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Antifungal and Antiaflatoxigenic Activities of Different Plant Extracts against *Aspergillus flavus*

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Abstract: In the current study, four organic solvents, including ethanol, methanol, acetone, and diethyl ether, were used to extract turmeric, wheat bran, and taro peel. The efficiency of three different concentrations of each solvent was assessed for their antifungal and anti-mycotoxin production against *Aspergillus flavus*. The results indicated that 75% ethanolic and 25% methanolic extracts of taro peels and turmeric were active against fungus growth, which showed the smallest fungal dry weight ratios of 1.61 and 2.82, respectively. Furthermore, the 25% ethanolic extract of turmeric showed the best result (90.78%) in inhibiting aflatoxin B_1 production. After 30 days of grain storage, aflatoxin B_1 (AFB_1) production was effectively inhibited, and the average inhibition ratio ranged between 4.46% and 69.01%. Simultaneously, the Topsin fungicide resulted in an inhibition ratio of 143.92%. Taro extract (25% acetone) produced the highest total phenolic content (61.28 mg GAE/g dry extract wt.) and showed an antioxidant capacity of 7.45 µg/mL, followed by turmeric 25% ethanol (49.82 mg GAE/g), which revealed the highest antioxidant capacity (74.16 µg/mL). RT-qPCR analysis indicated that the expression of *afl*D, *afl*P, and *afl*Q (structural genes) and *afl*R and *afl*S (regulatory genes) was down-regulated significantly compared to both untreated and Topsin-treated maize grains. Finally, the results showed that all three plant extracts could be used as promising source materials for potential products to control aflatoxin formation, thus creating a safer method for grain storage in the environment than the currently used protective method.

Keywords: plant extracts; antioxidant; *Aspergillus flavus*; aflatoxin B_1; maize storage; gene expression

1. Introduction

Several *Aspergillus* sp., including *A. flavus*, *A. nomius*, *A. parasiticus*, and *A. pseudotamarii*, produce toxins, such as aflatoxins B_1, B_2, G_1, and G_2, respectively. Meanwhile, patulin is produced by *A. terreus* and *A. clavatus*; *A. ochraceus* produce ochratoxin A; and cyclopiazonic acid is secreted by *A. versicolor* and *A. flavus* [1,2]. Nowadays, several aflatoxins produced by *Aspergillus* spp. are characterized as aflatoxin B_1 (AFB_1), a very toxic aflatoxin

to mammals [3,4]. Fungi cause severe damage to seed production and seed quality by deteriorating their formation, development, storage, and/or germination [5]. Aflatoxins are a big concern in cereal grains, such as maize, wheat, and their products, dried fruit, nuts, oilseeds, and spices [6,7]. However, maize, wheat, and groundnuts are humans' main sources of aflatoxin exposure worldwide [8]. AFB_1 is one of the most common mycotoxins, which is restricted in different countries if its concentration exceeds 20 ng/g. This restriction is due to the dangerous effects of exposure of humans to even a low dose of aflatoxins, which leads to immune suppression, cancer induction, and growth problems in children [9]. Therefore, controlling aflatoxins in stored grains is necessary for health security [10].

Plant extracts are of great importance since they are biologically active compounds that are easy to prepare and are usually used with a great deal of safety. Furthermore, no concerns for their residual effects have been raised, as they are biodegradable. Their stimulating effect on plant metabolism is evident [11,12]. Plant secondary products can afford new antibiotics against different biological agents with a potential new mode of action [13–15]. Their potential use as natural antioxidants affects the ability of mycotoxigenic fungi to grow and produce toxins [11,16]. For instance, plant extracts or essential oils of *Cymbopogon citratus*, *Thymus vulgaris*, and *Ocimum gratissimum* could replace synthetic fungicides to control fungi growth on seeds and grains, Tagne et al. [17]. Different strategies, such as physical and adsorption methods, have been adopted to remove aflatoxins from contaminated foods. Developed countries have recently increased the need for safer foods and cleaner production processes. On the other hand, fungi are widely used for bioremediation activities to remove toxic wastes from contaminated environments [18]. Hence, enhancing fungal growth for toxic material removal creates an urgent necessity for environmental remediation purposes [19]. Agro-food industrial byproducts, such as seed husks and fruit peels, usually produce a massive amount of agro-food waste [20,21]. The accumulation of such waste imposes a serious challenge to the environment after they rot due to microbial activity [22]. These wastes could be used as raw materials for high-added-value products, such as drugs or drug adjuvants, cosmetics, food constituents, antioxidants, and flavors. Therefore, researchers have spent huge efforts to reuse and recycle waste frequently in food and feed industries [23]. Plant wastes are discarded as if it is useless material, and hence, cause several waste-management and environmental problems [24]. Fruits and their peels, in particular, are rich in secondary products which produce different biological activities and/or medicinal properties [25,26]. Flavonoids, tannins, alkaloids, phenolic acids, and glycosides are secondary metabolites that usually exist in many plants, such as taro, wheat bran, and turmeric. Plant extracts containing rutin have shown in vitro antimicrobial, antioxidant, and anticarcinogenic properties [27–29]. In planta, linoleic acid, a substrate for the production of trihydroxy oxylipins [30], is known for being an antifungal metabolite. Linolenic acid and allylphenol have both been shown to decrease the growth of *Pythium ultimum* mycelia by 65% and *Rhizoctonia solani* mycelia by 74% at 1000 µM. Furthermore, they can decrease fungal biomass production and have been reported to act against a number of other plant pathogens [31,32].

Environmental conditions may also strongly affect the production of AFB_1 and its biosynthetic pathway. As one of the well-described pathways, 30 structural genes have been reported to be involved in aflatoxin biosynthesis, including *afl*D, *afl*G, *afl*H, *afl*I, *afl*K, *afl*M, *afl*O, *afl*P, and *afl*Q, along with two regulatory genes, *afl*R and *afl*S [33]. Several studies have reported that different plant secondary products cease or downregulate the expression of aflatoxin biosynthesis genes and may cause AFB_1 degradation [33–35]. For example, Liang et al. [36] showed that 0.40 mM/L cinnamaldehyde could suppress the biosynthesis of AFB_1 and the expression of some of its biosynthetic genes. Furthermore, Caceres et al. [37] and El Khoury et al. [38] investigated the effects of water extracts of hyssop, as well as piperine or eugenol (terpenes), on the gene expression level of twenty-five genes of an AFB_1 biosynthetic cluster (27 genes), and the expression of 15 regulatory genes was also shown to be affected. Therefore, we aim to evaluate the effect of using different plant extracts (turmeric, wheat bran, and taro peels) as antifungal treatments on

the production of aflatoxins on maize grains during storage compared to Topsin-treated grains. Furthermore, the potential of the peel extracts to suppress the expression of AFB_1 biosynthetic genes is evaluated.

2. Materials and Methods

2.1. Source of the Fungus and Maize Grains Used in the Study

An *Aspergillus* isolate was used, which had been previously evaluated for its ability to produce AFB_1 [39,40]. The maize grains (variety 2055 yellow hybrid) were purchased from Misr Hytech Seed Int. S.A.E. (Cairo, Egypt).

2.2. Preparation of Extracts

Extraction was performed from wheat bran (*Triticum aestivum*), turmeric (*Curcuma longa*), and taro peels (*Colocasia esculenta* L.) following the method published by Salem et al. [41], with slight modifications. Briefly, air-dried plant samples were ground to a fine powder using a commercial mill. Then, 100 mL of the extraction solvent (ethanol, methanol, diethyl ether, and acetone) was used to extract 20 g of plant powder at 25%, 50%, and 75% (v/v: solvent/water) concentrations. The mixtures were agitated at 200 rpm for 24 h on a bench shaker at room temperature; then, the cultures were filtered through Whatman filter paper (No.1). All the extracts were stored at 4 °C.

2.3. Total Phenolic Content Estimation in Plant Peels

Total phenolic content (TPC) was estimated using Folin–Ciocalteau reagent in all plant samples, as described by Turkmen et al. [42] and Farahmandfar et al. [43]; tests were conducted as follows. In a test tube, 0.5 mL of extract was added to 0.5 mL of 1 mol/L Folin–Ciocalteau reagent and 1 mL of distilled water. Three minutes later, 1.5 mL of 10% Na_2CO_3 was added, and then the mixture was incubated for 10 min. After incubation, absorbance at 725 nm was estimated for all samples by 6305 UV/VIS SPECTRO (Cole-Parmer, Stone ST15 0SA, United Kingdom). The TPC was calculated and presented as mg gallic acid equivalents (GAE)/gram (g).

2.4. Radical Scavenging Capacity of Plant Extracts

The antioxidant capacity was determined based on the radical scavenging ability of 1,1-diphenyl-2-picrylhydrazyl (DPPH). The free radical scavenging capacities of both extracts using ethanol and diethyl ether were estimated according to Asnaashari et al. [44]. The results were calculated using the following equation: Radical scavenging capacity % = $(AB - AA)/AB \times 100$, where AB = absorption of blank and AA= absorption of extract.

2.5. Plant Extracts Effects on Fungi Growth and Production of AFB_1

2.5.1. Antifungal Growth Estimations

Potato dextrose agar (PDA) plates containing 15 mL medium were prepared, inoculated with *A. flavus* fungus, and incubated at 30 °C for one week. After one week, *A. flavus* disks of 5 mm diameter were separated and used to inoculate the previously prepared conical flasks containing 50 mL yeast extract sucrose (YES) broth. Out of the three different plant extract concentrations, 1 mL of 25%, 50%, and 75% extracts was added to the YES broth and incubated for 15 days at 30 °C. After culture filtration, the fungal mats were collected and dried in an oven for four days at 50 °C. The mats' final dry weights (mass ratio%) were recorded for all treatments, and the filtrates were kept in the fridge at 4 °C to further determine aflatoxin B_1 production. Production ratios for aflatoxin were estimated as follows [45]:

$$(PR)\% = \left[\frac{\text{Aflatoxin conc. (control)} - \text{Aflatoxin conc. (treatment)}}{\text{Aflatoxin conc. (control)}}\right] \times 100) - 100$$

The production inhibition (PI%) was calculated with the same above equation, without the value of (−100).

2.5.2. Effect of Different Plant Extracts on the Storage of Maize Grains

Fifty grams portions of sterilized maize grains were distributed in sterile glass jars. Each jar was treated with the assigned plant extract, and Topsin fungicide was applied as a positive control. The jars were then inoculated with *A. flavus* discs and incubated at 30 °C for 30 days. After incubation, maize grains traits, including grain odor and shape, were recorded according to the criteria presented in Table 1. All the treated grains were kept at 4 °C for further aflatoxin production analysis.

Table 1. Maize grain approval criteria according to changes in grain shape/odor and trait scale.

Grain Shape Change	Odor Change	Approving	Scale
Whole grains (no change in shape)	No smell	Highly approved	5
Very simple	very simple	Very very approved	4
Moderate	Moderate	Very approved	3
Great	Great	Approved	2
Sever	Pungent	Unapproved	1

2.5.3. Extraction of Aflatoxin B_1 from Samples

AFB_1 was extracted from YES media, according to Alshannaq et al. [46]. The extraction was performed using chloroform as follows: A total of 2 mL of the broth culture was mixed with an equal volume of chloroform; the mixture was vortexed in 15 mL tubes and then centrifuged for 5 min at 10,000 rpm. Approximately 2 mL of the lower layer was taken into a new glass vial. Then, the solvent (chloroform) was evaporated under airflow. Finally, the dried portions were dissolved in 1 mL methanol [46]. Extraction of AFB_1 from contaminated grains was done as indicated by Hoeltz et al. [47], with a slight modification; samples (20 g of contaminated maize grains) were suspended in 12 mL 4% KCl and 100 mL of MeOH. The samples were spun down for 2 min at 10,000 rpm and then filtered. 100 mL of Copper Sulfate 10% (w/v) was added to the filtrate, mixed well, and filtered. Finally, an equal volume of dH_2O water was added, and AFB_1 was extracted twice using 15 mL chloroform. Then, in a water bath of 60 °C, the solvent was evaporated, and the pellet was dissolved in methanol. All the prepared samples were filtered into HPLC vials using a 0.5 µm syringe filter prior to performing HPLC analysis. Standard of AFB_1 was prepared at a concentration of 25 ng/mL AFB_1 (Sigma-Aldrich, St. Louis, MO, USA) in toluene–acetonitrile (9:1, v/v).

2.5.4. HPLC Analysis of AFB_1

Agilent 1260 Infinity-HPLC-Series (Agilent, Santa Clara, CA, USA) was used to analyze AFB_1 content. HPLC equipped with Zorbax Eclipse XDB-C18, 4.6 mm × 150 mm, 3.5 µm column, was used with a mobile phase of Water/MeOH/ACN; 50/40/10 ($v/v/v$) and a flow rate of 0.8 mL/min for separating the compounds. The injection volume was 10 µL [46]. A UV detector was used for detecting the analytes at 365 nm, and the temperature was adjusted to ambient.

2.5.5. Real-Time PCR Assay

The guanidium isothiocyanate reagent-based method was used to isolate RNA from plant peels with slight modification as described elsewhere [48]. A Nano SPECTRO star (BMG Labtech, Ortenberg, Germany) system was used to measure the concentration of the extracted RNA. Simultaneously, agarose gel electrophoresis was used to assure RNA integrity. 1.0 µg of DNase-treated RNA of each sample was for cDNA synthesis as described previously [49,50]. Real-time quantitative PCR (RT-qPCR) reactions were carried out in a Qiagen RGQ Rotor-Gene Q 2-Plex HRM real-time PCR system (Qiagen, Hilden, Germany). Table 2 illustrate all primer sequences that were used in this study. A β-tubulin internal reference gene transcript level was utilized for normalizing the amount of RNA discrepancy in each reaction. The total volume of the RT-qPCR reaction was performed in a 20 µL

volume using SYBR-Green PCR Master-Mix [51,52]. The relative levels of gene expression were calculated using the $2^{-\Delta\Delta CT}$ equation [53].

Table 2. Primers used in this study and their corresponding aflatoxin biosynthesis genes show the enzymes responsible for their functions.

Target Gene	Sequences (5′-3′)	Function in the Biosynthetic Pathway	Target Size (bp)
β-tubulin (*ben*A)	Forward: CTTGTTGACCAGGTTGTGGAT Reverse: GTCGCAGCCCTCAGCCT	Reference housekeeping gene	51
*afl*D (*nor*-1)	Forward: GTCCAAGCAACAGGCCAAGT Reverse: TCGTGCATGTTGGTGATGGT	Norsolorinic acid (NOR) → Averantin 9 (AVN)	66
*afl*P (*omt*A)	Forward: GGCCGCCGCTTTGATCTAGG Reverse: ACCACGACCGCCGCC	Sterigmatocystin (ST) → O-methylsterigmatocystin (OMST)	123
*afl*Q (*ord*A)	Forward: GTGTCCGCAGTGTCTAGCTT Reverse: GCTCAAAGGTCGCCAGAGTA	O-methylsterigmatocystin (OMST) → aflatoxin B_1 (AFB_1)	115
*afl*R	Forward: CTCAAGGTGCTGGCATGGTA Reverse: CAGCTGCCACTGTTGGTTTC	Pathway regulator	86
*afl*S	Forward: CTGCAGCTATATTGCCCACA Reverse: TAAACCCAGGCAGAGTTGGT	Pathway regulator	117

2.6. Statistical Analysis

A completely randomized statistical design was adopted to carry on the experiments [54,55], and analyses were performed using the Analysis of Variance (ANOVA) test, employing "CoSTAT" software. For analyzing the level of gene expression of the aflatoxin biosynthetic genes, gene expression values were expressed as means ±SD gene expression values were compared with the untreated samples and considered statistically significant when $p \leq 0.05$.

3. Results and Discussion

The aflatoxigenic *A. flavus* Af1 (#MG202161) isolate, previously identified on a molecular level, was used as a high producer of AFB_1 (26.79 ppb).

3.1. Effect of Plant Extracts on the Growth of A. flavus and Production of AFB_1

Wheat bran, turmeric, and taro plant peels were exposed to organic solvent extraction using ethanol, acetone, methanol, and diethyl ether in the extraction phase. Each solvent was used at different concentrations (25, 50, and 75%) to study its potential effect on *A. flavus* growth (dry weight mass ratio) and AFB_1 production (Figure 1). Regarding the effect of the extract on fungal growth, dry weight mass ratio results showed that the least significant values were 3.23, 2.82, and 1.61% when 75% methanolic extracts of wheat and taro, 25% ethanolic extract of turmeric, and 75% ethanolic extract of taro were applied, respectively, compared with control. Our results showed that the antifungal capability of turmeric extract is consistent with previous reports. For example, Hu et al. [56] reported the antifungal and antiaflatoxigenic properties of *Curcuma longa* L. essential oil against *A. flavus.* In another study, Hojo and Sato [57] reported that extract of licorice in 80% methanol exhibited significant antifungal effects when tested on *Arthrinium sacchari* M001 and *Chaetomium funicola* M002. Furthermore, less mycelial growth (about 100×) was observed after incubating licorice extract with *Aspergillus parasiticus* for 72 h [58].

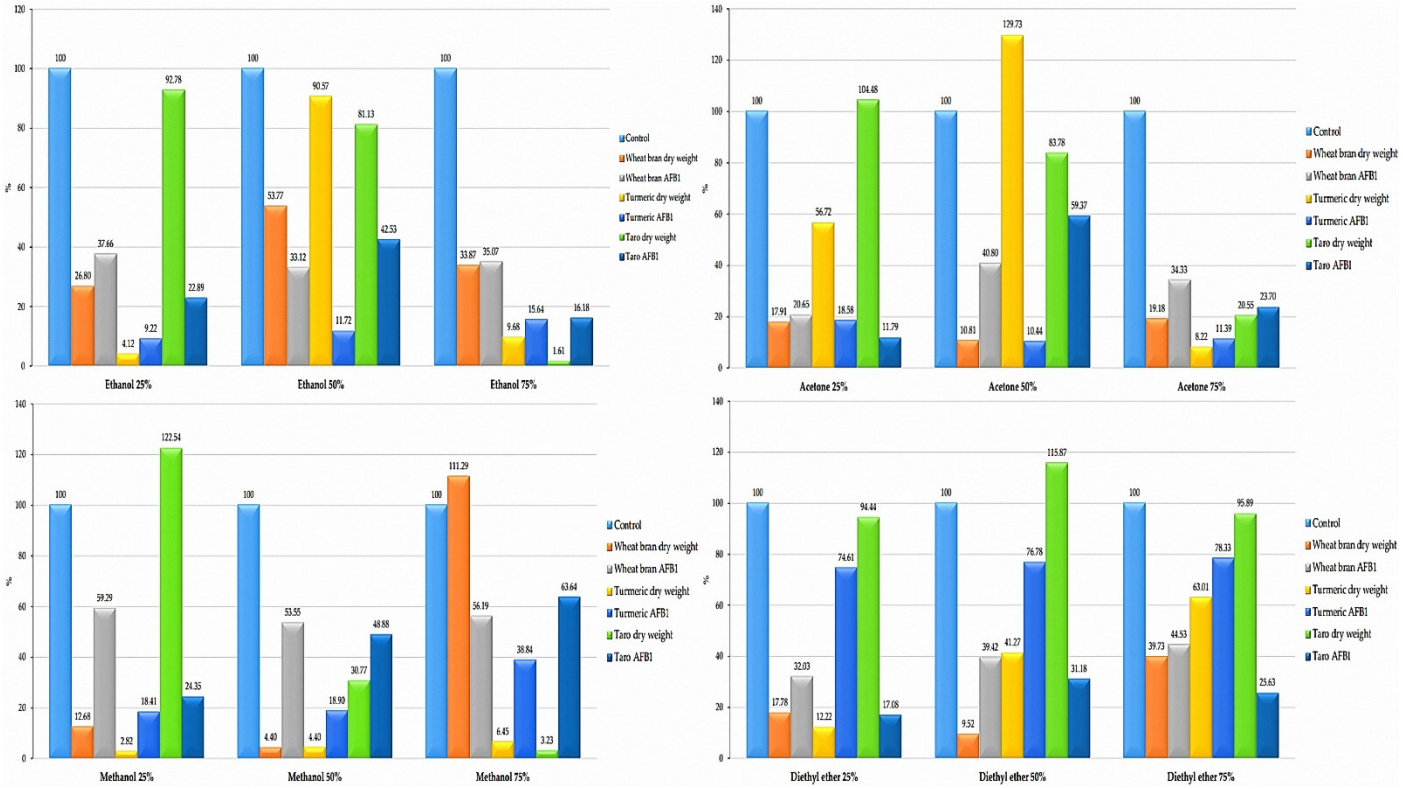

Figure 1. Effect of plant extracts on *A. flavus* mat dry weight (mass ratio %) and AFB_1 production ratio (PR%).

In the present study, most of the tested extracts using different extraction concentrations of the solvents showed high efficacy against aflatoxin production. Production ratios of AFB_1 (PR%) values ranged between 9.22% and 78.33% in turmeric, and PR% values in wheat bran ranged between 20.65 and 59.29%. PR% values, shown in Figure 1, indicated promising results of ethanol and acetone extracts of turmeric. It was found that the best PR% of 9.22% and the best production inhibition (PI%) of 90.78% were achieved with turmeric 25% ethanolic extract, followed by the 75% acetone extract, which produced a PR% of 10.44% and a PI% of 89.56%. The highest PR% values were obtained by 50% and 75% diethyl ether extracts (76.78% and 78.33%, respectively). Moreover, when taro peels were extracted using all solvents, the smallest PR% value was 11.79% with 25% acetone, which also produced a PI% of 88.21%. Meanwhile, the highest PR% values of 63.64% and 59.37% and the lowest PI% values of 36.36% and 40.63% for taro peel extracts were achieved when 75% methanol and 50% acetone were used in the extraction, respectively. Furthermore, wheat bran extract data presented in Figure 1 showed slight differences between the PR% values of the tested solvents. The lowest PR%, 20.65%, was achieved using 25% acetone treatment, which means there was a 79.35 % reduction in the AFB_1 production compared to PR% values of the other solvents. Meanwhile, a minor effect was observed when 25% and 50% methanol extracts were tested, for which the PI% values were 40.71 and 43.81%, respectively.

The results obtained in the current study regarding fungal growth and inhibition of AFB_1 production may be explained in light of the findings of Borges et al. [59]. The authors found that plant extracts act as antioxidants to inhibit aflatoxins via quenching free radicals and suppressing their propagation, converting them into less-toxic compounds. In addition, solvents showed different efficiency when different concentrations were used and the content of a given plant's content of secondary metabolites. Naik et al. [60] and Bernardo and Sagum [61] reported that eggplant (*Solanum melongena* L.) peels extract and sugar apple peels (*Annona squamosa*) using ethanol and methanol showed great free radical scavenging capacity towards human pathogens. Their findings are in harmony with our

findings in the current study. Two studies by Adom et al. [62] and Laddomada et al. [63] revealed that phenolic acids, cross-linked with plant cell wall polymers, such as in the case of wheat bran, play important antioxidant roles.

3.2. Maize Storage Experiment

3.2.1. Effect of Plant Extracts on Production of AFB_1

The results shown in Table 3 clarify that turmeric extract using 25% ethanol was the best treatment for inhibiting AFB_1 production (4.46 ppb), with a PI% of 98.95%. Meanwhile, wheat bran extracted with 25% acetone showed low AFB_1 content (51.18 ppb). Simultaneously, the value of AFB_1 in 25% acetone extract of taro peel was 69.01 ppb compared to the other treatments. These results are consistent with the results reported by Mohseni et al. [58], as they showed decreased aflatoxin production in *A. parasiticus* in the presence of 500 mg/mL of licorice extract.

Table 3. The ability of plant extracts to affect AFB_1 production from *A. flavus* in stored inoculated maize grains.

Treatments	Solvent Concentration	AFB_1 (ppb)	PI%	PR%
Healthy moistened control	-	0.00	-	-
Infected control	-	425	-	100
Wheat bran	Acetone 25%	51.18	87.96	12.04
Turmeric	Ethanol 25%	4.46	98.95	1.05
Taro	Acetone 25%	69.01	83.76	16.24
Topsin	2.5 mg/mL	143.92	66.14	43.96

3.2.2. Effect of Plant Extracts on Grain Shape and Smell

The results in Table 4 show significant changes in the appearance of plant-extract- and fungicide-treated grains compared to the control. Turmeric extract showed outstanding antifungal effects and maintained whole grain shape compared to the other treatments. Turmeric was followed by wheat bran extract and taro peel extract, which both showed a similar grain shape appearance. Topsin treatment using the recommended dose caused grain shape distortion, bad odors, and, consequently, unapproved grains. Similar results were obtained by Gemeda et al. [64] and El-Aziz et al. [65] when they tested reductions in fungal dry weight after treatment of *Aspergillus* with different essential oils. A similar reduction was observed in turmeric extract [66]. The current study's results also agree with Yazdani et al. [67], who illustrated that some plant metabolites (phenolics) could suppress aflatoxin production in *A. flavus*.

Table 4. Effect of plant extracts on grain appearance and smell compared to the control fungicide Topsin.

Treatments	Solvent Concentration	Grain Shape	Smell	Granted Grade
Healthy moistened control	-	5	0	5
Infected control	-	0	5	0
Wheat bran	Acetone 25%	4	3	4
Turmeric	Ethanol 25%	5	1	5
Taro	Acetone 25%	4	1	4
Topsin	2.5 mg/mL	1	5	1

3.3. RT-qPCR Analysis of AFB_1 Biosynthetic Genes

AFB_1 biosynthesis is a complicated pathway of the enzymatic production of aflatoxins [68]. In *A. flavus*, 25 genes are responsible for producing AFB_1, starting from acetyl CoA, in which the coding genes are allocated in a 75 kb cluster that controls 18 enzymatic biosynthetic steps [69,70]. Different regulatory and structural genes control such pathways [71]. In the current investigation, the effect of taro peel, turmeric, and wheat bran extracts, as well as the fungicide Topsin, on the relative gene expression of *afl*D, *afl*P, and *afl*Q (structural genes), as well as *afl*R and *afl*S (two regulatory genes) (Figure 2) was investigated. The *afl*D was found to play an important role in converting norsolorinic acid to averantin. Meanwhile, *afl*P and *afl*Q are necessary for converting sterigmatocystin to o-methylsterigmatocystin and AFB_1 during the final steps of the aflatoxin biosynthetic pathway [72,73]. The results indicated a 6.43-fold increase in the relative transcription level of *afl*D in the infected, untreated control maize grains. Topsin treatment showed a 3.62-fold increase. Meanwhile, taro peel and wheat bran extracts using 25% acetone extraction solvent and turmeric extract using 25% ethanol solvent reduced the expression level of *afl*D gene to relative expression levels of 2.48-, 2.06-, and 1.63-fold, respectively (Figure 2). Furthermore, the highest relative expressions levels of *afl*P and *afl*Q were detected in the infected control treatment (5.99- and 7.54-fold higher). Moreover, turmeric extract using 25% ethanol treatment showed the lowest transcriptional levels, producing relative expression levels of 1.51 and 1.78 for *afl*P and *afl*Q, respectively. The obtained results are consistent with those published by Mayer et al. [74], who observed the presence of an association between fungal growth kinetics and AFB_1 production when they studied *afl*D expression levels in wheat grains inoculated with an *A. flavus* isolate. The authors also reported that *afl*D, *afl*Q, and *afl*P gene expression levels could be used as markers to differentiate between aflatoxigenic and non-aflatoxigenic strains of *A. flavus* [75,76]. It was also reported that *afl*R and *afl*S are two important key regulatory genes that control the production of AFB_1. A significant correlation was found between the expression level of *afl*R and aflatoxin production by Sweeney et al. [77] using RT-qPCR data analysis. In the current study, transcripts abundant in *afl*R and *afl*S were found to be downregulated in all treated grains compared to untreated controls (Figure 2). The expression of *afl*S and *afl*D was beneficial in differentiating *Aspergillus* AFB_1-producing strains from the non-producing ones, Degola et al. [78]. Furthermore, Mohseni [58] found that *afl*R relative expression dropped significantly in experimental fungus trials that did not receive turmeric extract treatment compared to the control. Therefore, the obtained data show the high capability of the tested extracts to strongly inhibit *A. parasiticus* growth and aflatoxin production by reducing gene expression level in key limiting steps in the AFB_1 biosynthetic pathway.

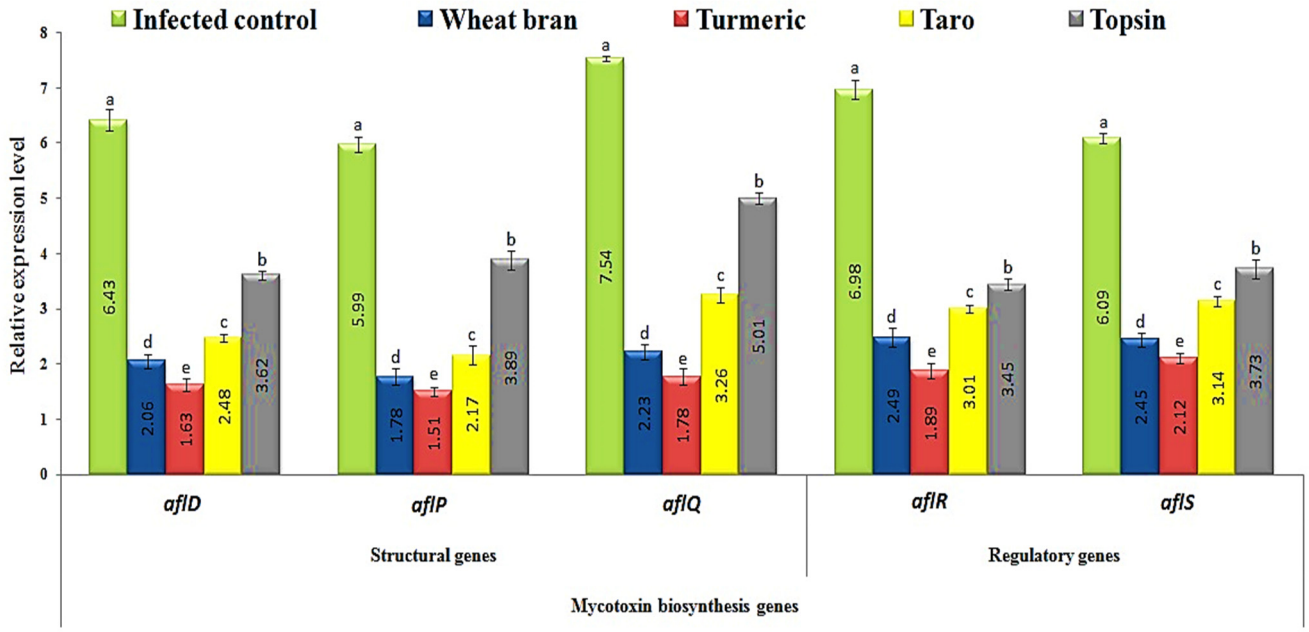

Figure 2. Expression of *afl*D, *afl*P, and *afl*Q (structural genes) and *afl*R and *afl*S genes control key limiting steps in the aflatoxin B_1 (AFB_1) biosynthetic pathway. The different letters above the columns mean the indicated values were significantly different at $p \leq 0.05$.

3.4. Total Phenolic Content (TPC) of the Studied Plant Extracts

The studied plant materials were analyzed for their TPC to determine their efficiency in affecting fungal traits. The plant TPC was estimated in mg GAE/g of dry extract weight. The results obtained in the current study clearly showed that all plant materials contain high-phenolic compound contents that ranged from 46.08 to 61.28 mg of GAE/g dry extract (Table 5). The TPC content was recorded to be highest with 25% acetone extracts of taro peels (61.28 mg of GAE/g dry extract wt.). On the contrary, the lowest TPC concentration was found in wheat bran extracts when 25% acetone was the extraction solvent (46.08 mg of GAE/g dry extract wt.). Thus, as previously reported, phenolic compounds are abundant secondary metabolites that have been the focus of many scientists due to their excellent antioxidant properties and their remarked roles in preventing oxidative stress-based diseases [79].

Table 5. Plant extracts total phenolic contents (TPC) and their antioxidant activity (AA).

Plant Extract	Solvent Concentration	TPC (mgGAE/g dry Extract wt) ± SD	AA (µg/mL)
Ascorbic acid	-	-	4.28
Wheat bran	Acetone 25%	46.08 ± 0.54	59.41
Turmeric	Ethanol 25%	49.82 ± 1.99	74.16
Taro	Acetone 25%	61.28 ± 0.64	7.45

3.5. Antioxidant Activities of the Extracts

DPPH is a method usually utilized to investigate a compound's free radical scavenging or hydrogen donating abilities and screen the antioxidant capacity of specific extracts [80]. This study estimated the plant extracts' antioxidant activities (AA) by comparing DPPH scavenging and IC_{50} (µg/mL) values. Table 5 illustrates the antioxidant activity values of the plants. Turmeric extract exhibited the highest AA value (74.16 µg/mL), while wheat bran and taro peel extract resulted in 59.41 and 7.45 µg/mL, respectively. In the meantime, taro peel extract proved to be a potent scavenger of free radicals and an excellent inhibitor of lipid peroxidation [81,82]. However, in the current study, taro peel extracts

did not show high antioxidant values. Furthermore, the turmeric plant extracts showed the highest antioxidant properties. It is well known that turmeric is rich in antioxidant and anti-inflammatory properties due to its plethora of free radical scavenging secondary metabolites [83].

The RT-qPCR analysis showed a clear downregulation for most of the aflatoxin biosynthetic genes. The results were in harmony with other published reports that have indicated a suppressing effect of some metabolites on the AFB_1 biosynthetic genes [84]. Furthermore, polyphenolic compounds were found to stop the biosynthesis pathway of AFB_1 in *A. flavus* by inhibiting norsolorinic acid accumulation, as reported by Hua et al. [85]. Moreover, Youssef et al. [86] detected several antimicrobial compounds, including 1-dodecanamine, hexadecanoic acid n, n-dimethyl, and *n*-hexadecanoic acid methyl ester, in methanol and ethanol beetroot extracts, which were suggested to produce potential activity against mycotoxin production. Finally, the results obtained in the current study suggest that taro, turmeric, and wheat bran extracts are promising sources for developing effective and environmentally friendly alternatives for controlling aflatoxin biosynthesis, thus, providing a new basis for the establishment of a new protective strategy for long-term grain preservation and storage.

4. Conclusions

Among different organic solvent extracts of turmeric, wheat bran, and taro peels, 75% ethanol extract of taro was extremely active against *Aspergillus flavus* growth, showing the best dry weight mass ratio of maize aflatoxigenic fungus. Meanwhile, the highest AFB_1 production inhibition ratio was achieved using the 25% ethanol turmeric extract. All tested plant materials were active against AFB_1 biosynthesis after one month of maize storage compared to Topsin fungicide. The transcription levels of *afl*D, *afl*P, *afl*Q, *afl*R, and *afl*S showed a significant down-regulated gene expression effect compared to the untreated control and Topsin treatments. The extracts' antioxidant capacities proved their ability to be antifungal growth and antiaflatoxin biosynthesis agents.

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