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Molecular Characterization and Expression Analysis of *aflR*, *aflS*, and *aflD* in Non-Aflatoxigenic and Aflatoxigenic *Aspergillus flavus* Treated with Gallic Acid.

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Abstract

In this study, from five *Aspergillus flavus* strains, only three strains have been aflatoxin B producers. Gallic acid as antioxidant was used to assay its potential in aflatoxin diminishing. Gallic acid treated aflatoxin B producing isolates showed a slightly inhibition in aflatoxin production and a remarked diminishing in spore formation and growth as compared with untreated isolates. Three genes, *aflR*, *aflS*, and *aflD* were successfully amplified by a conventional polymerase chain reaction in aflatoxigenic and gallic acid treated *Aspergillus flavus* strains. These genes have been sequenced and deposited in Genbank under the accession numbers LC537158, MW055253, and LC537157, respectively. It has been demonstrated that there was no difference in nucleotide sequences in the amplified fragments of these genes in both aflatoxigenic and gallic acid treated *Aspergillus flavus* isolates, qRT-PCR was employed to test the effect of gallic acid on the transcription of *aflR*, *aflS*, and *aflD* genes and that ensured the negative effect of gallic acid on these genes transcription and therefore production of aflatoxin production

Keywords :*Aspergillus flavus*, conventional PCR, Gallic acid, qRT- PCR

1. Introduction

Production of acute toxic, mutagenic, teratogenic, or estrogenic responses in higher vertebrates was reported via mycotoxin exosure such as Aflatoxins (AF) (Jelinek*et al.*, 1989); they are secondary metabolites, poly-ketide that have been produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxins are carcinogenic and contaminate food and feed worldwide (Bhatnagar*et al.*, 1987). The complete elucidation of the gene cluster involved in aflatoxin biosynthesis in *A. parasiticus* was achieved by Yu and Ehrlich (2002) and Yu *et al.* (2004); another study conducted by O'Brian *et al.* (2007) demonstrated the role of microarray to explain the regulation of aflatoxin biosynthesis genes.

Aflatoxin Genes in *A. flavus* and *A. parasiticus* have highly homologous sequences and the same order within the cluster. Aflatoxin B1 (AFB1) and B2 (AFB2) are known to be produced by *A. flavus* strains, whereas *A. parasiticus* produces aflatoxins B1, B2, G, and G2 (Giorni*et al.*, 2007). Yan *et al.* (2012) reported the opportunistic and pathogenic infection of *A. flavus* for humans and animals besides its pathogenicity for plants. In the 1960s, aflatoxins, the basic reason for Turkey-X disease, were reported to be mainly produced by *A. flavus* (Nesbitt *et al.*, 1962). While aflatoxins are considered the first fungal secondary metabolites shown to have all genes organized within a DNA cluster, there have since been significant efforts to realize regulatio n mechanism involved in aflatoxin biosynthesis.

Several interconnecting networks are involved in the regulation of AF biosynthesis which can be divided into three parts; the most important one comprises the regulation in the AF biosynthetic by *aflR* and *aflS*, positioned adjacent to each other in a 70 kb DNA cluster (Desjardins and Proctor, 2007).These genes are differentially transcribed with independent promoters; with short intergenic regions that share binding sites for regulatory elements or other transcription factors (Ehrlich *et al.*, 2005). Woloshuk *et al.* (1994) and Yu *et al.* (1996) reported that a putative 47-kDa protein encoded by the gene of *aflR*, has a similar sequence to a zinc binucleate cluster DNA-binding protein, Todd and Andrianopoulos (1997) classified these proteins and renamed them as Zn (II) 2Cys6 proteins. *aflR* is a remarkable gene in AF biosynthesis for the following discoveries: Yu *et al.* (1996) take up *aflR* from *A. flavus* to drive ST cluster expression in an *A. nidulans* which lack *aflR* despite clear differences in AF biosynthesis pathway. Another study conducted by Lee *et al.* (2007) announced the presence of differences in PacC and AreA binding sites, promoter regulatory elements for *aflR* in *A. parasiticus* and *A. flavus* aflatoxin biosynthesis. Carbone *et al.* (2007) reported the presence of conserved domain of the *aflR* gene in both *A. nidulans* and *A. fumigatus*.

Another gene, *aflS*, has a precise role in AF biosynthesis. A study by Meyers *et al.* (1998) observed

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unchanged mRNA expression levels of genes, *aflC*, *aflD*, *aflM*, and *aflP* in an *aflS* disrupted strain, with all this; AF pathway intermediates could not be converted to aflatoxin. Regarding a relationship between AFLS (a protein encoded by *aflS*) and AFLR (a protein encoded by *aflR*), Chang (2004) showed that activation of AFLR requires AFLS binding in *A. parasiticus*. However, in *A. flavus*, there was no need of AFLS to activate AFLR in AF biosynthesis (Du *et al.*, 2007), while they explained the roles of *aflR* and *aflS* as follows: transcription of early and mid-aflatoxin pathway genes require AFLR, but AFLS enhances this pathway.

Several environmental and cultural conditions such as light, temperature, pH, nitrogen, carbon source, and metals can modulate AF biosynthesis (Calvo *et al.*, 2004; Price, 2005). Over the past decade, research proved the increase in AF production by oxidative stress (Reverberi *et al.*, 2008); antioxidants, for example, Gallic and caffeic acid reduce AF production via expression reduction or inhibition of some AF pathway genes such as *aflD* (nor-1) (Mahoney and Molyneux, 2004). Kim *et al.* (2008) announced the down-regulation of the most genes in AF biosynthesis due to caffeic acid treatment using microarray technique. Reverberi *et al.* (2008) also used glucans from Lentinulaedodes to induce the antioxidant enzymes which subsequently caused to delay in *aflR* transcription as well as AF cluster genes. Several studies conducted different genes assigned in aflatoxin biosynthesis, for example, The *nor*-1(*aflD*), *apa*-2, and *omt*-1 (*omtA*) genes by Shapira *et al*. (1996), The *omt*-1, *nor*-1, and *ver*-1 genes individually (Färber *et al.*, 1997) and *aflR*, *aflJ*, and *omtB* genes (Rahimi *et al.*, 2008).

The objective of this study was to investigate the *aflR*, *aflS*, and *aflD* genes profile in aflatoxigenic and non-aflatoxigenic isolates of the *A. flavus* using genomic DNA as a template by traditional PCR and assay the potential of gallic acid in aflatoxin suppression through down regulation of *aflR*, *aflS*, and *aflD* using Rti-PCR technique

2. Materials and Methods

2.1. Culture Conditions and Fungal Strains

Five strains of *A. flavus* isolated from contaminated food, used in this study, were friendly provided from the toxicology department, National Research Centre, Egypt. About 10^3 spores *A. flavus* were inoculated into 250 mL Erlenmeyer flasks containing 50 mL Potato dextrose broth (PDB), cultured for three days at 28^OC, 200 rpm incubator (thermoscientific, UK) and Potato dextrose agar (PDA Difco) at 28^OC for three for further analysis.

2.2. Aflatoxin Analysis Using High Performance Liquid Chromatography (HPLC)

A total of 10^3 *A. flavus* spores were inoculated on PDB medium and cultured at 28^oC, 200 rpm incubator (thermoscientific, UK) for three days. Aflatoxin was extracted from 50 mL culture medium for each sample by high performance liquid chromatography according to (Yu *et al.*, 2004 and Salim *et al.*, 2019).

2.3. Investigation the effect of gallic acid on aflatoxin production and colony diameter

A. flavus isolate 5 (the highest aflatoxin producer) was cultured on both PDB for aflatoxin analysis, and PDA media for measurement of colony diameter, supplemented with a concentration of 1% gallic acid (w/v), for three days at 28^oC, but only, 200 rpm shaking conditions in case of PDB (treatment) in parallel with media without gallic acid (control).

2.4. Isolation, Molecular Detection and Sequencing of aflR, aflS and aflD Genes

2.4.1. Extraction of Genomic DNA from Aspergillus flavus Isolates

One strain of aflatoxigenic and another of non-aflatoxigenic *A. flavus* strains were separately grown on a100 mL conical flask ((Pyrex, USA) containing 20 ml PDB without 1% (w/v) GA, (control) and with GA (treatment). Incubation was at 28 C for 3-7 days. About 100 mg mycelium was scraped off and used for genomic DNA extraction as follows: it was ground to a fine powder with liquid nitrogen, fine powder was subsequently put into a 1.5 mL sterile Eppendorf. Plant Genomic DNA Miniprep Kit (QIAamp DNA Mini Kit, QIAGEN, Germany) was used to extract DNA. DNA was used as a template for PCR amplification of *aflR*, *aflS*, and *aflD* for both aflatoxigenic and non-aflatoxigenic isolates.

2.4.2. Primer Design and PCR Optimization

Simultaneously, based on (http://frodo.wi.mit.edu/primer3/), all primers used for detection, and sequencing of aflatoxin genes were developed using NCBI reference sequences. These primers are listed in Table 1. The primers were synthesized by HVD life sciences GMBH, Germany. Genomic DNA of *A. flavus*, as a template, was conducted with GeneAmp PCR system (PerkinElmer, Norwalk, Connecticut, USA). According to the method described by Ausubel *et al.* (1990), amplification was carried out in a 50 µl reaction mixture using a PCR master mix kit (Qiagen, Germany). The following program was used: 94^oC for 3 min as initial denaturation step, 35 cycles start with 94^oC for 30 sec for denaturation, 55^oC for 30 sec for annealing and 72°C for 30 sec for extension, finally, an extension step at 72^oC for 10 min. The PCR products were separated on 1% agarose gel using TAE buffer 1X (pH = 8.3) and run at 80 V for 45 min, the bands were isolated and purified after agarose gel electrophoresis using a gel extraction kit (Qiagen, Germany). Purified gene fragments were sent to (Clinilab, colors lab, Egypt) for sequencing. The obtained sequences were compared to other known sequences found in Genbank database via the Blast program (http://www.ncbi.nlm.nih.gov/BLAST/).

Table1. The primers used in PCR amplification of *aflR*, *aflS*, and *aflD*

primers	Sequence (5´ →3´)	NCBI reference sequence
AFLR _F	GGATGAGGAAGACCAGCCGC	AY650938
AFLR _R	CCTGTCATCTGCTCCTGGCG	
AFLS _F	GGCCGAAGATTCCGCTTGGA	FN398168
AFLS _R	GAGCGAGGGCAACAACCAGT	
AFLD_F	CTGACGGCGTACGGAGTGTC	MH280091
AFLD_R	GAGCACAGATGCCTGCCACA	

Notes: ((AFLR _F and AFLR _R, are forward and reverse primers for *aflR* PCR amplification, AFLS _F and AFLS _R, are forward and reverse primers for *aflS* PCR amplification, and

AFLD _F and AFLD _R, are forward and reverse primers for *aflD* PCR amplification) .

The obtained Sequences were translated to amino acids using https://web.expasy.org/translate/. Deduced protein sequences of all genes were aligned using CLUSTAL multiple sequence alignment using MUSCLE 3.8 analysis according to Thompson *et al*. (1994).

2.4.3. Isolation of Total RNA

Total RNA was isolated from GA untreated aflatoxigenic *A. flavus* (control) and GA treated one (treatment) by following the manufacturer's instructions of standard TRIzol® Reagent extraction method (cat#15596-026, Invitrogen, Germany). Purity assessment of total RNA was done by the 260/280 nm ratio between 1.8 and 2.1(https://www.agilent.com/cs/library/applications). Additionally, visualization of 28S and 18S bands was used to assure RNA integrity via formaldehyde-containing agarose gel electrophoresis. For reverse transcription (RT), Aliquots of RNA were used immediately (Mahrous *et al*., 2020).

2.4.4. Reverse Transcription (RT) Reaction and cDNA Synthesis

According to the manufacturer's instructions of RevertAid^{TM} First Strand cDNA Synthesis Kit (MBI Fermentas, Germany), RNA isolated control and treated samples were reverse transcribed into cDNA in a total volume of 20 µl. The thermocycler (Biometra GmbH, Göttingen, Germany) was used for carrying out the RT reaction at 25°C for 10 min, followed by 1 h at 42 °C, and the reaction was stopped by heating for 5 min at 99 °C. Then, the reaction tubes were flash-cooled in an ice chamber until getting used for DNA amplification via qRT-PCR .

2.4.5. Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

StepOne™ Real-Time PCR System from Applied Biosystems (Thermo Fisher Scientific, Waltham, MA USA) was used to determine the control and treated samples of fungal genes transcripts. Sequencing results of different genes were used to design gene specific primers, for qPCR by using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). PCR reactions were set up in 25µL reaction mixtures containing 12.5 µL 1× SYBR® Premix ExTaqTM (TaKaRa, Biotech. Co. Ltd.), 0.5 µL of 0.2 µM sense primer, 0.5 µL of 0.2 µM antisense primer, 6.5 µL distilled water, and 5 µL of cDNA template.

The reaction program was allocated to 3 steps. Firstly, it started at 95.0°C for 3 min. Secondly, 40 cycles in which each cycle divided to 3 steps: (a) at 95.0°C for 15 sec; (b) at 55.0°C for 30 sec; and (c) at 72.0°C for 30 sec. thirdly, 71 cycles started at 60.0°C and every 10 sec, it increased about 0.5°C until reach to 95.0°C. Each experiment included a distilled water control. GAPDH (housekeeping gene) was used as a control gene for differences in total cDNA input between samples. To check the quality of the used primers, a melting curve analysis was performed at 95.0° at the end of each qRT-PCR. We were careful to make three replicates for each experiment. The relative amount of the genes of interest was calculated according to the method described by Ruijter *et al*. (2009).

2.5. Statistical Analysis

All the wet-lab experiments were conducted in triplicate. The data were subjected to analysis of variance and Duncan's multiple rang test was used to differentiate means at 5% (Duncan, 1955). The error bars in all figures indicate the standard error of the mean.

3. Results

3.1. Identification of Aflatoxin production Using High Performance Liquid Chromatography (HPLC) in A. flavus

Direct extraction of aflatoxins from *A. flavus* cultures filtrates via chloroform and subsequent analysis by (HPLC), enables us to identify of AFB1, and AFB2 from three *A. flavus* isolates. The total AF of these strains are reported in (table 2) as follows; isolate 1 (11.17 ug/ml), isolate 2 (26.65 ug/ml), and isolate5 (34.97 ug/ml). The concentrations of aflatoxin (ug/ml) were calculated in three replicates of cultures average for every isolate and expressed as mean ± SEM (Standard Error of the Mean). Simultaneously, these toxins were not detected in isolate 3 and isolate 4. In this research, Isolate 5, as aflatoxigenic and isolate 3 as non-aflatoxigenic samples were taken as examples to elucidate the molecular differences on the level of *aflR*, *aflS*, and *aflD* genes in both of them and investigate the effect of GA on aflatoxin biosynthesis in *A. flavus* strain no.5.

Table 2. Aflatoxins production from *Aspergillus flavus* isolates

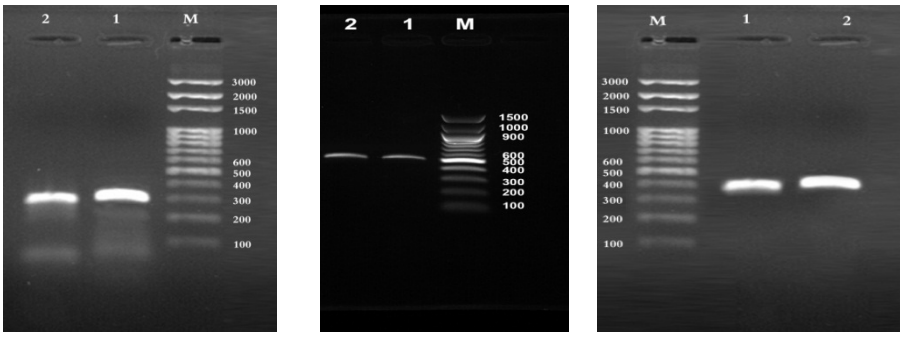
Fungal isolates	sample name	Production of mycotoxins	Mycotoxins types	Mycotoxin production ug/ml	Total Mycotoxins production ug/ml
A. flavus	isolate 1	+	AFB1, AFB2	8.63± 2.1	11.17
		+		2.54± 0.56	
	isolate 2	+	AFB1, AFB2	18.22±3.2	26.65
		+		8.43±1.7	
	isolate 3	-	--	ND	ND
	isolate 4	-	--	ND	ND
	isolate 5	+	AFB1, AFB2	23.30±3.12	34.97
		+		11.67± 2.54	

ND: Not Detectable, +: present, -: not present

3.2. Molecular Identification and Sequencing of aflR, aflS and aflD in Aflatoxigenic and non-Aflatoxigenic A. flavus isolates

Interestingly, unexpected results were observed, whereas AFLR _F and AFLR_R specific primers succeeded in *aflR* amplification at ~320 bp in both aflatoxigenic and non-aflatoxigenic *A. flavus* isolates figure (1a), the same situation occurred in *aflS*, ~550 bp using AFLS _F and AFLS_R specific primers figure (1b), and *aflD*, ~420 bp using AFLD _F and AFLD_R specific primers figure (1c) using genomic DNA as a template.

These amplified bands were isolated, purified from agarose gel, and subjected to nucleotide sequencing. The amplified regions of the isolated genes using the previously mentioned primers showed no differences in nucleotides sequences in the case of aflatoxigenic and non-aflatoxigenic isolates. Nucleotide sequences of genes were submitted to (https://www.ncbi.nlm.nih.gov/) and took accession numbers LC537158, MW055253, and LC537157 for *aflR*, *aflS* and *aflD*, respectively.



a **b** **C**

Figure 1. Agarose gel electrophoresis of PCR amplification (a): *aflR*, ~320 bp in aflatoxigenic isolate 5 (lane1) and non-aflatoxigenic isolate 3 (lane 2). (b): *aflS*, ~550 bp in aflatoxigenic isolate 5 (lane 1) and non-aflatoxigenic isolate 3 (lane 2). (c): *aflD*, 420 bp in non-aflatoxigenic isolate 3 (lane 1) and aflatoxigenic isolate 5 (lane 2)

CLUSTAL multiple sequence alignment (https://www.ebi.ac.uk/Tools/msa/) was used to align the deduced protein sequences of the isolated genes. Figure (2) showed that deduced protein sequence of *aflR* (our isolated gene) that consists of 86 amino acids, had 98.8%, and 100.0% identity in 86 residues overlap With AFLR deduced amino acids sequence accession numbers, AAM02997 and AAM02991, respectively, while figure (3) illustrated 94.7% and 96.5% identity between *aflS* (our isolated gene) and that of AFLS accession numbers FN398166 and AF077975, respectively. Identity of 100.0% and 90.6% were detected in 138 residues overlap between *aflD* (in this study) and that of AFLD accession numbers AXG50934 and CAZ61375 as shown in Figure (4)

```
AAM02997.1      MVVLIVLKVLAWYAAAAGTQCTSTAAGGETNSGSCSNSPATVSSGCLTEERVLHLPSMMG    360
AFLR            ------------------------------------------------------MG      2
AAM02991.1      MVVLIVLKVLAWYAAAAGTQCTSTAAGGETNSGSCSNSPATVSSGCLTEERVLHLPSMMG    360
                                                                        **

AAM02997.1      EDCVDEEDQPRVAAQLVLSELHRVQSLVNLLAKRLQEGGDDAAGIPAHHPASPFSLLGFS    420
AFLR            EDCVDEEDQPRVAAQLVLSELHRVQSLVNLLAKRLQEGGDDAAGIPAHHPASPFSLLGFS     62
AAM02991.1      EDCVDEEDQPRVAAQLVLSELHRVQSLVNLLAKRLQEGGDDAAGIPAHHPASPFSLLGFS    420
                ************************************************************

AAM02997.1      GLEANLRQRLRAVSSDIIDYLHRE        444
AFLR            GLEANLRHRLRAVSSDIIDYLHRE         86
AAM02991.1      GLEANLRHRLRAVSSDIIDYLHRE        444
                *******:*****************
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Figure2. Alignment of deduced amino acid sequences of the AFLR accession no. BCD52745, AFLR, Accession no. AAM02991 and AFLR, Accession no. AAM02997

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AFLS            ------------------------------MSETLAPSASAMGTQTRRFGASEQAEDSA     29
FN398166.1      FLCEPSPGHVAHSVLSKQFVTQPALLDAILFMSETLAPSASAMGTQTRRFGASEQAEDSA     78
AF077975.1      FLCEPSPGHVAHSVMSKQFVTQPALLDAILFMSETLAPSASAMGTQTRRFGASEQAEDSA    180
                                ********************************

AFLS            WNMAVGSDSPFAACLQQRLKVKRQLGDYLSYVSSSIDAGVEDTLTRMNWQNLGMATVVHV     89
FN398166.1      WNMAVGSDSPFAACLQQRPKVKRQLGAYLSYVSSSIDAGVEDTLTRMNWQNLGMATVVHV    138
AF077975.1      WNMAVGSDSPFAECLQQRPKVKRQLGAYLSYVSSSIDAGVEDTLTRMNWQNLGMAT---V    237
                ************ ***** ******* ****************************   *

AFLS            RILSPDRGIQPMT---NLIFM-------------------------------------     107
FN398166.1      GAQSPSLVVALAPQFPSLRFLVQTEAKAESGGHQPCLDNHGISALKLASIPLHLRARITW    198
AF077975.1      GAQSPSLVVALAPQFPSLRFLVQTEAKAESGGHQPCLDNHGISALKLASIPLHLRARITW    297
                   **.  :       .* *:
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Figure 3. Alignment of deduced amino acid sequences of the AFLS (under submission), AFLS, Accession no. FN398166 and AFLS, Accession no. AF077975

```
CAZ61375.1      TVYLVTGASRGIGRGLIEAFLQRPKSTVVACVRNVATATPALSALTVAEGSRMIIVQLNC   60
AFLD            ----------------------------------------------------MIIWHLNC    8
AXG50934.1      -VYLVTGASRGIGRGLIEAFLQHPKSTAVPCVPNVATATPALSALTYAEGSQMIIWHLNC   59
                                                                   *** :***

CAZ61375.1      DSETDAQAAVQTLREEHGVTHLDVVVANAAMATNFGPASTMPLEHLQAHMMVNMYAPVLL  120
AFLD            YSKTDAQASVQTLREEHGVTHLDLEVANAAMATNFGPASTMPLQHLQAHMMVNMHAPVLL   68
AXG50934.1      YSKTDAQASVQTLREEHGVTHLDLEVANAAMATNFGPASTMPLQHLQAHMMVNMHAPVLL  119
                 *:*****:***************: ******************:**********:*****

CAZ61375.1      FQATRLMLQQSKQQAKFVLIGAPISTITNMHDYARAPLTAYGVSKLAANYMVRKFHFENK  180
AFLD            FHATRLMLQQAKKQAKFVLIGAPIITITNMHDYARAPLTAYGVSKLAANYMVRKFHFENK  128
AXG50934.1      FHATRLMLQQAKKQAKFVLIGAPIITITNMHDYARAPLTAYGVSKLAANYMVRKFHFENK  179
                *:*********:*:************ ************************************

CAZ61375.1      WLTAFIIDPGHVQTDMGDQGARLMGRPQAPTTVADSVAGICARIDE  226
AFLD            WLTAFIIDPG-------------------------------------  138
AXG50934.1      WLTAFIIDPGHVQTDMGDQGARLMGRPQAPTTVADSVAGICARIDE  225
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Figure 4. Alignment of deduced amino acid sequences of the *aflD* gene accession no. BCD52744, AFLD, Accession no. AXG50934 and AFLD, Accession no. CAZ61375.

3.2.1. Effect of GA on Growth of Aflatoxigenic A. flavus and Aflatoxin Production

Treatment of *A. flavus* strain no. 5, as the highest aflatoxin producer, with 1% (w/v) GA caused colony diameter reduction, besides the decrease in the formation of spores after three days of cultivation figure (5A) as compared with its growth on PDA medium not containing GA figure (5B). Simultaneously, production of aflatoxin in PDB Medium containing GA with 1% concentration (w/v) was significantly reduced from 34.97 ug/ml to 8.12 ug/ml as follow; AFB1yield decreased from 23.30 to 6.09 and that of AFB2 reduced from 11.67 to 2.03 (figure 6). We concluded that AF production was directly proportional to colony diameter in *A. flavus*, i.e. GA affected the growth and AF production negatively.

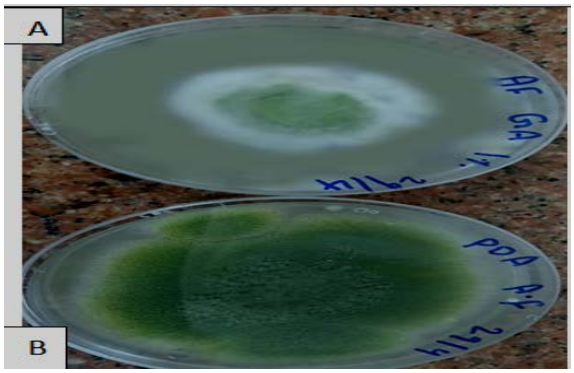


Figure 5. The effect of gallic acid on *A. flavus* growth, A: Growth of *A. flavus* on PDA containing 1% gallic acid (treatment). B: Growth of *A. flavus* on PDA (control) at 28 °C after three days.

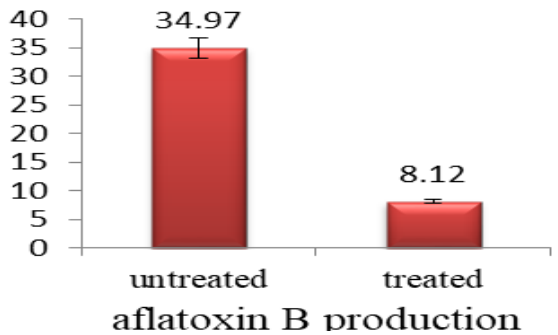
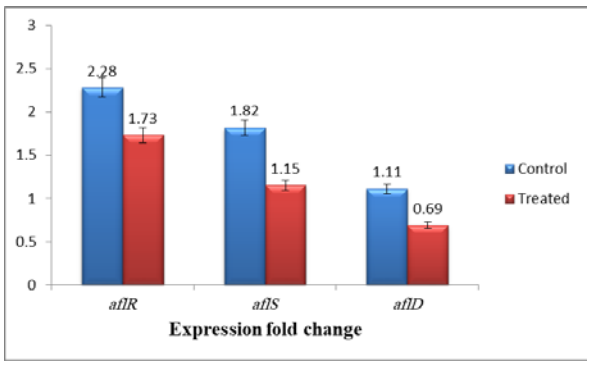


Figure 6. The effect of gallic acid on aflatoxin production from *A. flavus* isolates 5 in PDB containing 1% gallic acid (treated) compared with control at 28 °C after three days .

3.2.2. Analysis the Effect of GA on aflR, aflS and aflD Expression in Aflatoxigenic A. flavus using qRT-PCR

The increase in genes expression required for AF biosynthesis mainly depending on *aflR* transcription, supports the hypothesis that for AF-pathway induction, AFLR as transcription regulator is important, therefore, the blocking of undesirable AF production through designing approaches such as utilization of antioxidant substances requires understanding the mechanisms that are being followed by environmental and physiological factors and its effect on *aflR* transcription. From figure (7), expression analysis for transcripts of genes (*aflR*, *aflS* and *aflD)* in 1% gallic acid-treated aflatoxigenic *A. flavus* strain no. 5 indicated that these transcripts expression have to correlate GA treatment. Interestingly, all three assigned genes expressions significantly down regulated due to GA



treatment of concentration of 1% (w/v).

Figure 7. The effect of gallic acid on *aflR*, *aflS* and *aflD* expression in aflatoxigenic *A. flavus* using qRT-PCR

4. Discussion

Many Egyptian researchers directed their efforts for exploring the fungal contamination and their role in aflatoxin production in Egyptian crops. El-Shanshoury *et al*, (2014) investigated *A. flavus* in soybean, peanut seeds, wheat maize, and rice. As mentioned in the result section, the metabolic profile of mycotoxins in the aflatoxigenic *A. flavus* strains was similar to that reported in the study conducted by Rank *et al*, (2012), they announced the production of only B-types in *A. flavus*. Interestingly, the metabolism of mycotoxigenic fungi and its molecular genetics study is a good strategy to deal with mycotoxin contamination in crops (El-Kad and Youssef, 1993). It is

of interest to discover and understand the genetic differences between aflatoxigenic and non-aflatoxigenic strains. This conclusion has been employed to direct these differences to control aflatoxin biosynthesis. In this study, traditional PCR technique was used to investigate the presence of three genes; the first is *aflR*, which is considered as an indicator for aflatoxin production in *A. flavus* and its homolog in *A. parasiticus* (apaR) that regulate aflatoxin biosynthesis (Woloshuk *et al.*, 1994). Its role is to control nor-1 and ver-1 genes expression (Yu *et al.*, 2004). These genes are sufficient to stimulate transcription of early, mid, and late AF pathway biosynthesis genes. The second is *aflS* that positively regulates biosynthesis of AFB2 and the third gene is *aflD*, (formally, nor-1) as an important structure gene in aflatoxin cluster genes encodes norsolorinic acid reductase to convert norsolorinic acid to averantin (Payne *et al.*, 1993). To connect the presence of *aflR*, *aflS*, and *aflD* genes profiles in aflatoxigenic and non-aflatoxigenic, a single set of PCR primers was used to detect these genes and reliably succeed in amplification of these genes in aflatoxigenic and non-aflatoxigenic *A. flavus* strains. These conflicting results can be explained as follows: specific mutation may occur in *aflR* , and resulted in malfunction gene and subsequently failure in aflatoxin production in non-aflatoxigenic isolates even though in the presence of functional copies of *aflD* and *aflS*. In a similar study conducted by Bok and Keller, (2004), they investigated and explained the block in *omtA* (a structural gene in AF biosynthesis expression in *A. flavus* although presence of AFLR, a protein encoded by *aflR* as follows: the presence of specific mutation in *aflR* resulted in nonfunctional AFLR, therefore, *omtA* expression did not take place. However, Liu and Chu, (1998) explained this phenomenon by the presence of a mutation in DNA binding site in *omtA* gene caused to not producing *omtA* mRNA. Similar nucleotide sequences of detected genes between aflatoxin producing and nonproducing strains told no information about the characterization of these genes. Consequently, this research concluded that successful PCR amplification of aflatoxin genes should not be considered as a proof of aflatoxin synthesis due to undetected mutation external to the amplicon sequence that subsequently caused cryptic and not expressed genes. The study conducted by Patterson (2006), demonstrated that the *aflR* gene may be present in a number of non-aflatoxigenic and functions for conserved regulation of aflatoxin precursors. Twelve strains of *A. flavus* were investigated by Shapira *et al.* (1996), nine of them were non-aflatoxin producers in which some genes involved AF biosynthesis were successfully PCR amplified in varying band patterns; however, only three strains were aflatoxin producers in which the expected amplicon bands were produced. From the previous notifications, the presence of un-expressed genes in non-aflatoxigenic strains may be explained by the occurrence of non-functional gene products due to base-pair substitution mutations.

Our attempts were directed to inhibit the AF production by reducing the oxidative stress using antioxidant such as Gallic acid which proved its efficiency in diminishing of AF production in GA treated *A. flavus* isolate. In order to gain insight into the mechanism by which GA inhibits aflatoxin synthesis, we employed qRT-PCR to assess the expression of *aflS*, *aflR*, and *aflD*. Down regulation of these genes in GA treated sample as compared with untreated one explain the reason of diminishing of aflatoxin production. Mahoney and Molyneux, (2004) illustrated the role of GA in the inhibition of expression of several genes in AF pathway such as aflM (ver-1). Another study was conducted by Zhao *et al*, (2018) , they used gallic acid as an antioxidant agent to reduce aflatoxin production. Addition of gallic acid in different doses (0.5%, 0.8%, and 1%) slightly inhibited aflatoxin and growth of *A. flavus*; they showed the inhibition of AF by 0.8% of GA on farB gene encodes a factor for transcription of β-oxidation of peroxisomal fatty acid which contributes in AF biosynthesis. Another gene, creA, the carbon repression regulator encoding gene and necessary for aflatoxin biosynthesis, was also inhibited by GA treatment. Total inhibition for AF by gallic acid treatment was through the control of polyketide synthase (fatty acid synthase) required for the formation of norsolorinic acid, the first intermediate in the biosynthesis pathway of aflatoxin. It is striking that we noticed incompatibility between our results and the results of Zhao *et al*, (2018) in terms of the effect of GA on the expression of *aflR* and *aflS*, gallic acid concentration of 0.8% (w/v) inhibited nearly all the genes of aflatoxin except *aflR* and *aflS*.

In our research, taking these results together, we proposed that the down regulation of *aflR* as a master gene in AF regulation may result in down regulation of other genes such as *aflS* and *aflD*; this explanation is agreed with Cotty (2006) who acknowledged that introduction of an additional correct copy of the *aflR* compensates for the disrupted one in *A. flavus* and caused the transcription of AF biosynthesis structural genes and aflatoxin intermediates production. Clevstrom *et al*. (1983) also studied the inhibition of the biosynthesis of AF caused by *aflR* transcription delay as well as other cluster genes of AF by induction anti-oxidant enzymes via b-glucans from Lentinulaedodes. The previously reported studies, besides our research, proved the potential of gallic acid in biosynthesis inhibition of aflatoxin in *A. flavus* through the expression modulating of *aflR*, *aflS* and *aflD*.

5. Conclusion

Successful PCR amplification of specific genes such as *aflR*, *aflS*, and *aflD* should not be considered evidence of biosynthesis of aflatoxin due to the lack of gene expression as a result of cryptic form and undetected mutation outside to the amplicon sequence and this conclusion explained presence of *aflR*, *aflS*, and *aflD* genes in aflatoxigenic and non-aflatoxigenic *A. flavus* isolates by traditional PCR using genomic DNA as a template. In addition, upon finding that GA as antioxidant, it was used to effectively suppress aflatoxin synthesis. Interestingly, qRT-PCR, a potential tool was employed to investigate the effects of GA on the transcription of aflatoxin genes, *aflR*, *aflS*, and *aflD*, as these genes were down-regulated.

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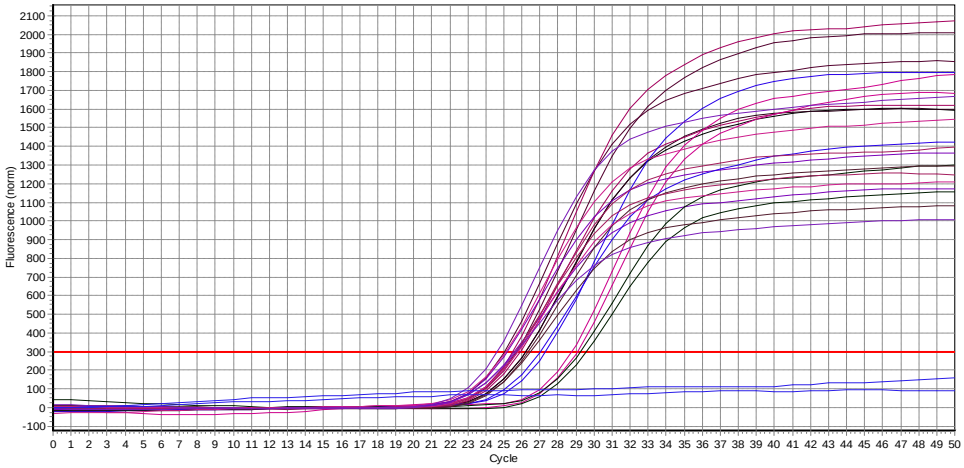
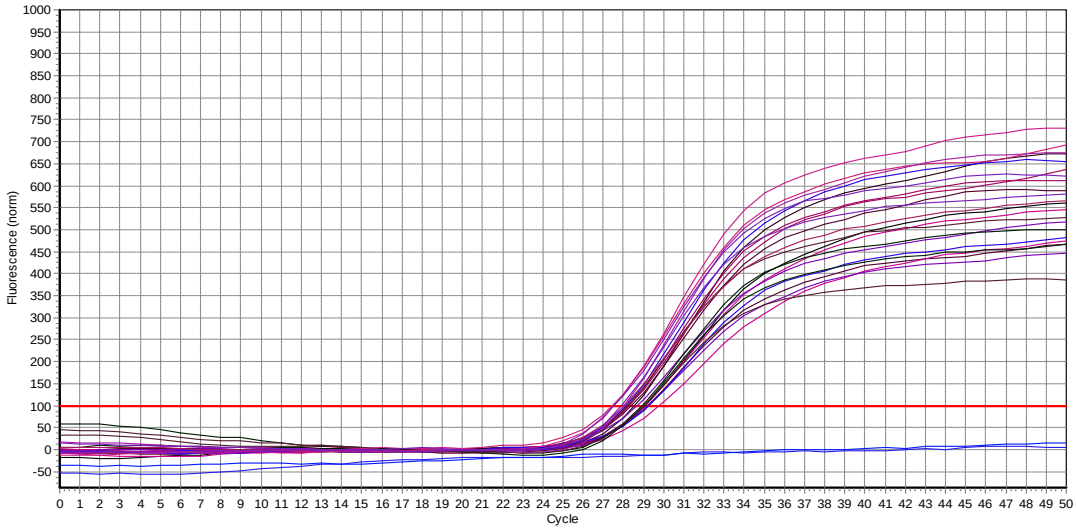
Appendix

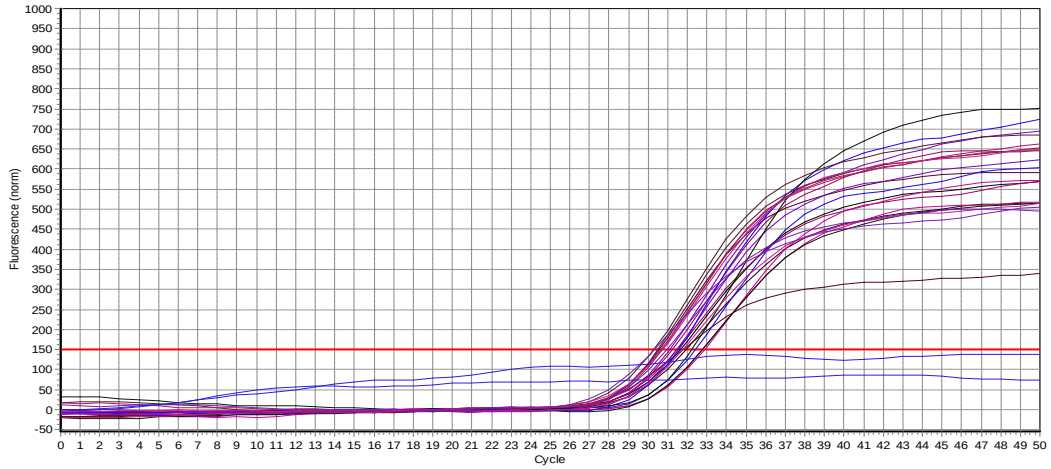


Threshold: 300 (Adjusted manually)
Baseline settings: automatic, Drift correction OFF

The linear and log amplification curves representing the Ct values of *aflR* gene.



Threshold: 100 (Adjusted manually)
Baseline settings: automatic, Drift correction OFF



Threshold: 150 (Adjusted manually)
Baseline settings: automatic, Drift correction OFF