V. levels. LSD= 5.86 at P ≤ 0.05**

**Platelet count (PLT)**

The results of platelet count (Figure 7) showed no significant differences among positive and negative control comparing to the rest of experiment treatments included AF+DMSO treatment. The only significant difference was between BSS4+AF (760.22×$10^9$/L) and PFMst (496.67×$10^9$/L). These results were similar to Sharma *et al.* (2011) results on mice as they found no significant differences in platelet count in blood of mice when they exposed to 2ug/kg of body weight of AFB1 comparing to control treatment.

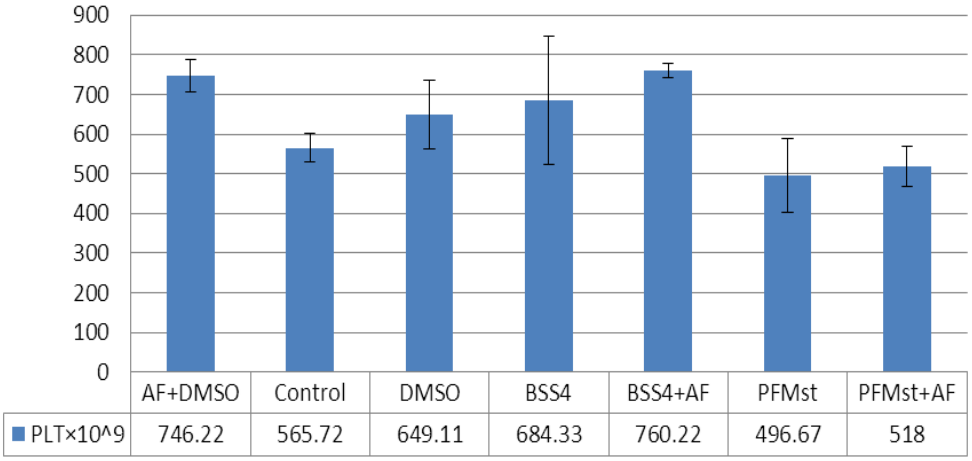


**Fig. 7: The effect of interaction of AFB1×biocontrol agents on platelet count. LSD=252.03 at P≤0.05**

**Mean corpuscular volume (MCV)**

The results in figure (8) showed that MCV parameter under AF+DMSO treatment (61.466 fL) was significantly exceeded all other treatments included (positive and negative) control treatments followed by PFMst and PFMst+AF (59.9667 and 59.2667 fL), respectively with no significant differences between them. BSS4+AF (52.667 fL) displayed no significant difference either with control or DMSO treatments. The increase in corpuscular volume known to be related to folic acid deficiency (Golder, 2007). This result accompanying with reduction in WBC, RBC, HB and P.C.V may led to believe that AFB1 caused a megaloplastic anemia to the treated rats (Golder, 2007).

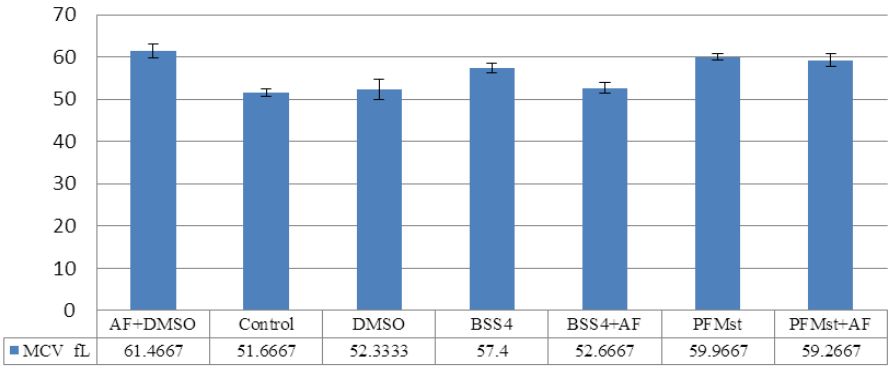


**Fig. 8: The effect of interaction of AFB1×biocontrol agents on MCV levels. LSD= 4.392 at P≤0.05**

**Blood biochemical measurements**

**Urea**

A significant increases in urea levels were observed in all treatment compared to (-) control and DMSO treatments (26.133and 31.933 mg/dl) respectively (Figure 9) giving an indication of renal malfunction as the rate of renal clearance becomes lower than blood urea production which in turn can be considered as nephrotoxicity indicator (Mandal *et al.*, 2013). AF+DMSO treatment (42.933 mg/dl) exceeded BSS4+AF treatment (33.666 mg/dl) significantly whereas it was retracted significantly against PFMst+AF and PFMst (46.066 and 45.133 mg/dl) respectively and that may be returned to the ability of *P.fluorescens* to produce toxic compound and/or that the degradation residues had a toxic potential on kidney. PFMst+AF, PFMst, BSS4 were surpassed BSS4+AF treatment significantly with no significant differences among them. Those results agreed with the past studies that reported a high urea levels after treating with AFB1 (El-Shewy and Ebrahem., 2004; Devendran and Balasubramanian, 2011).

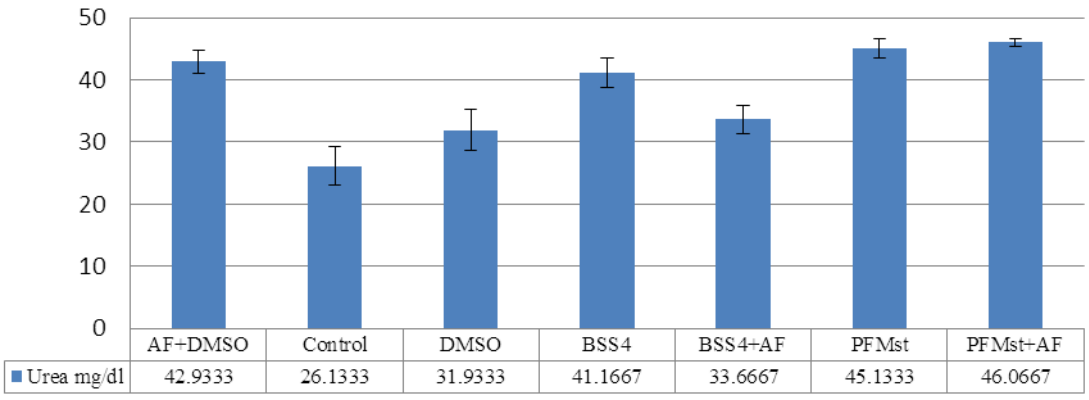


**Fig. 9: The effect of interaction of AFB1×biocontrol agents on Urea levels. LSD=7.408 at P≤0.05**

**Glutamic pyrovate transaminase (GPT) and Glutamic oxaloacetic transaminase (GOT)**

The GPT results displayed in Figure (10) revealed the superiority of AF+DMSO (39.433 U/L) over all other treatments significantly followed by BSS4+AF (27.833 U/L ) respectively while (Figure 11) demonstrated that GOT levels in AF+DMSO (402 U/L) treatment was exceeded all the other treatments. These results reflected the toxic effect of AFB1 to the liver tissue as it is lyses the hepatic cells membrane that resulting in the releasing of GPT and GOT to the blood which leads to elevate their levels. Moreover, aflatoxins may affect other organs like kidney and endoplasmic reticulum that contained those enzymes which may lead to same results (Al-Saadi, 2012). The results of our study corresponded with the earlier studies that showed similar effect of AFB1 represented by the elevation of GPT and GOT levels (El-Shewy and Ebrahem., 2004; Mishra and Kishore, 2009; Al-Saadi, 2012).

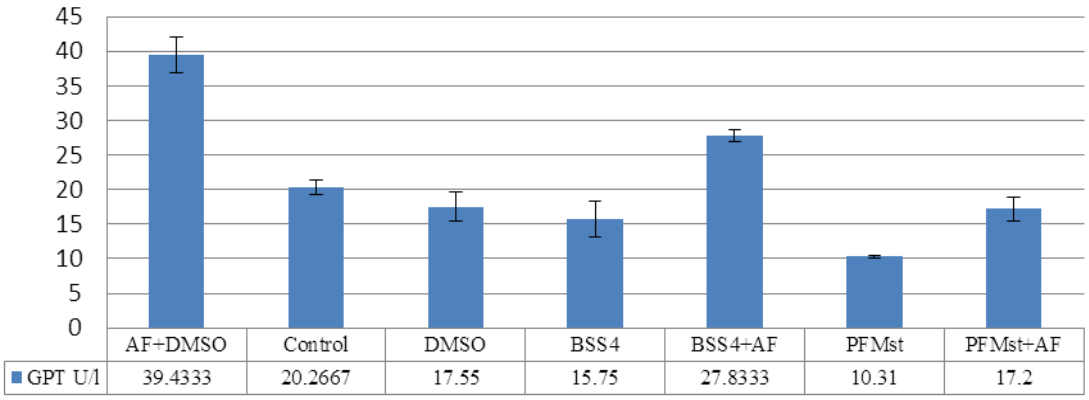


**Fig. 10: The effect of interaction of AFB1×biocontrol agents on GPT levels. LSD=6.302 at P≤ 0.05**

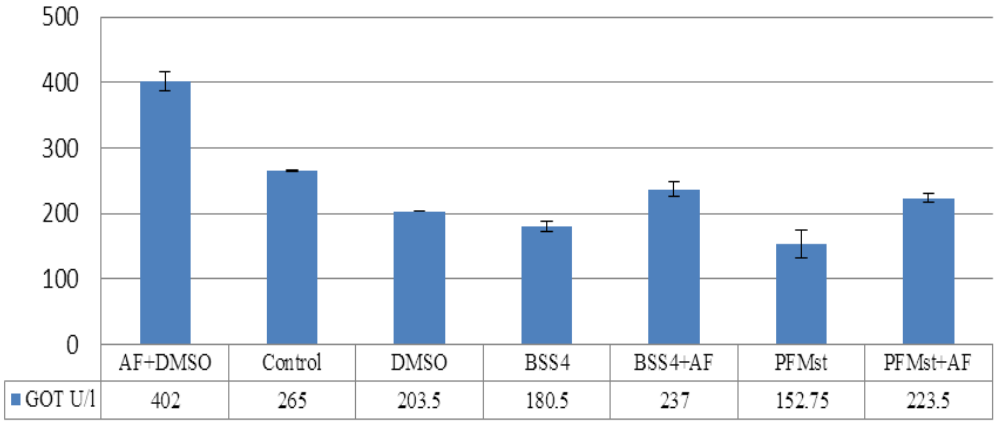


**Fig. 11: The effect of interaction of AFB1×biocontrol agents on GOT levels. LSD=39.129 at P<=0.05**

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