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The Inhibitory Effect of Different Ephedra Plant Extracts on the *Aspergillus flavus* Growth and Aflatoxin B_1 Gene Expression

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Abstract

This study was conducted to investigate the effects of *Ephedra sinica* extracts (ESE) on *Aspergillus flavus* growth, AFB_1 production and *O*-methyltransferase A (*Omt-A*) gene expression. The following various extracts such as Ethanol (EtOH), Ethanol: water (EtOH: H_2O 1:1, v/v), Methanol (MeOH), and Methanol: water (MeOH: H_2O 1:1, v/v) at concentration of 1.0, 2.0, 3.0, 4.0, and 5.0% have been used. AFB_1 production and gene expression were measured using HPLC and real time PCR, respectively. Our findings demonstrated that all ESE has significant inhibition (73.3- 94.6 %) for *Aspergillus flavus* growth based on the dose. Production of AFB_1 was markedly decreased or totally inhibited within range of 10– 99.3% depends on extract type. Supported the effect of ESE at concentrations of 1-5% were suppressed significantly ($P<0.05$) on aflatoxin (*Omt-A*) gene expression. The nucleotides sequence of *Ephedra* plant was submitted in GeneBank under accession number KY310531.1. These outcomes might be used to control the growth of toxigenic fungi and to inhibit the following contamination of feed, food, and agricultural supplies by carcinogenic aflatoxins.

Keywords: Ephedra extracts, Aspergillus, AFB_1 Production, HPLC, gene expression, Omt-A, qRT-PCR.

1. Introduction

AFB_1 is a highly toxic metabolite of food and agricultural resources formed by *Aspergillus species* (Leontopoulos et al., 2003). The disease associated with chronic aflatoxin ingestion is hepatocellular carcinoma (HCC or liver cancer) which is the third-leading cause of cancer death globally according to a World Health Organization report (2008), with about 550,000– 600,000 new cases emerging each year. About 83% of these deaths occur in East Asia and sub-Saharan Afrianca (Parkin et al. 2005, Liu and Wu 2010). Hepatotoxic and hepatocarcinogenic effects of aflatoxins are well known (Wogan, 1999). Aflatoxin exposure has disrupted the synthesis of DNA, chromosome separation, and mitosis progression (Deabes et al., 2012). Aflatoxin B_1 metabolism is divided into 2 phases (Diaz and Murcia 2011). Phase I consists of enzyme mediated oxidation, hydrolysis, reduction reactions. Phase II metabolism consists of conjugation reaction of the compounds modified from the phase I metabolism. Most of the phase I reactions are oxidation or hydroxylation reactions which are mostly catalyzed by cytochrome P450 enzymes. Cytochrome P450, 1A2 and 3A4 enzymes in the liver metabolize aflatoxin into aflatoxin-8,9-epoxide, which then binds to a protein or to DNA and initiates liver carcinoma (HCC) (Wu and Khlangwiset 2010, Eaton and Gallagher 1994). It is evident that Aflatoxin B_1-8,9- epoxide can induce activating mutations in the ras (small GTPase family protein) oncogene in experimental animals. In the presence of water, the epoxide gets hydrolyzed and becomes available to be linked to serum proteins (Groopman et al. 1985). The conjugation step is mostly the detoxification step where aflatoxin undergoes phase II biotransformation (Neal et al. 1998). The resulting aflatoxin conjugates get extracted in the bile. In the deconjugation stage, the bio-transformed aflatoxin gets reabsorbed in the body.

In *Aspergilli*, DNA information is organized into 8 chromosomes, where the genes responsible for the production of aflatoxin are located in the 54th cluster, 80 kb from the chromosome 3 telomere. There are 30 genes in this cluster and its activation is primarily controlled by *aflR* and *aflS*. The aflatoxin gene cluster has been extensively studied in *Aspergillus species*. In *Aspergillus flavus*, the AflR protein binds to at least 17 genes in the AF cluster, resulting in the activation of the enzyme cascade and leading to the production of different AFs. Interestingly, the over-expression of aflR in *Aspergillus flavus* regulates several AF genes, increasing the production of aflatoxin by 50-fold (Montibus et al., 2013). Recently, it has been demonstrated that different inhibitors of aflatoxin production are capable to down-regulate *aflR* expression as the key regulator of the aflatoxin biosynthetic pathway. Disruption of *aflR* completely blocks the expression of the genes in this biosynthetic pathway and aflatoxin/ sterigmatocystin

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production (Caceres et al., 2020). Shimizu and Keller (2001), De Souza et al. (2013) reported that G-protein receptors, cAMP, and PKA have roles in regulating the aflatoxin biosynthetic pathway. In addition, two global regulators of secondary metabolism, *laeA* and *veA* genes regulate the activation of the aflatoxin gene cluster have been documented (Bayram et al., 2008, Sarikaya-Bayram et al., 2015). The LaeA (nuclear protein) contains S-adenosylmethionine binding motif which can activate transcription of a gene cluster in aflatoxin secondary metabolite (Brakhage, 2013). Lae Amethylates histone proteins are associated with cluster for secondary metabolism and make the region accessible to gene transcription. The VeA gene was reported to be essential for the light dependent growth condition. In the absence of light, VeA migrates from cytoplasm into the nucleus to form a complex with LaeA that is essential for both developmental and secondary metabolism (Yin and Keller 2011). Significant decrease in the production of aflatoxin intermediates was reported during the elimination of V*eA* gene from *Aspergillus* strains (Calvo et al., 2004).

On the other hand, the decontamination of mycotoxins by natural products using plant extracts is one of the well-known methods for controlling mycotoxins in foods and feed. Essential oils and aqueous plant extracts have been used as fungal growth inhibitors and to avoid contamination of aflatoxins in food (Abou El-Soud et al., 2012; Ponzilacqua et al., 2018; Deabes et al., 2020). The *Ephedra species* are widely used for their medicinal properties and considered as a potential antimicrobial substitution agent (Ehrlich, 2014; Elhadef et al, 2020a,b). Actually, their organic, aqueous and spice extracts, as well as their essential oils, have shown fungicidal activities (Kuma et al., 2010; Abou El-Soud et al., 2012; Abou El-Soud et al., 2015; Deabes et al., 2020; Elhadef et al, 2020c). So, the present study is conducted to investigate the ability of *Ephedra sinica* extracts to inhibit *Aspergillus flavus* growth followed by investigate its AFB_1 production. Finally, examination of their effect on *O*-methyltransferase A (*Omt-A*) gene expression.

2. Materials and Methods

2.1. Fungal strain, chemicals and Medium.

An *Aspergillus flavus* toxigenic strain (ATCC 28542) was purchased from the Microbial Research Center (MIRCEN), Faculty of Agriculture, Ain-Shams University of Cairo, Egypt. We purchased potato dextrose agar (PDA), yeast extract sucrose (YES) and Na_2SO_3 (Sigma-Aldrich, France). Aflatoxin type B_1 (AFB_1) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Trifluroacetic acid (TFA), methanol, acetonitrile of HPLC grade were produced by BDH, Chemicals Ltd., Poole, England. The double distilled water was used by Millipore water purification system (Bedford, M A, USA).

2.2. Preparation of Ephedra sinica and Extracts (ESE)

The *Ephedra* plant was obtained from the mountain in Sinai, Egypt. The aerial parts was washed and then dried in the sunshade at room temperature until all of the aerial parts well dried up. The plant materials were then well powdered using a grinder and put in a container that was well sealed. 10 grams of *Ephedra sinica* powder were extracted by using the solvents Ethanol (EtOH), Pure Ethanol: water (EtOH:H2O 1:1v/v), Pure Methanol (MeOH) and Methanol: water (MeOH:H_2O 1:1 v / v) in a room temperature shaker for 24 hours, then under vacuum filtrated (Rustaiyanet al. 2011 and Deabes et al. 2020). Eventually, the extract was concentrated under vacuum using a rotary evaporator and sterilized using a Nylon Syringe Filter, 0.22 μm.

2.3. Antifungal activity

100 ml of yeast extract sucrose medium were seeded in 500 ml flasks and autoclaved at 120°C for 20 min. The inoculation was added by 1 ml of a spores suspension (10^5 spores) of a aflatoxigenic fungi *A. flavus* ATCC28542 strain with or without of ESE as (1.0 , 2.0,3.0, 4.0 and 5.0%) to the medium. The flasks then were incubated for 14 days at 28°C (Munimbazi and Bullerman 1998). Dry film was used for the determination of AFB1 by (HPLC) according to (Nada et al., 2010, Deabes *et al.*, 2011, Abou El-Soud et al., 2012, Eshak et al., 2013, Abou El-Soud et al., 2015, Deabes *et al.*, 2018). The percentage of inhibition of AFB_1 and mycelia dried weight were calculated using equation: % inhibition = (control - treatment / control) X 100.

2.4. Extraction of Nucleic Acids

For DNA, the DNA was extracted from stem sample of *Ephedra* Plant by using a DNAeasy Plant Mini Kit (QIAGEN, Santa Clarita, CA) according to (Moawed and Ibrahim, 2016). Also, the genomic DNA was isolated from harvested mycelia of *A. flavus* (25 mg) with or without the treatment with (ESE) following instruction of the protocol for DNA Tissue purification mini kit by grinding the liquid N2 frozen tissue in a mortar. Concentration and purity of the total genomic DNA yield were determined using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA), then preserved at -20°C for further use.

For mRNA, total RNA was isolated from selected *A. flavus* strain without treatment (control) or treated with *E. sinica* extracts using RNA Isolation Kit and the mRNA was reverse transcript using cDNA synthesis kit (Roche Applied Science) (Salem et al., 2017; kalill et al., 2018).

2.5. PCR and Sequencing

2.5.1. PCR for rbcL and ITS1

The PCR reaction was carried out as reported by (Ibrahim et al., 2016) in a total volume of 50 μL consisted of the following: 25 μL PCR master mixture (Promega, USA), 20 pcoml of each primer (Invitrogen, USA), 40 ng DNA and ultra-pure water to the final volume. Two primer pairs were designed based on the conservative coding sequences of rbcL and ITS1 as shown in table (1).

Table 1. List of Primer used in this study

Primer	Sequence 5'-3'
rbcL- F	ATGTCACCACAAACAGAGACTAAAGC
rbcL-R	TCGCATGTACCTGCAGTAGC
ITS-1L	CCGCYGAGTAAGTTCGCTCTC
ITS-1R	CCRTTGCCAGATTGCTTCCT
Omt –F	GACCAATACGCCCACACAG
Omt –R	CTTTGGTAGCTGTTTCTCGC

2.5.2. PCR for Aflatoxin B_1 gene

The amplification reactions were carried out in a T100-Bio-Rad Gradient Thermal cycler in mixture of 20 µl total reaction volume consisting of 10 µl of 2 X Go Taq master mix (Promega Corporation, Madison, WI) and 10 pmol of each primer of 50ng DNA and following amplification conditions consisting of initial denaturation at 94°C for 5 min (1 cycle), followed by 35 cycles of 94°C for 1 min, annealing at 59°C for 1 min and extension at 72°C for 1 min, with a final amplification of 72°C for 10 min. Aliquot of amplicon (10 µl) was separated on agarose gel (1.5%) and stained with ethidium bromide (0.1 mg/l) then photographed under Gel Documentation System (Gel DocTM XR+) against size standard consisting of 100 bp DNA Ladder.

2.5.3. PCR Products Purification and Sequencing

PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, USA). The sequencing of the PCR product was carried out using the dideoxynucleotide chain termination method with a DNA sequencer (ABI 3730XL, Applied Biosystems) (Microgen, Korea) and a BigDye Terminator version 3.1 Cycle Sequencing RR-100 Kit (Applied Biosystems, USA) following the protocol supplied by the manufacturer. The data obtained were analyzed using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) and aligned using Align Sequences Nucleotide BLAST then submitted to GenBank.

2.5.4. Gene Expression by using qRT-PCR

To assess the copy of the cDNA of *A. flavus* strain treated with *E. sinica* by different solvent extraction EtOH:H_2O(1:1v/v), EtOH and MeOH, MeOH:H_2O (1:1v/v) at concentration 1.0, 2.0, 3.0, 4.0 and 5%**,** a Step One Real-Time PCR System (Applied Biosystem, USA) was used**.** The detection of the expression values of the tested gene was performed according to El-Baz et al., (2016).

2.6. Statistical analysis

Data obtained were analyzed statistically using the Statistical Product and Service Solutions IBM SPSS version 22. Waller–Duncan k-ratio was used for determining degree of significance in the differences among all treated groups (Walter et al 1969). The difference was considered significant based on the probability of $P<0.05$.

3. Results

3.1. Identification of Ephedra sinica plant on molecular level

The conserved regions (rbcl) were amplified, which is primarily required for establishing the DNA barcodes for identification of species. In Ephedra species, it was noticed that good amplicons were obtained with both universal primers for rbc land ITS1.

Both primers resulted in efficient amplification (Figure 1). In this study, samples containing an Ephedra species were sequenced with PCR-amplified fragments of both the rbcL gene and ITS1 regions. Neighbor joining methods were used to establish phylogenetic trees of the rbcL and ITS1 sequences.

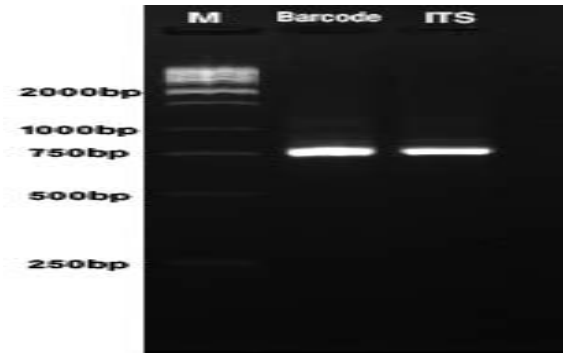

Figure 1. PCR result of DNA barcode regions rbcL and ITS. M; 1kb DNA ladder.

The rbcL sequence phylogenetic tree (Fig. 2) showed that five clades of the ten species formed, *E. sinica*, used in this analysis, have been clustered into *E. Fragile, E. Aphylla & E. Altissima* which suggested a similar genetic relationship between the three species, but *E. sinica* had a distant relationship with the other clade species.

The Basic Local Alignment Tool (BLAST) used for detecting the homology of sequences amplified. The sequence length of rbcL was 750 bp with Ephedra similarly with ITS1, the sequence homology of Ephedra was 99%-100%.

During the present study, it was suggested that the universal primers (rbcL and ITS) for DNA bar coding successful for amplification, identification and discrimination of above mentioned indigenous plant species.

The NJ tree of rbcL DNA sequences correctly placed for the species with the correct family of high statistical support (bootstraps of 99–100%). Due to higher sequence divergences, the rbcL regions can distinguish plant species and simply place these species in the correct family and genus (Fig. 2).

Based on the obtained data found that DNA barcoding can provide accurate plant identification type. The nucleotides sequence of Ephedra plant used in our study was registered in Gene Bank under accession number KY310531.1

Figure 2. Molecular phylogenetic analysis using rbcL for 10 taxa by Neighbor joining (NJ) method.

3.2. Antifungal Activity

3.2.1. Effect of Ephedra sinica extracts (ESE) on growth of Aspergillus flavus ATCC28542 strain and aflatoxin B_1 production in YES medium.

The effect of *Ephedra sinica* extracts was used by different solvents, (MeOH), (MeOH: H_2O 1:1 v/v) (EtOH), (EtOH: H_2O 1:1, v/v) at concentration 1.0, 2.0, 3.0, 4.0 and 5% individual of each showed reduce or inhibition the growth. These results were presented in table (2). Delayed or prevented spore germination, growth inhibition, and decreased of *A. flavus* growth rate at 28 ^oC in YES broth medium were observed. Increasing concentration of ephedra plant extracts resulted in a decreasing in total mycelial mass and reducing or preventing production of AFB_1 by *A. flavus* within incubation at 28 ^oC for 14 days. Compared with other extracts, MeOH/ H_2O *extract of Ephedra sinica* recorded the lowest effect of inhibition of mycelial growth of *A. flavus*. By increasing concentrations of ephedra extract, the reduction of total mycelia mass was achieved (Figure 2).

Table 2. Percentage of reduction of mycelia dry weight of *Aspergillus flavus* (ATCC 28542) growth (mg/100ml) in YES liquid media treated with *Ephedra sinica* extracts (ESE)

Concentrations of extract (%) **	Type of extract*			
	MeOH:H_2O	MeOH	EtOH:H_2O	EtOH
1	73.3	70.6	80.0	76.0
2	77.3	73.3	82.6	78.6
3	80.0	76.0	86.6	82.6
4	82.6	81.3	89.3	85.3
5	86.6	84.0	94.6	90.6

*Mycelia dry weight in control (75mg/100ml)

Indeed, the growth initiation of *A. flavus* was treated by ephedra solvent extract the observed after 14 days incubation in all flasks. However, growth was slightly inhibited at the lower levels of ephedra solvent extract. The results in Table (3) and Figure (3) were shown the inhibition of AFB_1 production in YES medium. A concentration of ESE by solvents (EtOH: H_2O) 1.0, - 5.0% to YES medium inhibited mycelium and AFB_1 production of *A. flavus* by 80%, 94.6 and 42.8, 98.3% respectively. At the higher concentration 5 % *Ephedra sinica* extract (EtOH: H_2O) in YES average concentration of aflatoxin B_1 was 0.72 µg/100 ml YES medium, and *A. flavus* grew very poorly.

Table 3. Concentrations of AFB_1 (µg/100ml) in liquid media treated with *Ephedra sinica* extracts (n=3)

Concentration of extract (%) **	Type of extract*				
	MeOH:H_2O	MeOH	EtOH:H_2O	EtOH	Mean with extract
[1]	36.96±0.88	38.57±0.63	33.31±0.46	35.06±0.62	35.97±1.6A
2	30.78±1.4	32.5±1.5	22.9±1.3	28.0±0.78	28.56±1.2^B
3	22.5±0.37	23.25±0.98	16.42±0.52	20.6±0.52	20.68±1.01^C
4	10.66±0.34	14.9±0.76	2.97±0.3	9.52±0.64	9.52±1.3^D
5	5.4±0.75	8.5±1.09	0.72±0.25	3.5±0.68	4.52±0.91^E
Mean with concentration	21.25±3.18^a	23.54±2.9^b	15.27±3.3^c	19.33±3.1^d	

*AFB_1 in control (43.53µg/100ml)

**Mean values in the row (as a small letter) or Column (as a capital letter) with the same letter are not significant different ($P \leq 0.05$) level.

Data in Table (2) and Figure (3) shown the concentrations of AFB_1 were treated by Four (ESE) in YES medium .Which affected significantly differences to the type of (ESE) and also the concentration of type of their extracts. The extracts of *Ephedra sinica* by different solvent EtOH: H_2O (1:1v/v), EtOH, MeOH, MeOH: H_2O (1:1 v/v) at concentration 1.0, 2.0, 3.0, 4.0 and 5.0% individual of each showed inhibition the AFB_1 production by *A. flavus* in YES medium were determined by HPLC. The percentages of AFB_1 reduction were treated by EtOH: H_2O (42.8, 47.4, 62.3, 93.2 and 98.4 %, respectively) (Figure 4). From the statistical analysis in Table (3), it was found that the effect of mean value with the concentrations of (ESE) for all the treatments EtOH: H_2O (1:1, v/v), EtOH, MeOH and MeOH: H_2O (1:1 v/v), the inhibition percentage of AFB_1 production significantly increased in the YES liquid medium to record 64.9, 55.6, 45.9, and 51.9 %, respectively. Based on the results of current study, it was observed that the positive effect of Ephedra extract against AF production and the AFB_1 biosynthesis by *A.flavus* was limited at all experimented treatments, and the inhibition or prevention depend on the type of solvents extraction and concentration of Ephedra extracts.

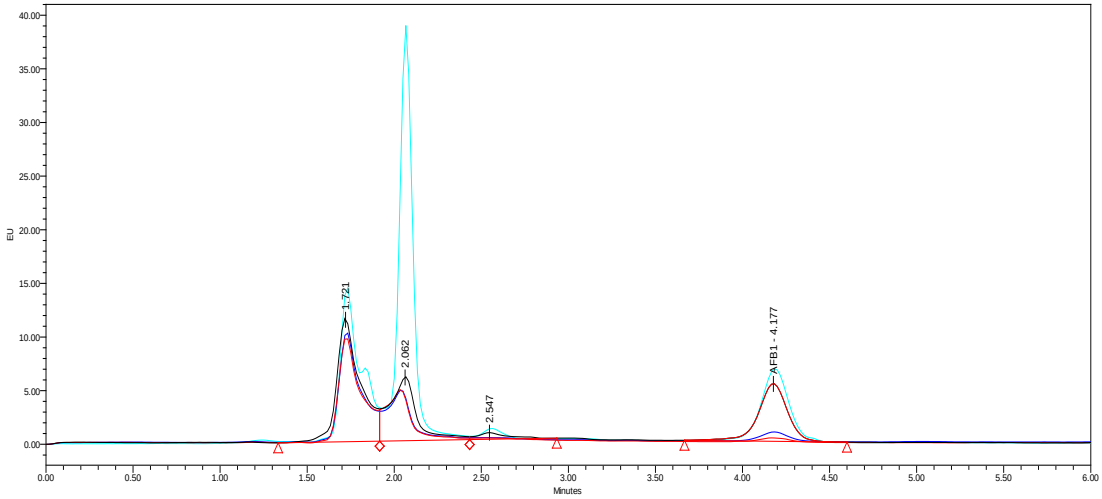

Figure 3. HPLC chromatogram of $AFB_1$with different concentrations of Ephedra (treated by 1-5 % EtOH:H_2O (1:1 v/v)/100 ml media .

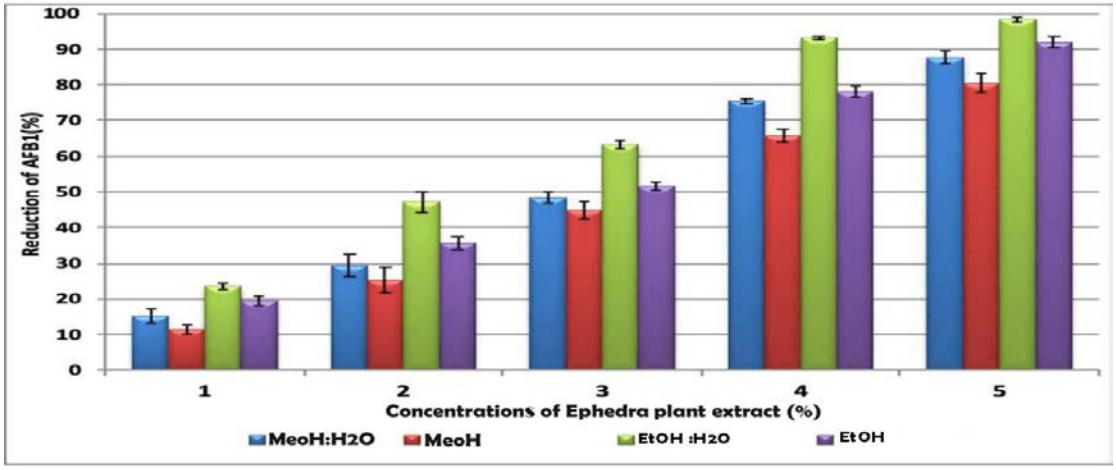

Figure 4 Percentage of reduction AFB_1 in liquid media treated by ESE.

3.3. Polymerase Chain Reaction (PCR) assay.

During the current study, the primers specific for *A. flavus* were designed based on the *omt-A* gene coding for one of the key regulatory enzymes in biosynthesis of AFB_1 (Table 1). Also, they were suitable to detect the fungus. The genomic DNA was extracted from *A. flavus* strain with and without the treatment with aflatoxigenic and then subjected to the PCR using *omt-A* primers. As expected, the DNA fragment with molecular size 300-bp was amplified from tested *A. flavus* isolate. It was found that the assays with *A. flavus* (with and without the treatment with (ESE) yielded a single amplified DNA fragment with expected size (300 bp) (Figure 5).

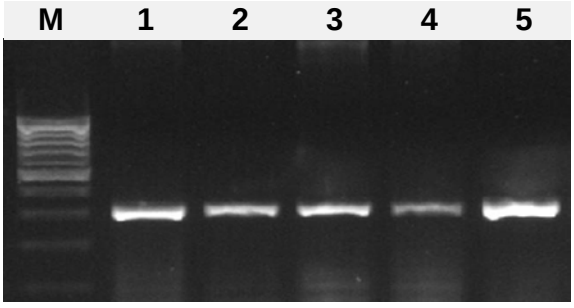

Figure 5. PCR assay using Omt A -primer of *A. flavus*, M .M Gene Ruler 1kb, lane 1; Cont, lane 2; EtOH, Lane 3; MeOH, Lane 4; EtOH:H_2O and Lane 5 MeOH:H_2O.

3.4. The effect ESE on Aspergillus flavus growth and aflatoxin B1 gene (Omt-A) expression on molecular level (quantitative by Real Time-PCR).

The gene (*Omt-A*) expression was analyzed and quantitative by real-time RT-PCR, and the results obtained are summarized in Fig (6). The gene encoding aflatoxin was determined in *A.flavus* fungus cells after treatment with three Ephedra plant extracts; EtOH: H_2O (1:1, v/v), EtOH, and MeOH: H_2O (1:1, v/v).

The results found that treatment of *A. flavus* cells with different plant extracts showed lower expression levels of aflatoxin gene than in control samples. Ethanol (EtOH) extract had a higher influence on aflatoxin gene (*Omt-A*) inhibition (80% decreased significantly compared to the control) followed by EtOH: H2O extract (78% decreased significantly compared to control). On the other hand, MeOH: H2O extract had a lower effect on aflatoxin gene expression than the other two extracts (22% decreased significantly compared to the control).

As for EtOH: H2O and EtOH, they were able to reduce *Omt-A* gene expression Figure (6) by 80 % and 73 % equally and had nearly the same effect on AFB_1 production confirmed by HPLC in (Figure 3) as they reduced it to 84.73 % and 80.76 % respectively in mean values (Table 3, Figures 3& 4).

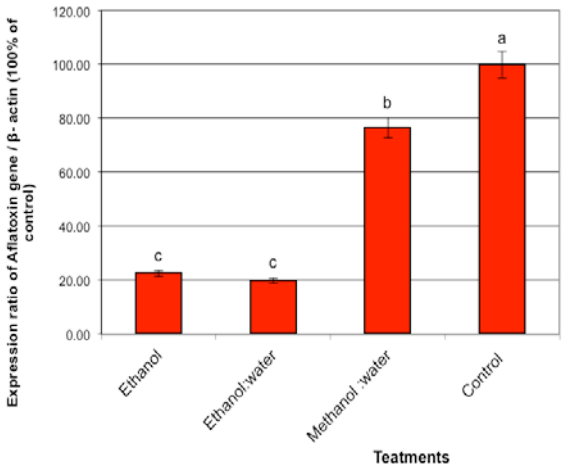

Figure 6. Expression of *Omt_A* gene in *A. flavus* treated with extracts of *Ephedra sinica* by different solvents.

EtOH, EtOH: H_2O (1:1v/v) and MeOHl: H_2O (1:1v/v) at concentrations 1.0, 2.0, 3.0, 4.0 and 5%. Data are presented as mean ± SE. a, b, c followed by different superscripts are significantly different (P≤0.05).

4. Discussion

Mycotoxigenic fungi invade the agriculture commodities and caused Food spoilage and poising causing hazards to humman and animals. So, the researchers looking for untraditional solution for the fungi contaminated food. The Ephedra species have a potent active compounds has the ability to inhibit the fungal growth and have to inhibit the DNA responsible gene of AFB1 produced by *A. flavus*. The current work was start by extraction a potent active compounds from Ephedra stems using different solvents. Then, the DNA were identified using PCR and the nucleotides sequence of Ephedra plant was registries in Gene Bank under accession number KY310531.1. In this respect order (Group et al., 2009) found that the multilocus DNA barcodes have been proposed to be very effective and precise in plant species identification. The rbcL has been proven to be an effective and precise barcode of DNA for plant species identification (Guo et al., 2011). The antifungal of *A. flavus* activity using ESE were studied and noted the efficiency as antifungal against *A. flavus* as well as AFB_1 production. In the same regard, Bagheri et al. (2009), Deabes et al. (2020) found that the Ephedra have antifungal effect. Based on inhibition of growth of *A. flavus* and AFB_1 production due to the potent active compounds such as alkaloids, phenols, glycosides, steroids, coumarins and tannins (Masako, et al., 1989; Ebana, et al., 1991, Deabes et al., 2020).

The obtained data demonstrates that the antifungal effect of ESE can regulate the cellular effects of aflatoxins, and this may be due to the aromatic organic compounds of spices or medicinal plants that can control the formation of secondary metabolites aflatoxins (Chatterjee 1990). Ephdra's antimicrobial effect was related to Cis-314-methanoproline (Caveney et al., 2001) and Heptadecane (Bagheri et al., 2009). Linked to Ephedra's antimicrobial activity, the study by Deabes et al. (2020) found that the extraction of *Ephedra sinica* using EtOH: H_2O solvent was established to be a valuable source for natural polyphenols and alkaloids against pathogenic (*K. pneumoniae, E. cloacae, S. marcescens, E. coli, A. flavus, A. ochraceus, A. niger* and Coxsackie B3 virus).

Due to flavonoid compounds including p-coumaric acid and quercetin in ESE causing on both growth inhibitor and AFB_1 producer by *A. flavus* and *A. parasiticus* (Aziz et al., 1998 ; Kim et al., 2004 , Deabes et al., 2020) .

Most AF biosynthesis inhibitors operate on one of three levels: altering the physiological environment or other signaling inputs perceived by the fungus, interfering with signal transduction and gene expression regulatory networks upstream of AF biosynthesis, or blocking enzymatic activity of a biosynthetic enzyme (Holmes et al., 2008). Several researchers studied the anti-microbial and anti-fungal properties of the species Ephedra. In the another studies, they analysis of plant Ephedra extracts or essential oil (EO) has shown to inhibit *A. parasiticus* or *A. flavus* growth. However, the fungal growth significantly inhibited at the maximum concentration (1000 µg/ml) (Bagheri-Gavkosh et al., 2009; Deabes et al., 2020). Ozdemir et al. (2004) reported that two heptadecane and tetradecane derivatives isolated from *Spirulina platensis* EO had antimicrobial effects on a broad range of microorganisms. Different studies suggest the ability of plant extracts and EOs to inhibit aflatoxigenic fungal growth and AF production. In agreement with our results, organic solvents extract of aerial parts and roots of Ephedra plant were able to inhibit *A. parasiticus* growth and AFB_1 production (Bagheri-Gavkosh et al., 2009). Some phenolic compounds such as acetosyringone, and syringaldehyde can inhibit aflatoxin biosynthesis in *A. flavus* efficiently (Hua et al., 1999). There are some studies on using plant extracts such as afoetida, turmeric and *Azadirachta indica* leaf extract to prevent aflatoxin production (Ghewande and Nagaraj, 1987). Eleven potent active compounds as antioxidant agents and anticancer were detected in ethanol/ water extract of *Saussurea costus* root, which used to mitigate the oxidative stress (Deabes et al., 2021). On the other hand, Holmes et al., (2008) found that norsolorinic acid (NOR), is stable intermediate in the AF biosynthetic pathway. The findings by Nazareth et al. (2020) indicate that allyl isothiocyanate may be used as a fumigant to prevent *A. flavus* growth and AFB_1 development, further confirming transcriptional alteration of AFB_1 genes.

5. Conclusions

Ephedra sinica extracts showed an antifungal activity and prevented the growth of A. *flavus* cultured on YES medium. EtOH/ H_2O extract was the most effective extract to control the biosynthesis of AFB_1 and *A. flavus* regulatory *Omt-A* gene, in combination with a drastic reduction in AFB_1 production. It is worthy to mention that the obtained findings mentioned that ephedra plant extracts have a positively potent effect on the growth and development of aflatoxigenic fungi in food, feed, and agricultural supplies by carcinogenic aflatoxins.

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