Depression of Aflatoxin Production by Flavonoid-Type Compounds from Peanut Shells

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ABSTRACT

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Peanut shells contain luteolin, eriodictyol, and 5,7-dihydroxychromone. These flavonoid-related compounds, as well as a mixture of them, were tested to determine whether they would affect aflatoxin production. Broth medium (50 ml) was amended with 0.01, 0.02, and 0.06 mg/ml of the individual and mixed compounds, inoculated with 0.1 ml of an *Aspergillus parasiticus* spore suspension (1×10^{10} spores/ml), and incubated at 27 C. At 4, 7, 11, and 14 days after inoculation, the mycelium was removed, dried, and weighed. The medium was extracted and tested for aflatoxin. No differences in the mycelial weights were observed among the controls and amended cultures. However, each individual compound, at all concentrations, depressed aflatoxin production as compared with the controls. The mixture of the compounds was the most effective on a percentage basis in reducing aflatoxin amounts.

Additional key words: flavonoidlike compounds, fungi, groundnuts, mycotoxins, peanut hulls.

Aspergillus parasiticus Speare, NRRL2999, a close relative of *A. flavus* Link, generally produces four aflatoxins, whereas *A. flavus* generally produces two. These fungi are ubiquitous in nature and have proven to be a serious problem in areas where peanuts are a major crop. The aflatoxin-producing fungi can invade peanuts (groundnuts) while developing in the ground, during postharvest drying, and even during storage.

Sanders et al (6) found that peanut hulls adjusted to 20% moisture and inoculated with *A. flavus* supported mycelial growth but not aflatoxin production. Three flavonoidlike compounds, luteolin (a flavone), eriodictyol (a flavonone), and 5,7-dihydroxychromone (a chromone), have been identified in peanut hulls (5). The purpose of our work was to discern whether these naturally occurring compounds retard fungal growth and/or aflatoxin production. Because the compounds occur together in peanut hulls, a combination of these compounds was also tested.

MATERIALS AND METHODS

Fungal strain. *A. parasiticus* was used in this study because it is a stable toxin producer that forms four aflatoxins: B_1, B_2, G_1, and G_2. *A. parasiticus* NRRL2999 was grown on freshly prepared potato-dextrose agar (PDA) slants and incubated at 25 C for 14 days, followed by storage at 4 C.

Experimental procedure. Erlenmeyer flasks (250 ml) containing 50 ml of Adye and Mateles (AM) medium (1) were sterilized at 121 C for 15 min. The flavonoidlike compounds were sterilized by filtration. Luteolin and eriodictyol were purchased from Sarsyntex (Merignac, France); 5,7-dihydroxychromone was isolated from peanut shells according to the method of Pendse et al (5) and purified by high-performance liquid chromatography (HPLC) in our laboratory.

The peanut shells were placed in a Waring Blendor at high speed for 1 min. They were next ground to a fine powder by a Wiley mill. Five grams of the powder in a solution (200 ml) of 80% methanol was refluxed for 4 hr by use of a water bath. This mixture was filtered and the filtrate evaporated at room temperature down to 20 ml. Ether (40 ml) was used three times to extract the phenolics. The ether was then evaporated at room temperature to dryness. The dried material was dissolved in 2 ml of HPLC methanol and filtered (45 μ filter).

A Waters Associates (Milford, MA) liquid chromatograph equipped with a model 165 Beckmann (Berkeley, CA) variable wavelength detector, two 6000A pumps, and a model 660 solvent programmer were used. The output from the detector was monitored on a Hewlett-Packard (Avondale, PA) 3380A integrator. The detection wavelength was 254 nm. The scanning wavelength range was 230–400 nm, and the ultraviolet scan output was recorded on a Kipp and Zonen (Delft, Holland) BB 40. The inside diameter of the column was 30 cm $\times$ 3.9 mm packed with μ Bondapak (Waters Associates) C_{18}, preceded by a microguard silica column.

Solvents were filtered using a glass Millipore (Milford, MA)

system with a 0.45-μm filter and degassed at room temperature under vacuum with magnetic stirring. The elution solvent was water:acetic acid (495 ml/5 ml) from pump A and methanol from pump B. The flow rate was 1.9 ml/min. A 15-min linear gradient, pump A provided 60–80% and pump B provided 20–40% of the solvent mixture used. The content of each chromone was determined by counts as measured by the Hewlett-Packard 3380A integrator in comparison with known quantities of the standard under the same conditions.

A filter-sterilized solution of the individual and a mixture of the test compounds were added to the sterile medium to provide for final test concentrations of 0.01, 0.02, and 0.06 mg/ml. These values equaled, respectively, 35, 70, and 210 μM/ml for luteolin and eriodictyol, and 61, 122, and 366 μM/ml for the chromone. In the mixture of compounds tested, the values for luteolin, eriodictyol, and the chromone equaled, respectively: in the 0.01 mg/ml mixture, 23, 6, and 10 μM/ml; in the 0.02 mg/ml mixture, 47, 12, and 20 μM/ml; and in the 0.06 mg/ml mixture, 140, 36, and 61 μM/ml. A mixture of the flavonoidlike compounds was also tested, because it is in this state that the compounds are found in peanut hulls. The ratio, in which they exist in peanut shells, used was luteolin:eriodictyol:5,7-dihydroxychromone (4:1:1, wt/wt), respectively (6). Unamended AM medium was used as the control. The fungal spore inoculum (1×10^{10} spores/ml) was prepared by adding 10 ml of a sterile 0.05% Tween 20 solution to a slant of *A. parasiticus* and diluting to that concentration before adding 0.1 ml of the spore suspension to each flask. The inoculated flasks were incubated at 25 C as stationary cultures. Duplicate flasks of each concentration as well as controls were assayed at 4, 7, 11, and 14 days for aflatoxin content and mycelial dry weight. Separate triplicate runs of the duplicate flasks with each compound and controls were made. A total of 1,536 separate data points were generated for the various test compounds (four total), replicate runs (three total), duplicate flasks (two total), compound concentrations (four total), days assayed (four total), and aflatoxin types (four total).

At the appropriate time, each culture was poured through a funnel lined with Whatman no. 4 filter paper (Whatman, Ltd., England) which retained the mycelium and allowed the culture fluid liquid to be received in a flask. The mycelia were not extracted, because we were interested in only the excreted aflatoxins which would contaminate the seed in an intact pod. Because peanut seeds do not contain any of the tested compounds, in nature only the intact hull with these compounds would prove inhibitory to toxin production. The mycelium was rinsed with 50 ml of distilled water and the washings were added to the culture fluid. Aflatoxin was partitioned into methylene chloride from the culture in a separatory funnel. An initial 15-ml aliquot of methylene chloride was used. After shaking and removal of the organic solvent layer, the aqueous portion was extracted again with a 10-ml aliquot of methylene chloride. The sample was evaporated to dryness under nitrogen and then redissolved with an appropriate amount of methylene chloride (0.5 or 5.0 ml) required for fluorescent visualization of aflatoxin spots after thin-layer chromatography (TLC). Aliquots of 5 and 10 μl were spotted on a silica gel (Absorbosil-Plus-1) TLC plate. Five and 10 μl of the aflatoxin standards (B_1 and G_1, 1 μg/ml; B_2 and G_2, 3 μg/ml) were also spotted (standards prepared at the Southern Regional Research Center, USDA-ARS). The plate was developed in a mixture of ether:methanol:water (96:3:1). The intensity of the fluorescent spots on the developed plate was recorded by a Shimadzu CS-930 dual wavelength TLC scanner (Shimadzu Corp., Kyoto, Japan) using an excitation wavelength of 365 nm and emission wavelengths of 420–460 nm. Aflatoxin concentrations of the aliquots were calculated by comparing the peak areas of the samples with those of the aflatoxin standards. The concentration of aflatoxins in the cultures amended by the tested compounds were obtained by averaging the amounts of toxin produced in the duplicate flasks of the three runs. Percentage of control for the four aflatoxins caused by the tested compounds was determined by dividing the average aflatoxin concentration in the amended medium by that in the controls and multiplying by 100. These values, in turn, were averaged with those of the other two runs. The rinsed mycelial pad was dried for 2 hr at 100 C in a forced air oven and mycelial dry weights were determined. The mycelial dry weights for cultures grown in amended and control media were obtained by averaging the dry weights from the respective duplicate flasks in each run, followed by averaging these data between the respective runs. The mycelial dry weights of amended cultures were calculated as percentages of the control mycelial dry weights as described above for aflatoxin concentrations.

The data on the percentage of aflatoxin reduction by the flavonoidlike chemicals were analyzed using analysis of variance (ANOVA, SAS Institute, Inc., Cary, NC). Separate ANOVA analyses were made for each of the four aflatoxins and each of the four flavonoidlike compounds. Day (4, 7, 11, 14) and concentration (0.01, 0.02, 0.06 mg/ml) were considered to be fixed effects. Due to the problem of inflated probability estimates (i.e., increased chances of Type I error) resulting from 16 separate ANOVA analyses, a conservative alpha level of 0.01 was selected for the statistical cut-off point of statistical significance.

RESULTS

Mycelial dry weight. Table 1 describes the mycelial dry weights grown in the amended media as a percentage of the respective controls. The control and eriodictyol-amended media produced mycelia with varying weights. On day 4, the 0.02 and 0.06 mg/ml (70 and 210 μM/ml, respectively) eriodictyol-amended media were approximately 9% less than the control. However, during the remaining sampling dates, the values for all amended media were no more than 2.8% different from those of the control weights.

Cultures amended with 5,7-dihydroxychromone produced mycelia that varied only slightly from those of the controls. The 0.01 mg/ml (61 μM/ml) concentration treatment had mycelial dry weights that most closely paralleled those of the controls, with those for day 14 only 6.1% higher than the controls and those for the remaining days no more than 3.9% different from those of the controls. The 0.02 mg/ml (122 μM/ml) concentration treatment had one sample set (day 4) that had mycelial dry weights 14% greater and another set (day 11) that was 12.5% lower than the controls. The remaining sampling dates for the 0.02 mg/ml (122 μM/ml) treatment as well as all those for the 0.06 mg/ml concentrations produced approximately the same mycelial dry weights as those of the controls.

In the cultures amended with luteolin, all concentrations resulted in much greater mycelial weights than unamended

TABLE 1. Mycelial dry weight of *Aspergillus parasiticus* Speare, NRRL2999, in amended media[a] as a percentage of controls[b]

Compound concentration	(mg/ml)[c]	Percentage of controls			
		Day 4	Day 7	Day 11	Day 14
Eriodictyol	0.01	102.5	99.5	99.1	98.1
	0.02	90.9	100.2	101.1	97.2
	0.06	91.3	102.5	100.9	97.2
5,7-dihydroxychromone	0.01	101.0	97.8	100.2	106.1
	0.02	114.0	97.0	87.5	99.0
	0.06	108.3	94.1	93.4	100.5
Luteolin	0.01	139.4	102.8	96.4	90.4
	0.02	141.3	103.6	93.2	87.8
	0.06	130.3	113.1	100.2	99.4
Mixture of compounds	0.01	156.3	101.5	100.3	96.2
	0.02	179.3	100.6	102.3	95.2
	0.06	208.9	103.7	107.7	97.1

[a] Adye and Mateles medium.
[b] Mycelial dry weight of control culture = 100%.
[c] These values equalled, respectively, 35, 70, and 210 μM/ml for luteolin and eriodictyol, and 61, 122, and 366 μM/ml for the chromone. The values for luteolin, eriodictyol, and the chromone in the compound mixture equalled, respectively: in the 0.01 mg/ml mixture, 23, 6, and 10 μM/ml; in the 0.02 mg/ml mixture, 47, 12, and 20 μM/ml; and in the 0.06 mg/ml mixture, 140, 36, and 61 μM/ml.

cultures on day 4 (0.01 mg/ml [35 μM/ml], 139.4%; 0.02 mg/ml [70 μM/ml], 141.3%; and 0.06 mg/ml [210 μM/ml], 130.3%). However, the mycelial dry weights produced in the amended cultures on the remaining sampling dates were approximately the same as those of the controls. The rate of mycelial growth usually slows by day 7, so the rate of metabolism of luteolin probably also slowed, resulting in the loss of the stimulatory effect on the weight of the mycelium. This might result in the mycelial weights of treated cultures returning to those of the controls after day 4.

The final mycelial dry weights produced in media amended with a mixture of the three compounds were similar, on a sampling data basis, to those grown in luteolin-amended media. On day 4, the mixture produced mycelial weights higher than those of the controls (0.01 mg/ml, 156.3%; 0.02 mg/ml, 179.3%; and 0.06 mg/ml, 208.9%), whereas the remaining sampling dates gave values near those of the controls. The reason the treated cultures showed this difference on day 4 followed by similar weights to those of the controls on later days was probably the same as that suggested for luteolin-amended cultures.

Reduction of aflatoxin levels. Flavonoids reduced markedly the amount of aflatoxin produced (Table 2). Depression of aflatoxin was the greatest on day 11 among all test chemical concentrations as compared with the controls. The concentration of aflatoxins in the eriodictyol-amended cultures was highest on day 14 regardless of the concentration of eriodictyol. Even so, the highest aflatoxin level, the B_1 in AM medium with 0.01 mg/ml (35 μM/ml) eriodictyol, was only 33.66% of the control. Generally, B_2 and G_2 levels were lower than the respective B_1 and G_1 concentrations. Overall, no one concentration of eriodictyol proved to inhibit aflatoxin production more than another.

The flavonoid, 5,7-dihydroxychromone, also greatly reduced all aflatoxin production. A concentration of 0.02 mg/ml (122 μM/ml) caused the greatest reduction of all four aflatoxins, with 0.06 mg/ml (366 μM/ml) producing the next greatest reduction and 0.01 mg/ml (61 μM/ml) the least. The most effective concentration depressed the levels of the two most potent aflatoxins, B_1 and G_1, to 2.19–8.20% and 0.00–12.18% of the controls, respectively. B_2 and G_2 toxin levels were uniformly lower than the amounts of the corresponding B_1 and G_1 produced by the fungus.

Luteolin also displayed significant reduction of aflatoxin production. The concentration of luteolin that produced the greatest reduction of aflatoxin was 0.02 mg/ml (70 μM/ml); 0.06 mg/ml (210 μM/ml) and 0.01 mg/ml (35 μM/ml) were the next most effective aflatoxin-reducing flavonoid levels. Table 2 shows that the amounts of B_1 and G_1 produced in the cultures containing 0.02 mg/ml of luteolin were only 4.19–20.87% and 0.12–11.29%, respectively, of that produced by the controls. Surprisingly, the luteolin-amended media produced (as a percentage of the controls) more B_2 and G_2 than B_1 and G_1. Nonetheless, B_2 and G_2 levels were much lower than those of the controls.

The cultures grown on the AM medium amended with the mixture of flavonoids (luteolin:eriodictyol:5,7-dihydroxychromone; 4:1:1, wt/wt) had the most dramatic decrease in aflatoxin production. Initially, on day 4, little reduction, if any, in aflatoxin concentration occurred. However, on the remaining extraction dates, aflatoxin was reduced with all concentrations of the flavonoid mixture. The concentrations of B_1 were no greater than 6.28% of the controls. Aflatoxin G_1 concentrations were also very low, not exceeding 3.67% of control concentrations for any sampling date. The amended medium resulted in B_2 and G_2 concentrations that were generally similar to their respective B_1 and G_1 levels.

The results of the ANOVA (Table 3) indicated that some of the individual as well as the mixture of compounds greatly reduced aflatoxin levels. Significant reduction occurred for nine of the 16 combinations. Luteolin failed to significantly reduce aflatoxin G_1 and G_2 levels but it did significantly reduce B_1 and B_2 production. Eriodictyol failed to significantly reduce any aflatoxin level. The outstanding chemical tested was 5,7-dihydroxychromone, which displayed the strongest significance levels (Table 3). No other test compound showed such reductions. The mixture of compounds gave the second greatest reduction of aflatoxin by concentration levels, with ANOVA values equal to or slightly less than those for the chromone.

DISCUSSION

We found that addition of the flavonoid-type compounds to the culture medium did not reduce the amount of mycelial mass produced during incubation. However, addition of these compounds, singly or as a mixture, greatly reduced aflatoxin production. On a percentage basis, the mixture of compounds gave the greatest reduction in aflatoxin production. It is this

TABLE 2. Aflatoxin production by *Aspergillus parasiticus* Speare, NRRL2999, in amended medium[a] as a percentage of controls[b]

Test chemical	Concentration[c]	Day	Percentage of controls B_1	B_2	G_1	G_2
Eriodictyol	0.01	4	5.12	3.06	15.45	9.43
		7	2.02	11.18	3.07	1.20
		11	2.64	1.96	2.62	1.22
		14	33.66	6.26	18.47	0.00
	0.02	4	6.05	4.05	15.47	8.79
		7	15.54	19.46	4.87	1.80
		11	4.43	2.43	4.56	0.99
		14	20.50	12.81	7.27	0.37
	0.06	4	13.05	7.47	32.84	12.46
		7	2.57	12.56	15.16	4.73
		11	3.36	0.86	1.66	3.88
		14	13.01	7.08	4.94	2.81
5,7-dihydroxychromone	0.01	4	33.96	23.79	85.63	0.00
		7	23.37	20.85	36.46	23.37
		11	43.57	37.33	132.41	20.76
		14	58.90	34.15	55.26	54.09
	0.02	4	8.20	5.48	12.18	0.00
		7	2.19	1.66	0.58	0.00
		11	3.73	2.71	3.91	0.00
		14	6.98	1.89	0.00	0.00
	0.06	4	31.07	28.51	36.21	0.00
		7	7.10	6.57	7.50	0.00
		11	16.83	13.43	10.11	2.34
		14	14.83	8.31	0.00	19.63
Luteolin	0.01	4	40.89	46.09	0.99	109.33
		7	62.29	38.28	0.10	256.33
		11	9.85	19.47	0.08	16.34
		14	12.83	30.79	11.29	8.23
	0.02	4	20.87	63.97	3.01	72.09
		7	4.19	16.92	0.85	4.45
		11	4.52	15.38	0.12	3.14
		14	5.26	16.50	11.29	17.82
	0.06	4	35.01	43.94	2.39	63.36
		7	14.35	7.63	35.86	6.32
		11	2.06	15.59	0.13	1.68
		14	0.38	13.28	81.17	0.00
Mixture	0.01	4	82.15	52.60	85.14	0.00
		7	3.00	2.03	3.26	1.74
		11	6.28	3.67	3.67	2.24
		14	2.16	1.06	1.05	1.23
	0.02	4	98.30	73.61	131.76	0.00
		7	2.18	3.05	2.82	2.39
		11	3.37	1.79	1.79	1.73
		14	1.12	1.87	1.85	1.93
	0.06	4	178.47	56.04	203.68	0.00
		7	2.66	3.95	1.32	3.21
		11	3.71	3.28	3.28	3.66
		14	1.35	0.96	0.95	1.37

[a] Adye and Mateles medium.

[b] Aflatoxin concentrations of control cultures = 100%.

[c] These values equalled, respectively, 35, 70, and 210 μM/ml for luteolin and eriodictyol, and 61, 122, and 366 μM/ml for the chromone. The values for luteolin, eriodictyol, and the chromone in the compound mixture equalled, respectively: in the 0.01 mg/ml mixture, 23, 6, and 10 μM/ml; in the 0.02 mg/ml mixture, 46, 12, and 20 μM/ml; and in the 0.06 mg/ml mixture, 140, 36, and 61 μM/ml.

combination and ratio of compounds as tested that occur naturally in peanut hulls.

Although we did not observe this with each experiment, it appeared that the concentration of 0.02 mg/ml of two of the tested compounds in AM medium is the least amount necessary which resulted in the greatest inhibition of aflatoxin production. This was particularly true with 5,7-dihydroxychromone and to a lesser extent with luteolin. Eriodictyol best reduced aflatoxin production at the 0.01 mg/ml (35 μM/ml) concentration. Overall, there was little difference between the amount of aflatoxin produced with AM medium amended with 0.02 and 0.06 mg/ml (70 and 210 μM/ml, respectively) of the mixture. Cultures grown in these two concentrations produced slightly less aflatoxin than cultures grown in the 0.01 mg/ml (35 μM/ml) level. Because we did not have difficulty dissolving the compounds, even at the 0.06 mg/ml (210 μM/ml) level, a reduction of solubility with increasing concentration would not explain this lack of difference.

Statistical analysis showed that 5,7-dihydroxychromone gave the greatest reduction of all four aflatoxins. The mixture of compounds gave only slightly less significant results. Luteolin significantly reduced only B_1 and B_2, and eriodictyol failed to do so for any of the aflatoxins.

The question arose as to why the aflatoxin values for cultures amended with the mixture of compounds on day 4 should be very high compared with the controls but drop so precipitously on later assay dates. The raw data showed that B_1 produced on day 4 in medium amended with 0.01 mg/ml of the mixture averaged 1.15 μg/ml. The 0.02 mg/ml of mixture-amended medium averaged 1.38 μg/ml; the 0.06 mg/ml of mixture and control averaged 2.50 and 1.40 μg/ml, respectively. However, on day 7, the values for the 0.01, 0.02, and 0.06 mg/ml amended medium and controls averaged 1.40, 1.02, 1.24, and 46.62 μg/ml of culture, respectively. We believe that this decrease in aflatoxin compared with the control was due to amended cultures and controls being low and similar in aflatoxin concentration on day 4. However, by day 7, the controls had produced high concentrations of aflatoxin, and the amended cultures had little if any increase in aflatoxin concentration. Applebaum and Buchanan (2) showed that aflatoxin production coincided with a period of accelerated glucose utilization and is a function of exogenous glucose concentration. It was possible that by day 4, glucose utilization had not reached its maximum, as on day 7, so the production of aflatoxin was proceeding at a low level in all test flasks on day 4. However, by day 7, utilization of glucose and production of aflatoxin reached its maximum in the control cultures but was retarded in the amended cultures. Therefore, the percentage of control on day 4 was considerably greater than the percentage of control on day 7.

TABLE 3. Statistical (ANOVA) probability levels[a] of tested chemicals in reducing aflatoxin production by *Aspergillus parasiticus* Speare, NRRL2999

Tested compound	Aflatoxin			
	B_1	B_2	G_1	G_2
Luteolin	0.0087[a]	0.0048[a]	0.3159	0.2253
Eriodictyol	0.0381	0.0405	0.0660	0.0424
5,7-Dihydroxychromone	0.0001[a]	0.0001[a]	0.0001[a]	0.0010[a]
Mixture[b]	0.0001[a]	0.0011[a]	0.0002[a]	0.0105

[a] Using fixed affects analysis of variance. Probability levels of <0.01 indicate a significant reduction in aflatoxin production with increasing test compound concentrations (0.00, 0.01, 0.02, and 0.06 mg/ml).

[b] Mixture is composed of luteolin:eriodictyol:5,7-dihydroxychromone (4:1:1, wt/wt).

Several sources of experimental error were possible. Because the complete set of experiments took a long time, no one spore suspension was used as the inoculum source. Occasionally, *A. parasiticus* NRRL2999 was subcultured onto fresh PDA slants to ensure viability of the inoculum. A possible source of error was the use of different PDA slants of the same fungal isolate rather than a single inoculum source. Also, the numbers of spores could have varied slightly among inocula, thereby resulting in nonuniform inocula size for each experimental run. Nevertheless, the distinctly different aflatoxin values for control and amended media showed that the flavonoidlike compounds reduced the production of all four aflatoxins.

The tested compounds were different from other aflatoxin inhibitors of plant origin. Chloroform extracts of raw carrot tissue contain a compound that inhibits fungal differentiation and aflatoxin production (3). The carrot extract is believed to inhibit toxin production by preventing fungal growth. O'Neill and Mansfield (4) have shown that the antifungal activity of flavonoids and isoflavonoids depends on some common physiochemical attribute, such as lipophilicity, and an ability to penetrate fungal membranes.

Our data agreed with those of Sanders et al (6), which indicate that peanut hulls support the growth of *A. flavus* but with little corresponding production of aflatoxin. These toxins are secondary metabolites produced during the period of accelerated glucose utilization and are a function of exogenous glucose concentrations (2). The action of the tested compounds in this study appeared to affect only the secondary metabolic pathway producing aflatoxin and not the pathways necessary for fungal growth.

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