horticulturae

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The Application of Pomegranate, Sugar Apple, and Eggplant Peel Extracts Suppresses *Aspergillus flavus* Growth and Aflatoxin B1 Biosynthesis Pathway

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Abstract: Even though the green revolution was a significant turning point in agriculture, it was also marked by the widespread use of chemical pesticides, which prompted severe concerns about their influence on human and environmental health. As a result, the demand for healthier and more environmentally friendly alternatives to control plant diseases and avoid food spoilage is intensifying. Among the proposed alternatives, food by-product extracts, especially from the most consumed fruits in Egypt, eggplant, sugar apple, and pomegranate peel wastes, were largely ignored. Hence, we chose them to evaluate their antifungal and antiaflatoxigenic activities against maize fungus, *Aspergillus flavus*. All the extracts exhibited multiple degrees of antifungal growth and aflatoxin B1 (AFB1) inhibitory activities (35.52% to 91.18%) in broth media. Additionally, diethyl ether 50% eggplant, ethanol 75% sugar apple, and diethyl ether 25% pomegranate extracts exhibited the highest AFB1 inhibition, of 96.11%, 94.85%, and 78.83%, respectively, after one month of treated-maize storage. At the same time, Topsin fungicide demonstrated an AFB1 inhibition ratio of 72.95%. The relative transcriptional levels of three structural and two regulatory genes, aflD, aflP, aflQ, aflR, and aflS, were downregulated compared to the infected control. The phenolic content (116.88 mg GAEs/g DW) was highest in the 25% diethyl ether pomegranate peel extract, while the antioxidant activity was highest in the 75% ethanol sugar apple extract (94.02 µg/mL). The most abundant active compounds were found in the GC-MS analysis of the fruit peel extracts: α-kaurene, α-fenchene, p-allylphenol, octadecanoic acid, 3,5-dihydroxy phenol, hexestrol, xanthinin, and linoleic acid. Finally, the three fruit peel waste extracts could be a prospective source of friendly ecological compounds that act as environmentally safer and more protective alternatives to inhibit AFB1 production in maize storage.

Keywords: *Aspergillus flavus*; maize; peel extracts; AFB1; GC-MS; qRT-PCR

Citation: Ismail, I.A.; Qari, S.H.; Shawer, R.; Elshaer, M.M.; Dessoky, E.S.; Youssef, N.H.; Hamad, N.A.; Abdelkhalek, A.; Elsamra, I.A.; Behiry, S.I. The Application of Pomegranate, Sugar Apple, and Eggplant Peel Extracts Suppresses *Aspergillus flavus* Growth and Aflatoxin B1 Biosynthesis Pathway. *Horticulturae* **2021**, *7*, 558. https://doi.org/10.3390/horticulturae7120558

Academic Editors: Dasha Mihaylova and Aneta Popova

Received: 20 September 2021
Accepted: 3 December 2021
Published: 7 December 2021

1. Introduction

Mycotoxins, i.e., aflatoxins, are a type of fungal polyketide secondary metabolite that are produced mostly by *Aspergillus*, including *Aspergillus flavus* [1,2]. Currently, there are 18 types of aflatoxin produced by *Aspergillus* spp., of which the four principal kinds are Aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2) [3]. AFB1 is the most common mycotoxin in nature and is functionally carcinogenic in animal models as well as mammals if the toxicity level exceeds a certain threshold [4]. Aflatoxigenic fungi can cause damage loss in seeds growth, preservation, or viability [5]. Aflatoxin contamination primarily affects dried fruits (such as nuts and peanuts), cereal grains (maizes, etc.), some spices, and oils [6,7]. The primary sources of the world's exposure to aflatoxins are maize and peanuts because of their high consumption [8]. Consequently, solutions for the control of aflatoxigenic *A. flavus* in maize grains and food during storage are in demand worldwide [9]. Chemical treatments can effectively control aflatoxins, but they cannot be used on grains, cereals, or other food materials due to hazardous residues, teratogenicity, carcinogenicity, spermatotoxicity, and hormonal imbalances, as well as the development of resistance microbes against antimicrobial agents [10–12]. Currently, the use of plant-based natural antifungal agents is considered a beneficial and healthy practice in this regard [13,14]. Plant extracts could be employed as antimicrobial agents or for improved food storage and preservation due to their high activity, the simplicity of their production and utilization, their reliability, and their biocompatibility [15,16].

The release of large quantities of agro-by-product wastes such as peels and seed husks is one of the biggest problems facing society, as they are grave threats to the environment [17,18]. As a result, researchers continue to assess into the possibility of reusing these wastes. Such wastes encompass a wide range of compositions, including high levels of proteins, carbohydrates, and minerals. It was reported that many fruit peels offer a range of biological and medicinal properties and are known to contain them [19,20]. Pomegranate peels, lemon peels, and green walnut husks have been reported to be effective natural antimicrobials in various investigations [21–23]. Pomegranate peel extracts are high in functional molecules, such as flavones, phenylpropanoids, and alkaloids, which feature potent antioxidant properties [24,25]. Many investigators have reported the significant antifungal activity of pomegranate and eggplant peel extracts against many phytopathogens [26–29]. Linoleic acid is known to feature antifungal activity in larger plants as a substrate for producing a series of trihydroxy oxylipins [30]. The growth and biomass production of *Rhizoctonia solani* was reduced by 74% and *Pythium ultimum* by 65% when 1000 µM linolenic acid and allylphenol were applied together [31]. Several studies have reported that such natural compounds, including essential oils or extracts such as monoterpenoids and sesquiterpenes could suppress *A. flavus* growth and AFB1 formation by downregulating the transcription of genes involved in AFB1 synthesis [32,33].

Generally, AFB1 is produced from a complicated biosynthetic pathway, including at least 28 enzymatic steps. The structural genes encoding these enzymes are grouped in one gene cluster while two cluster-specific regulators, aflR and aflS, mainly regulate their expression [34,35]. It was reported that the decrease in the transcription levels of aflatoxin genes was associated with a reduction in AFB1 production. The ability of many plant-derived substances to stop AFB1 production or inhibit its expression has been observed [36–38]. Several investigations have shown that various doses of different plant extracts suppress the expression of 25 of the 27 studied genes in the AFB1 biosynthesis pathway [39,40]. The purpose of this study was to evaluate the effectiveness of different extracts of pomegranate, sugar apple, and eggplant peels to inhibit *A. flavus* growth; to test the ability of the peel extracts to suppress the expression of AFB1 biosynthesis genes in maize grains compared with Topsin fungicide; and to identify the different bioactive compounds of the best extracts using the GC-MS analysis technique.

2. Materials and Methods

2.1. Fungus Isolation and Identification

Aspergillus isolate was isolated from local maize grains, purified, characterized morphologically, and assessed for its ability to produce AFB1. The aflatoxigenic isolate was identified by sequencing the amplified ITS region [41,42].

2.2. Peel Extracts Preparation

The pomegranate (*Punica granatum* L.), sugar apple (*Annona squamosa* L.), and eggplant (*Solanum melongena* L.) edible fruits were purchased from local markets in Alexandria Governorate, Egypt. All the fruits were washed and surface sterilized; the peels obtained, air-dried, and pulverized to a fine powder [43]. Twenty grams of the fine powder for each fruit peel was mixed with 100 mL of each of the four solvents: ethanol, diethyl ether, methanol, and acetone, with three concentrations, of 25%, 50%, and 75% (solvent/water, v/v). The preparations were left overnight on an orbital shaker (Heidolph, Schwabach, Germany) at 200 rpm. All the mixtures were filtered using Whatman No. 1 and stored in a refrigerator (at 5 °C) until further use.

2.3. Total Phenolics Content

The Folin–Ciocalteau reagent (FCR) assay (Sigma-Aldrich, Taufkirchen, Germany) [44], was used to determine the total polyphenols content (TPC), with slight modifications. A total of 0.1 mg/mL of extract was dissolved in distilled water. Next, 0.5 mL FCR (1 mol/L) and 1.5 mL of sodium carbonate (10% w/v) were added to 0.5 mL of each extract. The final mixture was kept for 30 min in the dark, and the absorbance values at a wavelength (λ) = 725 nm were measured. The TPC was calculated according to a standard curve using gallic acid prepared in methanol with 12.5, 25, 50, 75, and 100 µg/mL concentrations. The concentrations of TPC were expressed in milligrams of gallic acid equivalents per gram of dry extract weight (mg GAEs/g DW) [45].

2.4. DPPH Radical Scavenging Ability

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (Sigma-Aldrich, Taufkirchen, Germany) was used to test antioxidant activity, and the capacities of the extracts to scavenge free radicals were determined as described by Asnaashari et al. [46]. The calculation equation was: $(\mathrm{DPPH})\ \% = [(\mathrm{Ab} - \mathrm{Abs})/\mathrm{Ab}] \times 100$ where Ab is the blank absorbance value and Abs is the sample absorbance value.

2.5. Gas Chromatography-Mass Spectroscopy Analysis

Thermo Scientific ISQ Quadrupole GC-MS with Trace GC Ultra (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a capillary column TG–5MS (30 m × 0.25 mm × 0.25 µm film thickness) was used as previously described [47,48]. The separation conditions were performed as outlined by Okla et al. [49]. For the identification of the different compounds in the fruit peel extract samples, the retention times and mass spectra databases were compared to those of authentic standards.

2.6. Effect of Fruit Peel Extracts on Fungal Biomass and Aflatoxin Production

2.6.1. Fungal Biomass Determination

Fifteen mL of potato dextrose agar (PDA) media was poured into a petri dish, and after the solidification, a 5 mm disc of the aflatoxigenic fungus was placed in the center of the PDA petri dish and incubated for 7 days at 30 °C. In total, 1 mL of each fruit peel extract, previously prepared as described in Section 2.2, was added to 50 mL yeast extract sucrose (YES) broth in a conical flask. Next, a fungus disc was placed in each conical flask and set for 15 days at 30 °C. Each treatment's fungal mat was oven-dried. The wet and dry weights (g) of the fungal mat were recorded in all the treatments, and the filtrates were

maintained at 4 °C for later aflatoxin B1 analysis. The following equation was used to calculate the aflatoxin inhibition (AI) percentage ratio [50]:

$$(\mathrm{AI})\% = \left[\frac{\text{AFB1 control} - \text{AFB1 treatment}}{\text{AFB1 control}}\right] \times 100$$

2.6.2. Maize Storage Experiment

Fifty grams of maize grains were treated with the fruit peel extracts, yielding the most significant results, as in the previous section. The treated grains were placed in sterilized-glass bottles. The fungicide treatment (2.5 mg/mL) was treated with Topsin (Thiophanate methyl, 70% wettable powder, United Phosphorus, Inc., King of Prussia, PA, USA), which was used as a control. Subsequently, each bottle was inoculated with a 5 mm disc of the aflatoxigenic fungus and kept for 30 days at 30 °C. The shape and odor of the maize grains were assessed after the storage period, using the scale developed by Youssef et al. [15]. All of the analyzed grains were crushed and refrigerated at 4 °C until they were used for further aflatoxin studies.

2.6.3. Aflatoxin B1 Extraction

Aflatoxin B1 (AFB1) was extracted by mixing 2 mL of fungal filtrate YES broth medium with chloroform (1:1 v/v). The mixture was centrifuged at 10,000 rpm for 5 min; a total of 2 mL of the bottom layer was transferred to a fresh glass vial. After evaporating under a moderate air stream, the dried chloroform extracts were re-dissolved with 1 mL methanol [51]. To extract the AFB1 from the contaminated maize grains, about 20 g of crushed grains was mixed with 100 mL of methanol and 12 mL of 4% potassium chloride (w/v), according to Hoeltz et al. [52], with some adjustments. The samples were filtered after a spin for 2 min at 10,000 rpm. The filtrate was then added to 100 mL of 10% (w/v) $CuSO_4$, mixed, and filtered. To extract the AFB1, 15 mL of an equal volume of chloroform and distilled water (1:1 v/v) was mixed with the filtrate in the separating funnel; this process was repeated twice. The solvent extracts were collected and evaporated. Before high-performance liquid chromatography (HPLC) analysis, all the samples were filtered into HPLC vials using a 0.2 m syringe filter (Thermo Fisher Scientific, Waltham, MA, USA).

2.6.4. Preparation of AFB1 Standard and HPLC Conditions

To prepare the AFB1 standard (Merck, MO, USA), 1 mg was dissolved in 100 mL of toluene: acetonitrile (9:1, v/v) to obtain a final concentration of 10 µg/L. A working standard solution was prepared with a sample diluent (7% methanol + 92% 0.01 phosphate-buffered saline + 1% dimethylformamide) at concentrations of 5, 2, 1, 0.5, 0.2, and 0 µg/L [53]. The limit of detection and quantification for AFB1 as detected by the UV detector were 0.01 µg/L and 1 µg/L, respectively. Agilent HPLC (Santa Clara, CA, USA) was used to analyze the AFB1 using a Zorbax Eclipse Plus C18 column (4.6 mm 150 mm, 3.5 m) and a UV 365 nm detector. The mobile phase ratios were water, methanol, and acetonitrile (50:40:10, $v/v/v$). The flow rate was 0.8 mL/min, at ambient temperature, and the injection volume was 10 µL, with a concentration of 0.044 mg/mL [51].

2.6.5. RNA Extraction, cDNA Synthesis, and qRT-PCR Assay

The guanidium isothiocyanate technique was used to isolate whole-plant RNA, with certain modifications [54]. As previously described, the reverse transcription procedure was carried out [55,56]. The real-time PCRs (Qiagen Rotor-Gene Q2, Qiagen, Hilden, Germany) were carried out with designated primers targeting the aflatoxin biosynthesis pathway (Table 1). For normalization, the β-tubulin gene was served as an internal reference. As previously indicated, 20 µL SYBR Green qPCR reactions were performed [57,58]. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative gene expression levels from the threshold cycle [59].

Table 1. Primer sequences were used in this study.

Gene	Primer Sequences (5′-3′)	Function in the Biosynthesis Pathway of AFB1
β-tubulin	F: CTTGTTGACCAGGTTGTGGAT R: GTCGCAGCCCTCAGCCT	Reference gene
aflD	F: GTCCAAGCAACAGGCCAAGT R: TCGTGCATGTTGGTGATGGT	Norsolorinic acid (NOR) → Averantin (AVN)
aflP	F: GGCCGCCGCTTTGATCTAGG R: ACCACGACCGCCGCC	Sterigmatocystin (ST) → O-methylsterigmatocystin (OMST)
aflQ	F: GTGTCCGCAGTGTCTAGCTT R: GCTCAAAGGTCGCCAGAGTA	OMST → AFB1
aflR	F: CTCAAGGTGCTGGCATGGTA R: CAGCTGCCACTGTTGGTTTC	Regulator gene
aflS	F: CTGCAGCTATATTGCCCACA R: TAAACCCAGGCAGAGTTGGT	Regulator gene

2.7. Statistical Analysis

All the statistical analyses were performed with the CoStat program, version 6.303, and the analysis of variance technique (CoHort software, Monterey, CA, USA). The data from the expression analysis of the aflatoxin biosynthesis genes were expressed as means standard deviation (S.D.), and the values were considered statistically significant when $p \leq 0.05$.

3. Results and Discussion

3.1. Identification of the Aflatoxigenic Fungal Isolate

The aflatoxigenic isolate was found to produce AFB1 at a rate of 25.67 µg/L. The revealed sequence of the ITS region of the aflatoxigenic isolate was submitted to NCBI using a blasting tool given the high similarity with *Aspergillus flavus* fungus [60]. A coded name was given to the isolate as *A. flavus* f2 and GenBank accession no. (# MG202160).

3.2. Fruit Peel Extracts Effect on A. flavus Biomass and AFB1 Production

To evaluate the influence of different fruit peel extracts prepared with varying solvents at three different concentrations on *A. flavus* fungal biomass (dry and wet weight) and AFB1 production, the pomegranate, sugar apple, and eggplant fruit peels were extracted with diethyl ether, acetone, ethanol, and methanol at concentration percentages of 25%, 50%, and 75% (Table 2). In comparison to the control, the 75% ethanol pomegranate peel extract (0.09 and 0.03 g), 25%- methanol sugar apple peel extract (0.12 and 0.01 g), and 75%- methanol eggplant peel extract (1.89 and 0.24 g, respectively) produced the least substantial wet and dry weight values. These results agree with those previously reported for pomegranate peel extract, which has been shown to be intensely active against *A. flavus*, *A. parasiticus*, *A. fumigatus*, *Fusarium proliferatum*, and *F. verticillioides* isolates with minimal inhibitory concentration (MIC) values ranging between 1.25 and 5 mg/mL [61]. Furthermore, similar results were recorded by Oliveira and Furlong [29], according to which eggplant peel phenolic extract inhibited the growth of *A. flavus* after 72 h of incubation with 84.80%. In comparison, Basudan [62]'s findings proved that black and white eggplant peel extracts had no fungal effect against the *A.flavus* strain.

Table 2. *Aspergillus flavus* biomass and AFB1 inhibition ratio (AI%), as affected by applying pomegranate, sugar apple, and eggplant peel extracts.

Solvent Concentration (%)	Control AFB1 Production (µg/L)	Control		Weight of *A. flavus* Mat (g)											
				Pomegranate				Sugar Apple				Eggplant			
		Wet	Dry	Wet	Dry	AFB1 (µg/L)	AI%	Wet	Dry	AFB1 (µg/L)	AI%	Wet	Dry	AFB1 (µg/L)	AI%
Ethanol															
25	23.58	6	0.97	4.56	0.62	2.19	90.70	1.16	0.08	8.52	63.86	6.16	0.88	2.88	87.79
50	24.74	6.97	1.06	4.59	0.44	3.18	87.13	1.19	0.17	5.32	78.49	4.66	0.63	6.79	72.55
75	25.20	3.59	0.62	0.09	0.03	3.69	85.35	0.16	0.05	4.48	82.23	5.60	0.75	2.52	90.60
Acetone															
25	22.49	4.58	0.67	3.78	0.69	2.18	90.32	0.17	0.02	5.97	73.45	3.30	0.62	9.04	59.79
50	20.12	5.05	0.74	4.33	0.93	3.46	82.79	0.14	0.02	6.58	67.29	5.57	0.56	9.60	52.30
75	17.56	4.33	0.73	5.12	1.00	2.57	85.37	0.26	0.01	3.52	79.97	5.19	0.82	10.77	38.66
Methanol															
25	17.00	7.49	0.71	4.34	0.72	6.42	62.26	0.12	0.01	10.50	38.23	5.16	0.57	8.09	52.41
50	13.85	7.16	0.91	4.84	0.74	3.44	75.15	0.32	0.13	8.93	35.52	7.19	0.65	8.69	35.97
75	14.08	5.27	0.62	0.17	0.04	2.60	81.50	5.83	0.75	8.85	37.12	1.89	0.24	4.16	70.43
Diethyl ether															
25	26.44	5.09	0.9	5.83	0.79	2.42	90.87	0.24	0.07	13.71	48.15	5.84	0.80	3.54	86.62
50	24.83	6.08	0.63	4.42	0.85	5.23	78.96	0.19	0.01	5.07	79.59	6.46	0.76	2.19	91.18
75	24.16	7.05	0.73	4.18	0.86	6.86	71.61	2.21	0.39	5.92	75.50	6.31	0.78	2.19	90.95
$L.S.D._{0.05}$		1.47	0.08	0.47	0.03	0.16		0.02	0.03	0.01		0.49	0.03	0.12	

Furthermore, most of the solvent extracts examined were highly efficient against the formation of AFB1 at various concentrations. The AFB1 inhibition ratio (AI%) ranged from 35.52% to 91.18%. Besides, AFB1 production was less affected by the methanol extracts, with the AI% values ranging from 35.52% to 81.5%. Table 2 shows that pomegranate peel extracts, diethyl ether 25%, ethanol 25%, and acetone 25% produced promising results, with AI values of 90.87%, 90.70%, and 90.32%, respectively. Still, methanol 25% and diethyl ether 75% exhibited the lowest AI values (62.26% and 71.61%, respectively). Table 2 demonstrates that the AI% values of the sugar apple extract with different solvent concentrations differed significantly. In comparison to the AI% values of the other solvents, the highest AI%, of 82.23%, was achieved at 75% ethanol treatment. The least effective ratio was obtained with the 25%, 50%, and 75% methanol sugar apple peel extracts (38.23%, 35.52%, and 37.12%), respectively. Table 2 demonstrates that the highest levels of AFB1 inhibition were obtained with eggplant diethyl ether extract at concentrations of 50% and 75% (91.18% and 90.95%, respectively), while the 50% methanol treatment produced the lowest AI% results (35.97%).

After 72 h of incubation, the pomegranate peel extract at the 1250 µg/mL concentration inhibited AFB1 production by 67% without affecting fungal growth [61]. According to other researchers, extracting antioxidants reduces aflatoxins by absorbing, neutralizing the free radicals, and preventing their proliferation chains, resulting in less dangerous compounds. Furthermore, the efficacy of solvents varies depending on their quantities and the components of a particular plant extract. Sugar apple and eggplant methanol and ethanol peel extracts demonstrated strong antioxidative properties against human infections, according to Bernardo and Sagum [63]; these findings endorse our results. According to Adom et al. [64] and Laddomada et al. [65], the essential antioxidants in maize bran are the phenolic acids, mainly the phenolics covalently bonded with cell wall structural components through ester bonds, which play a defensive role against plant fungal infection.

3.3. Maize Storage Experiment

3.3.1. AFB1 Production

Table 3 demonstrates that 50% diethyl ether eggplant peel extract was the most effective treatment, with the most negligible value of AFB1 (20.72 µg/L) and an AI of 96.11%, followed by 75% ethanol sugar apple peel extract with an AI of 94.85% (27.39 µg/L). Com-

pared to the other treatments, the value of AFB1 in the 25% diethyl ether pomegranate peel extract was 112.64 µg/L. These findings are similar to those of Oliveira and Furlong [20], who found that the presence of 30µg phenol/mL agar eggplant bulb extract can reduce *A. flavus* AFB1 production by 87.80%.

Table 3. AFB1 production (µg/L) and aflatoxin inhibition ratios (AI) after the storage period correspond to peel extract treatments.

Treatments	AFB1 (µg/L)	AI%
Healthy control	0.00	100
Infected control	532	0.00
25%-Diethyl ether pomegranate peel extract	112.64	78.83
75%-Ethanol sugar apple peel extract	27.39	94.85
50%-Diethyl ether eggplant peel extract	20.72	96.11
Topsin fungicide (2.5 mg/mL)	143.92	72.95

3.3.2. Grain Shape and Odor as Affected by Applied Fruit Peel Extract Treatments

Table 4 demonstrates that the tested fruit peel extracts and Topsin fungicide dramatically modified the grains' appearance compared to the control. The eggplant peel extract, followed by the sugar apple and the pomegranate peel extracts, demonstrated outstanding antifungal efficacy and a high-grain appearance compared to the control. The Topsin fungicide treatment of inoculated grains at authorized quantities resulted in rotting, deformation, foul odors, and unapproved grains. Our findings are consistent with those of Gemeda et al. [66] and El-Aziz et al. [67]. They reported similar reductions in *Aspergillus* fungal dry weight after using essential oils. Doum, banana, and licorice peel extracts exhibited similar decreases [15]. The findings were similarly consistent with those of Yazdani et al. [68] and Oliveira and Furlong [29]. They found that some phenolic compounds could reduce aflatoxin production AFB1.

Table 4. Grains' shape and odor correspond to peel extract treatments.

Treatments	Scale	Odor	Shape
Healthy control	5	0	5
Infected control	0	5	0
25% Diethyl ether pomegranate peel extract	4	2	4
75% Ethanol sugar apple peel extract	5	1	5
50% Diethyl ether eggplant peel extract	5	1	5
Topsin fungicide (2.5 mg/mL)	0	5	0

3.4. Transcriptional Levels of AFB1 Biosynthesis Genes

The biosynthesis of aflatoxin compounds, particularly AFB1, is a complicated enzymatic pathway [69]. AFB1 is generated in *A. flavus* from acetyl CoA by a 75 kb cluster of genes that encodes more than 18 enzymatic steps [70,71]. Such pathways are organized by different structural and regulatory genes [72]. These regulatory genes include many genes such as aflR and aflS, while structural genes contain more than 20 genes, such as aflD, aflG, aflH, aflI, aflK, aflM, aflO, aflP, and aflQ [73,74]. In the present study, the influence of the three fruit peel extracts, as well as Topsin fungicide, on the relative expression of two regulatory genes (aflR and aflS) and three structural genes (aflD, aflP, and aflQ) was investigated (Figure 1). The aflD enzyme is required to convert norsolorinic acid to averantin in the early stages of AFB1 biosynthesis. In the late stages of the AFB1 pathway, the aflP and aflQ genes encode enzymes that convert sterigmatocystin to o-methylsterigmatocystin and AFB1, respectively [75,76].

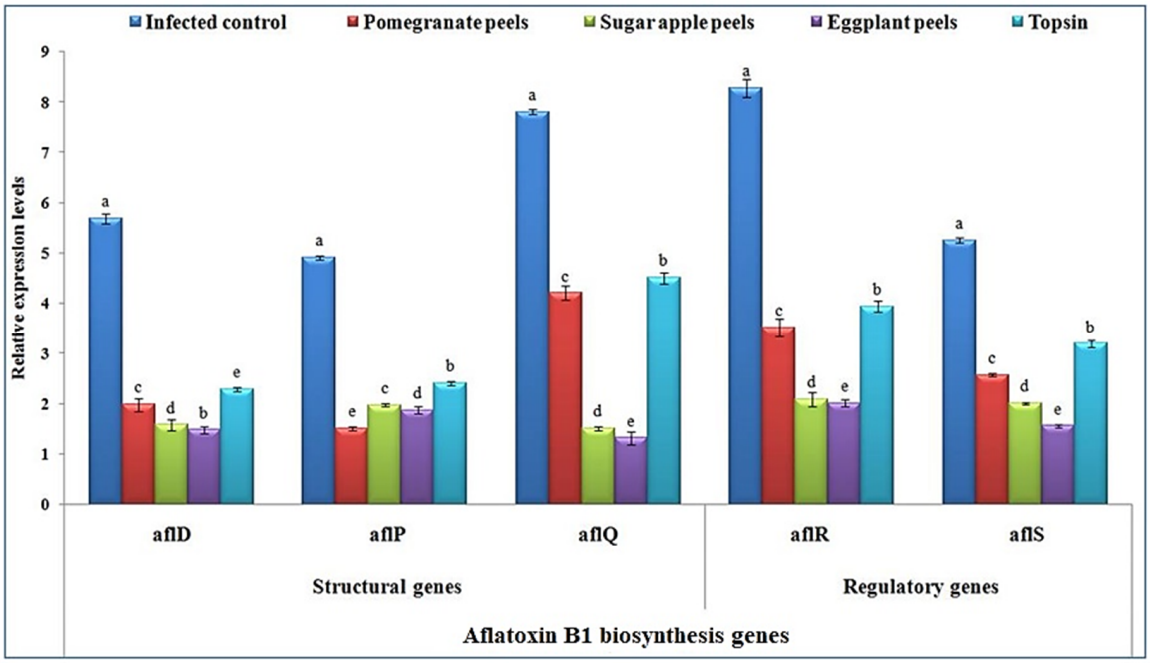

Figure 1. The expression levels of regulatory genes (aflR and aflS) and structural genes (aflD, aflP, and aflQ) of the AFB1 biosynthesis pathway. Columns with the same letters among each gene are not significantly different at $p \leq 0.05$.

The untreated maize grains (infected control) demonstrated the highest relative transcription level of aflD (5.69-fold), followed by the Topsin-treated grains, with a relative expression level 2.29-fold higher than the control (Figure 1). In addition, the acetone 25% pomegranate, diethyl ether 75% sugar apple, and eggplant peel extract treatments showed decreasing transcriptional of aflD, with relative expression levels of 1.98-, 1.58-, and 1.48-fold, respectively (Figure 1). Similarly, the infected control treatment presented the highest relative expressions levels of aflP and aflQ, which were 4.91- and 7.81-fold, respectively, higher than the control (Figure 1). Interestingly, the two-aflP and aflQ genes exhibited the lowest relative expression levels, of 1.51-fold, with acetone 25% pomegranate and diethyl ether 75% eggplant peel extract treatments, respectively (Figure 1). The findings are consistent with those of Mayer et al. [77], who reported that the relative expression level of aflD in wheat experimentally infected with an aflatoxin-producing *A. flavus* was linked with AFB1 production and fungus growth kinetics. As a result, the expression of the aflD, aflP, and aflQ genes can support valuable distinguishing between aflatoxigenic and non-aflatoxigenic *A. flavus* strains [78,79]. Generally, the aflR and aflS are essential regulatory genes for AFB1 synthesis. Many reports assed significant correlations between the transcriptional levels of the two regulatory genes and AFB1 production [15,80]. The results showed the downregulation of two genes, aflR, and aflS, for all treatments compared to infected (untreated) controls. The diethyl ether 75% eggplant peel extract treatment exhibited the lowest aflR and alfS relative expression levels of 2.01- and 1.56-fold, respectively (Figure 1). The untreated infected control exhibited relative expression levels of 8.28- and 5.26-fold for aflR and alfS, respectively (Figure 1). Similar to the structural genes, it was demonstrated that the expression levels of aflS and aflD could be used to distinguish AFB1-producing *Aspergillus* strains from non-producing *Aspergillus* strains [81]. The results indicated that all the tested extracts exert an inhibitory effect on growth and aflatoxin production and could be used as antimycotoxigenic agents against AFB1-producing *Aspergillus* strains.

3.5. Total Phenolics Content of the Fruit Peel Extracts

The TPC was calculated as mg GAEs/g DW for each of the plant materials evaluated. The fruit peel extracts tested presented TPC levels ranging from 30.26 to 116.88 mg of GAEs/g DW (Table 5). The 25% diethyl ether pomegranate peel extract (116.88 mg GAEs/g

DW) featured the greatest TPC. The TPC of the 75% ethanol sugar apple peel extract was 35.09 mg GAEs/g DW, and 50% diethyl ether eggplant peel extract featured the lowest TPC value (30.26 mg GAEs/g DW). It was shown that TPC values in various 70% ethanol pomegranate peel fractions ranged from 78.10 to 200.90 mg GAE/g. Meanwhile, the ethyl acetate fraction featured the highest TPC, whereas the petrol-ether fraction presented the lowest. In the ethyl acetate fraction, gallic acid and ellagic acid were found in the highest quantities. However, in the water fraction, punicalin and punicalagin were found in the highest concentrations [82]. Manochai et al. [83] found that TPC ranged from 33.80 to 140.40 mg GAEs/g in ten Thailand sugar apple peel ethanol extracts, while Petch Pakchong cultivar featured the lowest TPC, of 33.80 mg GAEs/g. In another study, Ji et al. [84] found that the total phenolic content of eggplant peel water extract was more than four times higher than that of eggplant pulp. Malviya et al. [85] reported that the highest value of TPC was detected in a 100% aqueous pomegranate extract. At the same time, the lowest TPC was found in the 70% ethanol extracts. Phenolic contents are the most common secondary metabolites in plants. Their high antioxidant activities and significant impact in preventing oxidative stress-related disorders have drawn increasing attention [86].

Table 5. Total phenolics (TPC) and total antioxidant activity (TAA) of the fruit peel extracts.

Fruit Peel Extract	TPC (mgGAEs/g DW) ± SD	TAA (μg/mL) ± SD
25% Diethyl ether pomegranate	116.88 ± 1.44	86.76 ± 0.22
75% Ethanol sugar apple	35.09 ± 1.79	94.02 ± 0.08
50% Diethyl ether eggplant	30.26 ± 1.76	52.94 ± 0.15

3.6. DPPH Scavenging Ability

DPPH is a method for evaluating the antioxidant potential of extracts and testing the ability of substances to serve as free radical scavengers or hydrogen donors [87]. For DPPH scavenging IC_{50} values, total antioxidant activities (TAA) were evaluated in all the investigated fruit peels. The results revealed that most of the extracted peels examined featured reasonably strong antioxidative activity values (Table 5). The highest TAA was found in the sugar apple peel extract (94.02 μg/mL), followed by 86.76 and 52.94 μg/mL in pomegranate and eggplant peels, respectively. The findings were similar to those of Ji et al. [84], who observed a higher amount of ascorbic acid in eggplant peel (51.88 mg/100 g). Meanwhile, Jayaprakasha and Rao [88]'s results suggested that methanol pomegranate peel extract offered the highest antioxidant activity among all the tested extracts in scavenging or preventive capacity against superoxide anion, hydroxyl, and peroxyl radicals [89]. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was found to be the highest for the methanol pomegranate peel extract and the 70% aqueous ethanol extract (79.50; 94.60), respectively [85]. Ten Thailand cultivars of sugar apple peel extracts featured antioxidant activity ranging between 0.42 and 3.06 mg/mL, and the "Nhur Thong" cultivar peel ethanol extract featured the highest antioxidant capacity [83].

3.7. Bioactive Compounds Identified in Fruit Peel Extracts

The GC-MS analysis of the pomegranate, sugar apple, and eggplant peel extracts revealed many bioactive components in each extract. Table 6 presents the main extract compounds with peak area percentages (%) at different retention times (RT). The pomegranate peel extract included a total of 29 components. High relative abundance concentrations were observed in p-allylphenol (20.78%), 3,5 dihydroxy phenol (9.37%), linoleic acid (7.35%), xanthinin (7.05%), sorbitol (5.66%), ethylnorbornane (4.93%), levoglucosenone (4.76%), D-mannose (4.70%), α-himachalene (4.33%), and octadecanoic acid (3.65%). The sugar apple peel extract included a total of 32 components, with a high relative abundance concentration in α-fenchene (11.03%), octadecanoic acid (10.34%), alpha-kaurene (6.33%), hexestrol (5.87%), longipinene (5.83%), methyl isopimarate (5.67%), rhodopin (5.61%), valproic acid (5.04%), (S)-(-)-citronellic acid (4.76%), 4-methylcatechol (4.75%), and 1,16-hexadecanedioic acid (4.20%). As stated previously, the polyphenol content of the pomegranate peel extract

included ellagic and punicalagin compounds, or their derivatives [90]. The eggplant peel extract included a total of 43 components, with a high relative abundance concentration of alpha-kaurene (25.67%), p-allylphenol (8.50%), methyl isopimarate (6.01%), linoleic acid (5.22%), α-fenchene (4.67%), phyllocladene (4.29%), rhodopin (4.19%), octadecanoic acid (3.98%), dimrthoxydurene (3.76%), and (+)-beyerne (3.03%). The most relative abundance of octadecanoic acid, the ester of linoleic acid, which operates as a signaling molecule, was 10.34%, at an RT of 15.70 min.

Table 6. GC-MS phytochemical analysis of pomegranate, sugar apple, and eggplant peels extracts.

Retention Time	Compound	Class	Peak Area (%)		
			Pomegranate	Sugar Apple	Eggplant
3.70	α-Fenchene	Bicyclic monoterpene	-	11.03	4.67
4.90	p-Allylphenol	Phenylpropene	20.78	2.11	8.50
5.10	Valproic acid	Saturated fatty acids	-	5.04	0.79
5.31	Ethylnorbornene	Cyclic hydrocarbons	4.93	1.14	1.68
5.60	Phenylglyoxylic acid	Carboxylic acids	2.26	1.43	0.62
6.20	4-Methylcatechol	Polyphenols	0.76	4.74	0.93
6.34	Sorbitol	Sugar alcohols	5.66	-	-
6.60	7,8-Dihydro-α-ionone	Carotenoids	-	2.79	0.51
6.63	4-Ethylbezaldehyde	Aldehydes	3.07	-	-
6.88	Dimethoxy durene	Alkylbenzene	0.57	0.77	3.76
7.05	Dimethyl caffeic acid	Phenolic acids	1.66	-	-
7.15	Scopoletin	Phenylpropanoids	0.62	-	-
7.45	Farnesol	Sesquiterpenes	0.29	1.87	0.46
7.70	Hexestrol	Nonsteroidal estrogen	0.35	5.87	0.29
7.91	*p*-Cymene	Monoterpenes	1.00	-	0.37
8.04	α-Terpineol	Monoterpenes	-	-	0.59
8.11	Stevioside	Diterpene glycosides	1.29	0.43	0.43
8.27	γ-Terpinene	Monoterpenes	-	3.24	0.32
8.55	Levoglucosenone	Heterocyclic ketones	4.76	-	-
8.59	6-Hydroxyflavone	Flavonoids	0.78	0.39	0.35
8.94	Resveratrol	Polyphenols	-	0.69	0.61
9.49	N-Acetylneuraminic acid	Alpha-keto acid sugars	-	3.01	-
9.76	D-mannose	Carbohydrates	4.70	-	-
9.90	Xanthinin	Sesquiterpene lactones	7.05	3.80	0.53
10.10	3,5-Dihydroxyphenol	Phenoles	9.37	-	-
10.63	3,5,7-Tri-O-methylgalangin	Flavonoids	-	0.50	-
11.00	*p*-Menthone	Monoterpenes	0.96	-	-
11.20	δ-Elemene	Sesquiterpenes	-	-	2.14
11.47	β-lonol	Sesquiterpenes	1.35	-	-
11.67	α-Selinene	Sesquiterpenes	-	-	0.70
12.00	Caryophyllene	Sesquiterpenes	-	-	0.69
12.26	Kaempferol	Flavonoids	-	-	0.26
12.34	5,7,3′,4′-Tetrahydroxflavanone	Flavonoids	-	-	0.54
12.47	β-Patchoulene	Polycyclic hydrocarbons	-	-	1.09
12.55	β-Gurjunene	Sesquiterpenes	-	-	0.67
12.76	γ-Muurolene	Sesquiterpenes	-	-	0.74
13.01	Quercetin 7, 3, 4—Trimethoxy	Flavonoids	1.50	-	-
13.20	α-Himachalene	Sesquiterpenes	4.33	-	-
13.70	Longipinene	Epoxides	-	5.83	0.72
14.95	Apigenin 8-C-glucoside	Flavonoids	-	0.65	-
15.10	4-Hydroxy-2-methoxybenzaldehyde	Methoxyphenols	-	0.19	-
15.42	Methyl 17-methyloctadecanoate	Fatty acid methyl esters	0.67	0.23	-
15.70	Octadecanoic acid	Fatty acids	3.65	10.34	3.98
15.85	Glycitein	Isoflavones	0.74	-	-
15.85	Stearic acid	Fatty acids	-	2.17	0.91

Table 6. *Cont.*

Retention Time	Compound	Class	Peak Area (%)		
			Pomegranate	Sugar Apple	Eggplant
16.19	Phyllocladene	Diterpenoids	-	-	4.29
16.52	Luteolin 6,8-C-diglucoside	Flavonoids	2.03	-	1.42
16.91	(S)-(-)-Citronellic acid	Monoterpenes	-	4.75	-
16.91	Linoleic acid	Polyunsaturated fatty acids	7.35	-	5.22
17.04	5β, 7Bh, 10α-Eudesm-11-en-1α-ol	Sesquiterpenes	0.78	-	
17.05	Quinine	Alkaloids	-	-	0.28
17.10	1,16-Hexadecanedioic acid	Fatty acids	-	4.20	1.40
17.79	Tetrahydroisovelleral	Sesquiterpene dialdehydes	-	-	2.29
18.26	2β-hydroxy-9-oxoverrucosane	Terepnoides	-	-	1.04
18.60	(+)-Beyerene	Diterpenes	-	1.92	3.03
18.70	Abietic acid	Diterpenes	-	-	1.84
19.22	α-Kaurene	Diterpenes	-	6.33	25.67
19.89	Methyl isopimarate	Diterpenes	-	5.67	6.01
21.31	Isosteviol	Diterpenes	-	0.36	2.54
21.62	7α-Hydroxymanool	Diterpenes	-	0.33	1.15
23.08	Sclareol	Diterpene alcohols	-	1.56	1.08
23.65	Zeaxanthin	Carotenoids	2.09	-	-
23.71	Rhodopin	Carotenoids	-	5.61	4.19

It was reported that linolenic acid and p-allylphenol (syn. chavicol) possess antifungal properties [91,92]. They may help to manage plant infections, such as *Botrytis cinerea*, which increases fungal oxygen consumption by 19.58% at 5 ppm and features fungicidal properties against various taxa, including *Alternaria* and *Sclerotinia* species [91,92]. Hydroxychavicol derivative found in betel leaf chloroform extract inhibited *Aspergillus* species with minimum fungicidal concentration (MFC) ranging from 125 to 500 µg/mL employed with broth microdilution method [93]. Similarly, several linoleic acid derivatives have been shown to inhibit the production of AFB1 by various *Aspergillus* species [68].

The second abundant phenolic compound found in the pomegranate peel extract was 3,5-dihydroxy phenol (syn., phloroglucinol). Acylated phloroglucinol is employed as an antifungal agent against *A. flavus* and *A. niger* at a low minimum inhibitory concentration (MIC), 1.0 µg/mL [94]. Flavones, catechins, tannins, and quinines are phenolic compounds that interact with proteins and inactivate them by modifying their structure. Different phenolic compounds, such as dihydrochalcone and chavibetol, were identified from *Piper betle* extract, as reported by Ali et al. [93] and Yazdani et al. [68]. Polyphenolic compounds could halt the *A. flavus* production pathway of AFB1 by reducing norsolorinic acid accumulation, according to Hua et al. [95]. Pomegranate peel extract also contains xanthinin, which acts as a plant growth regulator and features phytotoxicity. It was purified from *Xanthium macrocarpum* fruit and evaluated against *A. fumigates*; it exhibited antifungal activity with MIC > 250 µg/mL. A possible metabolization leading to an unsaturated carbonyl group by eliminating an acetate ion could thus explain its antifungal activity [96]. Furthermore, the pomegranate and eggplant peel extracts contain the energy source linoleic acid, which is crucial for maintaining the membrane fluidity of the epidermis' transdermal water barrier. The biological inhibitory effect of hexestrol was investigated in a study by Inamori et al. [97], who found potent antifungal activity on the growth of plant pathogenic fungi, *Fusarium oxysporum f.* sp. *lycopersici* and *Botrytinia fuckeliana* with MIC 5 and 10 µg/mL, respectively.

Terpenes are the most diverse category of bioactive molecules identified in many plant extracts, with significant antibacterial activities that can be boosted synergistically through the interplay of multiple compounds (from the plant's crude extracts). The biochemical composition of these extracts varies based on the plant species and the plant part used [98]. The compound α-fenchene, also known as (-)-7,7-dimethyl-2-methylene bi-cycle [2.2.1]

heptane, was the most organic compound in sugar apple peel extract considered bicyclic monoterpenoids. The α-kaurene (ent-kaurene) was the most diterpene chemical compound detected in the eggplant peel extract. Low antimicrobial properties of ent-kaurene against *E. coli*, *P. aeruginosa*, *S. aureus*, *C. albicans T. mentagrophytes*, and *T. rubrum* were observed; in addition, no activity was observed against *A. niger* at 30 µg/mL [99,100]. Since monoterpenes, diterpenes, and sesquiterpenes were detected in the three studied fruit peel extracts, they could be effective alone or synergistically at killing fungi or preventing aflatoxin production in media or stored grains. Similarly, Bisht et al. [101] found that *Origanum vulgare* hydro-distilled oil (the main constituent is the oxygenated monoterpene *p*-cymene) strongly inhibited both fungi *A. flavus* and *A. niger*, with the highest inhibition zone of 30 mm. Linalool and citral terpenes are effective against *C. albicans*, and when combined with fluconazole, they produce tremendous synergistic action against a fluconazole-resistant *C. albicans* [102].

Even though eggplant peel extract includes a high amount of polyphenols, terpenoids, and fatty acids, it suppresses *A. flavus* AFB1 production by up to 95%. By contrast, a combined treatment (low dosages of pomegranate peel extract and the azole fungicide prochloraz) resulted in the total prevention of toxin synthesis over 72 h. A qRT-PCR analysis revealed the downregulation of most aflatoxin biosynthetic cluster genes [61]. Youssef et al. [48] reported that phytochemical compounds, such as fatty acids or their esters (octadecanoic acid, n-hexadecanoic acid, and hexadecanoic acid methyl ester), as identified in beetroot extracts, offered potential activity against the mycotoxin produced by *Alternaria alternata*. Overall, these findings suggest that fruit peel extracts could be promising as efficient and sustainable green sources of antioxidants, inhibit aflatoxin production, and potentially become protective grain storage saver applications instead of the chemicals currently used.

4. Conclusions

Among three concentrations of four different extracts of pomegranate, sugar apple, and eggplant peels applied as inhibitors for aflatoxigenic maize fungus *A. flavus*, the diethyl ether 50% eggplant extract displayed the highest AFB1 inhibition ratio (91.18%). After one month of maize grain storage, all the studied peel extracts were effective against AFB1 production, with average inhibition ratios ranging from 78.83% to 96.11% compared to Topsin fungicide (72.95%). The relative levels of aflD, aflP, aflQ, aflR, and aflS expression were considerably down-regulated compared to the untreated maize grains. GC-MS phytochemical analysis of fruit peel extracts suggests that compounds such as; α-kaurene, α-fenchene, *p*-allylphenol, octadecanoic acid, 3,5-dihydroxy phenol, hexestrol, xanthinin, and linoleic acid could provide antioxidant capacity, antifungal properties, and finally suppress aflatoxin production.

Author Contributions: Conceptualization, I.A.I. and S.I.B.; methodology, N.H.Y., N.A.H., A.A., S.H.Q., R.S., I.A.I., M.M.E., E.S.D., I.A.E. and S.I.B.; software, A.A. and S.I.B.; validation, A.A., S.H.Q. and R.S.; formal analysis, A.A., R.S. and M.M.E.; resources, N.A.H. and S.I.B.; data curation, S.I.B.; writing—original draft preparation, I.A.I., N.H.Y., M.M.E., R.S., A.A., I.A.E. and S.I.B.; writing—review and editing, I.A.E., I.A.I., S.H.Q., R.S., E.S.D., A.A. and S.I.B.; visualization, A.A. and S.I.B.; supervision, S.I.B.; project administration, I.A.I.; funding acquisition, I.A.I. All authors have read and agreed to the published version of the manuscript.

Funding: The current work was funded by Taif University Researchers Supporting Project number (TURSP-2020/120), Taif University, Taif, Saudi Arabia.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All the data reported here are available from the authors upon request.

Acknowledgments: The authors extend their appreciation to Taif University for funding the current study through the Taif University Researchers Supporting Project number (TURSP-2020/120), Taif University, Taif, Saudi Arabia.

Conflicts of Interest: The authors declare no conflict of interest.

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