

## Aflatoxigenesis induced in *Aspergillus flavus* by oxidative stress and reduction by phenolic antioxidants from tree nuts

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### Abstract

Almonds, pistachios, and walnuts grown in California have an aggregate value of over \$3.3 billion, with a large proportion of the crop being exported. Mycotoxin contamination affects this value, because importing nations, and in particular the European Community (EC), apply strict regulatory levels for aflatoxins, formed by infection of the product by *Aspergillus* species. Conformance with such limits can best be achieved by capitalising on natural resistance factors inherent to the nuts. Differential resistance of tree nut species to aflatoxin formation, with walnuts being least susceptible, led to research which established that the hydrolysable tannins in walnut pellicle were responsible for this effect. This observation has now been extended to show that the addition to *Aspergillus flavus* growth media of natural antioxidants present in tree nuts, such as hydrolysable tannins, flavonoids and phenolic acids, decrease maximum aflatoxin by as much as 99.8%. There is evidence that aflatoxin formation is exacerbated by oxidative stress on the fungus and therefore compounds capable of relieving such stress should limit aflatoxin accumulation. The addition of *tertiary*-butyl hydroperoxide to the media induced oxidative stress in *A. flavus* and stimulated peak aflatoxin production with high levels being maintained, rather than declining as in control samples. Aflatoxin formation was significantly inhibited by incorporation into the media of the antioxidant, tannic acid. A similar effect was seen for drought-stressed cultures, with increased aflatoxigenesis being reduced in the presence of gallic or caffeic acids. Increasing antioxidant natural products in tree nuts may restrict the ability of *A. flavus* to biosynthesise aflatoxins, thus achieving conformity with regulatory limits.

**Keywords:** aflatoxins, hydrolysable tannins, almonds, pistachios, walnuts

### 1. Introduction

Almonds, pistachios and walnuts, collectively classified as tree nuts, are among the most valuable crops (ca. \$3.34 billion in 2008) in the State of California, where virtually all of the US commercial production is located (USDA-NASS, 2009). The volume of these crops, especially almonds, has increased rapidly over the past decade, with 40-70% of production entering the export market, in particular to the European Union (USDA-FDA, 2009). However, income from this source is jeopardised by increased food

safety concerns arising from potential contamination by mycotoxins, concurrent with a rise in the number of alert and information notifications for incoming shipments issued by the European Commission (EC) Rapid Alert System for Food and Feed (RASFF) (EC, 2009). In 2008 the highest number of notifications (931) concerned mycotoxins, with 902 involving aflatoxins, of which 710 were in nuts, nut products and seeds; fumonisins, ochratoxin A, patulin, and zearalenone were not found in nut crops. Aflatoxins are metabolites produced by certain strains of the fungi *Aspergillus flavus* and *Aspergillus parasiticus* which

can infect many agricultural crops, with tree nuts being particularly susceptible. The European Community (EC) and a number of other countries have set tolerance levels as low as 2 ng/g for aflatoxin B<sub>1</sub> and 4 ng/g total aflatoxins because of a presumed carcinogenic potential (EC, 2006a).

In response to the rise in RASSF notifications, import controls for US almonds and tree nuts from other countries were imposed in early 2007, requiring that all shipments to Europe after September 1, 2007 be subject to 100% inspection (EC, 2006b). To address this issue, a Voluntary Aflatoxin Sampling Plan (VASP) was created by the Almond Board of California (2007). The VASP protocol is designed to have a level of detection equivalent to that currently used by the European Union so that only 5% of consignments accompanied by a VASP certificate would be subject to control on entry. Since warehousing and replacement could cost \$10,000 for each rejected consignment, and even more if the almonds have to be reprocessed or destroyed, it is obviously desirable to ensure that the product is essentially aflatoxin-free, whether analysis is done at point of shipment or point of entry. The nature of the crop and its consumption (i.e. with minimal processing) requires that aflatoxin control be acceptable to consumers. Thus, the use of fungicides, or processing to destroy aflatoxins once they are formed, are not likely to be approved and natural methods of control must be applied. Procedures that reduce aflatoxin levels prior to the VASP are therefore economically advantageous in reducing costs associated with sorting to reduce potentially contaminated kernels.

In previous research we have shown both species and varietal differences in the susceptibility of tree nuts to aflatoxin contamination (Mahoney *et al.*, 2003). In California, tree nuts in the retail trade have been found to be infected primarily by *Aspergillus* species, especially *A. flavus* and *A. niger*, and the microorganism profiles are very similar in almonds, pistachios and walnuts (Bayman *et al.*, 2002). However, *in vitro* experiments with walnuts showed that they were much less capable of supporting aflatoxin production by *A. flavus* than almonds. This is confirmed by the EC sampling program, in which almonds comprise the majority of notifications issued for tree nut shipments from the US. For example, notifications for almonds increased steadily from 28 in 2005 to 66 in 2007; pistachios remained fairly constant at 13 and 11 notifications for the same two years; and there was only a single notification for walnuts in the three-year period from 2005-2007. This led us to hypothesise that specific constituents in these tree nuts, with variable degrees of bioactivity, were capable of inhibiting aflatoxin formation. In the case of walnuts, hydrolysable tannins present in the pellicle (seed coat) and their hydrolysis products, gallic and ellagic acid, were found to suppress aflatoxin formation *in vitro* (Mahoney and Molyneux, 2004). Since all of these compounds are phenolic antioxidants a reasonable hypothesis is that biosynthesis of aflatoxins in the fungus is induced by oxidative stress, and

relief of such stress by natural antioxidants suppresses or eliminates aflatoxin formation. The objectives of this study were therefore to investigate levels of aflatoxin and the time-course of production by *A. flavus* cultured *in vitro* under oxidative stress induced by *tertiary*-butyl hydroperoxide (*t*-BuOOH) or low humidity, and the counteracting effect of phenolic antioxidants known to occur in tree nuts. Aflatoxin control by constituents inherently present in crops offers considerable advantages because no artificial compounds or organisms are required; it is independent of multifactorial vectors of infection and contamination; it can be manipulated through breeding, selection, or agronomic practices; and the bioactivity is generally present throughout growth, harvest, processing, and distribution.

## 2. Materials and methods

### Instrumentation

NMR spectra were obtained at 25 °C from samples dissolved in CD<sub>3</sub>OD with TMS as an internal standard on a Bruker ARX400 spectrometer (Bruker Optics, Billerica, MA, USA) at frequencies of 400.13 MHz (<sup>1</sup>H) and 100.62 MHz (<sup>13</sup>C). A 90° pulse at a 7-8 s repetition rate was used for <sup>1</sup>H experiments, and a 30° pulse at a 2.3 s repetition rate was used for <sup>13</sup>C experiments. The number of attached protons for <sup>13</sup>C signals was determined from DEPT90 and DEPT135 assays.

### Chemicals

Pentagalloyl-*O*-glucose was prepared by methanolysis of tannic acid as previously described (Chen and Hagerman, 2004).

3,4-di-*O*-galloylquinic acid was isolated from hulls of 'Kerman' pistachios. In brief, fresh hulls were separated by hand from mature pistachios and lyophilised. Dry hulls (675 g) were sequentially extracted with 4 litre each of hexane, ethyl acetate, acetone, methanol, and water using a Polytron homogeniser (Kinematica, Riverview, FL, USA) followed by filtration through #4 filter paper (Whatman, Piscataway, NJ, USA). The water extract was centrifuged and the supernatant stirred overnight with 2 kg Amberlite XAD-2 (Acros Organics, Morris Plains, NJ, USA). The XAD-2 resin was filtered and washed with 4 litres methanol, followed by solvent removal under reduced pressure and lyophilisation (yield 18 g). The dried, XAD-retained fraction was dissolved in 0.1% acetic acid and applied to a 60×4.8 cm Sephadex G25 fine column (GE Healthcare, Piscataway, NJ, USA), followed by elution with 0.1% acetic acid and detection at 254 nm. The eluate containing 3,4-di-*O*-galloylquinic acid was collected and lyophilised (430 mg yield).

The <sup>1</sup>H NMR (CD<sub>3</sub>OD) spectrum obtained was as follows: δ 2.15, *J*=14.0, 6.0 Hz, (1H, dd, H-6ax), 2.31, *J*=6.3 Hz (2H,

br d, H-2ax, 2eq), 2.35,  $J=13.9$ , 2.9 Hz (1H, dd, H-6eq), 4.44,  $J=6.0$ , 3.0, 3.0 Hz (1H, H-5), 5.22,  $J=8.4$ , 3.0 Hz (1H, H-4), 5.68,  $J=14.7$ , 6.8 Hz (1H, H-3), 6.99 (2H, s, H2' or 6'), 7.09 (2H, s, H2' or 6'). The  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OD}$ ) spectrum was:  $\delta$  38.5 (C-6), 39.0 (C-2), 68.9 (C-3), 69.4 (C-5), 75.5 (C-4), 75.8 (C-1), 110.3 (2C, C-2'), 110.4 (2C, C-6'), 121.2 (2C, C-1'), 140.0 (2C, C-4'), 146.4 (4C, C-3;5'), 167.4 (-COO), 167.8 (-COO).

The  $\text{C}^{13}$ -NMR spectrum was consistent with literature values for 3,4-di-*O*-galloylquinic acid isolated from *Quercus stenophylla* and with partial data reported for the  $^1\text{H}$  spectra (Nishimura *et al.*, 1984). Comparison of the spectra reported for other isomers, especially with respect to the signals for the quinic acid moiety, clearly established that the data did not correspond to that of the 1,4-, 3,5-, or 4,5-di-*O*-galloylquinic acids (Ishimaru *et al.*, 1987; Bouchet *et al.*, 1996).

Chlorogenic acid was purchased from Indofine Chemical Co., Inc. (Hillsborough, NJ, USA). All other compounds (quinic acid, caffeic acid, vanillic acid, 4-hydroxybenzoic acid, protocatechuic acid, gallic acid, ellagic acid, catechin, lauryl gallate, tannic acid, and *t*-butyl hydroperoxide) were obtained from Sigma-Aldrich (Milwaukee, WI, USA) and used without further purification.

### Fungal strains

*A. flavus* strain 3357 was obtained from the NRRL culture collection (Peoria, IL, USA) and strain 4212 (NRRL 25347) was isolated from pistachio kernels.

### Preparation of media

Pistachio medium was prepared as follows: commercial raw, organic, shelled pistachios were ground in a blender with dry ice. The frozen, ground pistachio kernels were passed through a sieve with a 1 mm screen and lyophilised overnight in a freeze dryer. The pistachio kernel medium, consisting of 5% (w/v) ground pistachio kernels and 1.5% (w/v) Select agar (Sigma, St. Louis, MO, USA) in purified water, was autoclaved and 10 ml of the preparation was poured per 60 mm Petri dish. Antioxidant compounds were incorporated into the medium at 12 mM and autoclaved with the media. Petri dishes were prepared in triplicate. Control samples contained none of the added compounds.

Oxidatively stressed *A. flavus* was grown on media incorporating 100  $\mu\text{M}$  or 1000  $\mu\text{M}$  *t*-butyl hydroperoxide.

### Preparation of fungal cultures

Spore suspensions were prepared from *A. flavus* strain 4212 and strain 3357, grown on potato dextrose agar for 7 days. These strains produce aflatoxin  $\text{B}_1$ , with only trace levels of aflatoxin  $\text{B}_2$  (8,9-dihydro-aflatoxin  $\text{B}_1$ ) and no aflatoxin  $\text{G}_1$

or  $\text{G}_2$ . Spores were collected on a swab and transferred to 0.05% Tween 80. Spore concentration was calculated using a Neubauer counting chamber. Spores (200 per 5  $\mu\text{l}$  0.05% Tween 80) were inoculated in a single point (5  $\mu\text{l}$ ) onto pistachio medium (10 ml per 60 mm Petri plate). Plates were prepared in triplicate and incubated for 7 days at 30  $^\circ\text{C}$  in the dark. For the time-course experiment, triplicate sets of plates incubated under the same conditions were analysed daily from day 0 (control) to day 10.

### Fungal weights

Fungal weights were determined by inoculating spores in the centre of a 0.2  $\mu\text{m}$ , 50 mm diameter polycarbonate membrane filter (GE Osmonics, Minnetonka, MN, USA) placed on top of the pistachio medium. Pre-weighed membrane filters with fungal mats were removed from the media, dried at 50  $^\circ\text{C}$  for 48 h, and weighed after being cooled in a desiccator for 24 h.

For studies under controlled humidity, cultures were grown either in an incubator with 2 litres purified water in a humidity pan for high humidity, or in an incubator with 2 kg silica gel indicating desiccant (EMD, Gibbstown, NJ, USA) for low humidity. Incubator humidities were monitored using HOBO U12 dataloggers (Onset, Bourne, MA, USA).

### Sclerotial counts

Sclerotia were counted using a stereomicroscope (Zeiss, Thornwood, NY, USA) at 8 $\times$  magnification.

### Analysis for aflatoxin

The fungal mat, including spores and media from each of the three replicate Petri plates were extracted with MeOH (50 ml) and analysed individually. MeOH was removed from an aliquot (1 ml) by evaporation with  $\text{N}_2$  at 40  $^\circ\text{C}$  and the residue derivatised by treatment with hexane (200  $\mu\text{l}$ ) and trifluoroacetic acid (200  $\mu\text{l}$ ) (Pierce Chemical Co., Rockford, IL, USA) at room temperature for 10 min. The sample was evaporated to dryness with  $\text{N}_2$  at 40  $^\circ\text{C}$  and redissolved in  $\text{H}_2\text{O}-\text{CH}_3\text{CN}$  (9:1; 1 ml). Aliquots (20  $\mu\text{l}$ ) were analysed for aflatoxin by reversed-phase HPLC and fluorescence detection, with excitation at 365 nm and detection of emission at 455 nm (Mahoney and Rodriguez, 1996; Rodriguez and Mahoney, 1994). The lower detection limit was 0.02  $\mu\text{g}$  per Petri dish or 10 ml media. Aflatoxin  $\text{B}_2$  was detected at levels which were insignificant relative to aflatoxin  $\text{B}_1$  (ca. 0.1%) and was therefore not quantitated. Each data point was calculated as the average of three replicates and standard deviations (SD) determined.

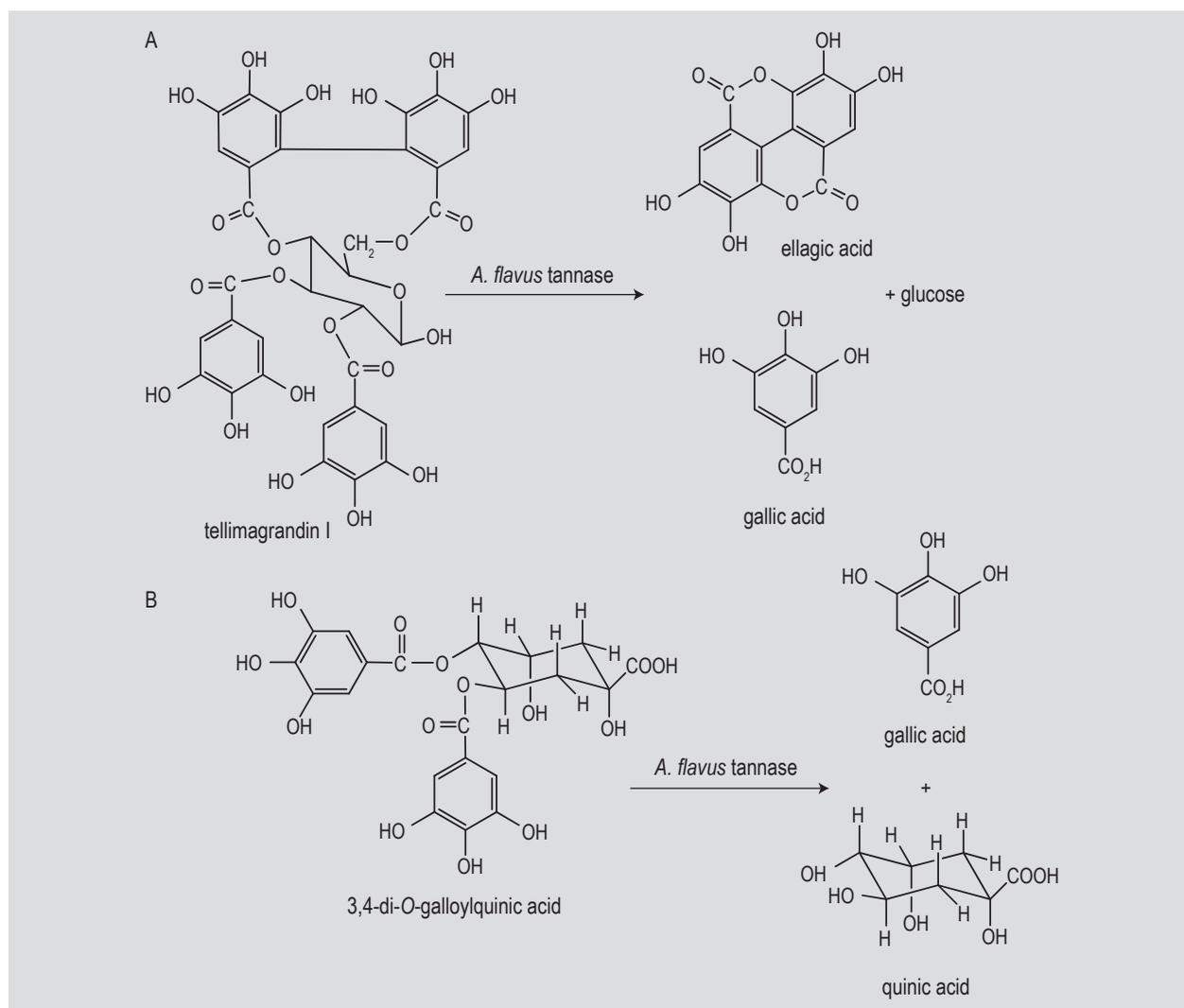
### 3. Results and discussion

#### Reduction of aflatoxin formation by phenolic antioxidants from tree nuts

The involvement of oxidative stress in stimulating aflatoxin production has been demonstrated by comparison of oxygen requirements and antioxidant status in toxigenic and atoxigenic strains of *A. parasiticus*, with increased oxidative stress resulting in enhanced lipid peroxidation (Jayashree and Subramanyam, 2000). This was in accord with earlier work showing that lipoperoxides from polyunsaturated fatty acids are capable of inducing overproduction of aflatoxins *in vitro* and *in vivo* (Castoria *et al.*, 1989). It was subsequently shown that *in vitro* the pro-oxidant cumene hydroperoxide increased levels of lipoperoxidation and aflatoxin biosynthesis by *A. parasiticus* whereas the inclusion in the growth medium of the commercial antioxidant, butylated hydroxyanisole

(BHA), inhibited lipoperoxidation and markedly reduced aflatoxin production (Reverberi *et al.*, 2006).

In previous work (Mahoney and Molyneux, 2004) we showed that the resistance of walnuts to aflatoxigenesis relative to pistachios and almonds correlated with the levels of hydrolysable tannins in the pellicle. These tannins consist of a diverse mixture of at least 30 compounds (Fukuda *et al.*, 2003; Ito *et al.*, 2007), all possessing the common structural feature of a glucose core, esterified with gallic acid and diphenic acid moieties, and biosynthetically derived from pentagalloyl glucose. The tannins exhibited superoxide dismutase (SOD)-like activity and particularly potent radical-scavenging activity, consistent with the reported antioxidant activity of high molecular weight plant phenolics (Hagerman *et al.*, 1998). The major individual compound, comprising 0.007% of the weight of the undried kernel, was tellimagrandin I (Figure 1), a structural type representative of the walnut tannins in general. However, the tannins are



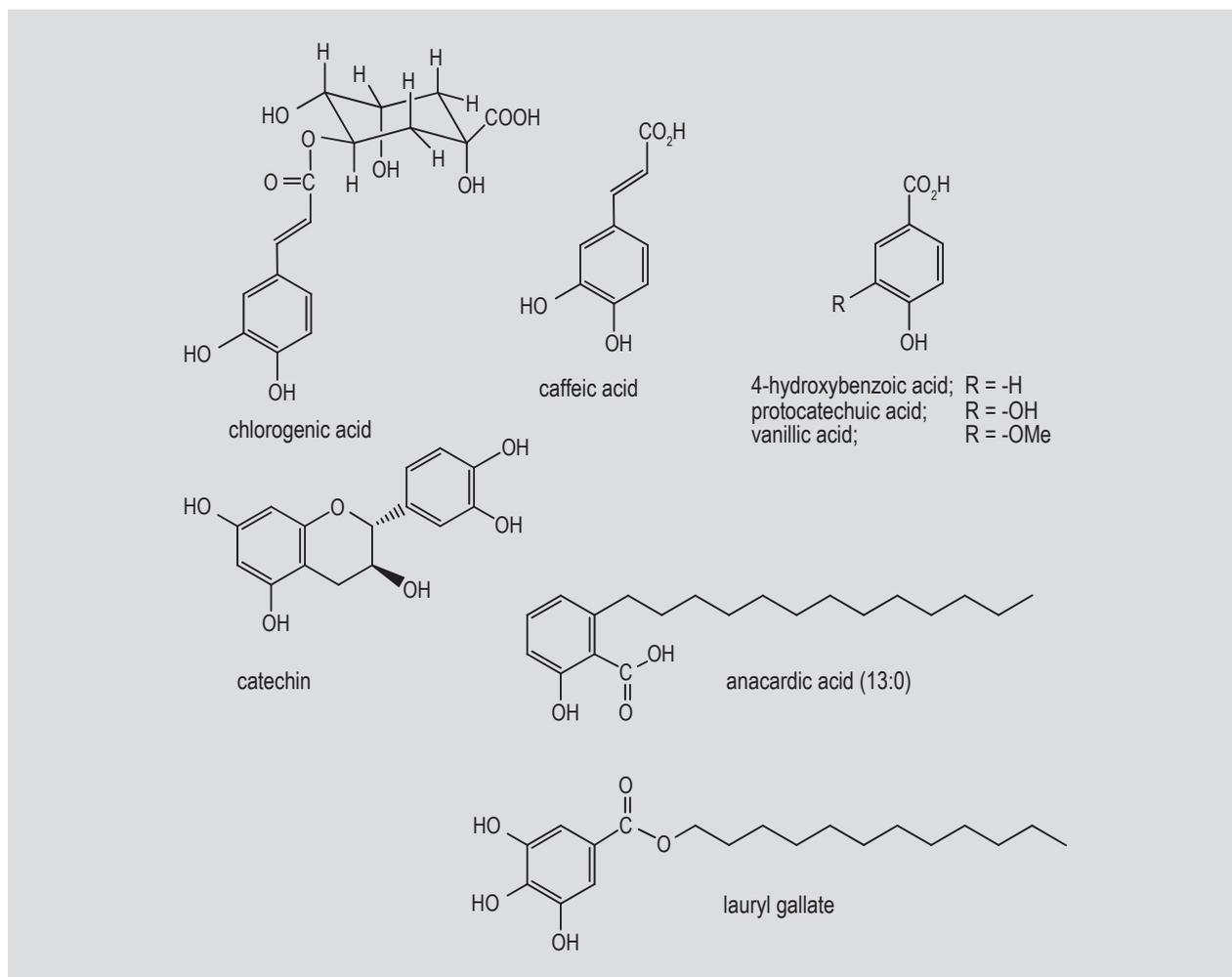
**Figure 1. (A) Structure of tellimagrandin I, a major hydrolysable tannin of walnut pellicle. (B) Structure of 3,4-di-O-galloylquinic acid, a hydrolysable tannin of pistachio seed coat. The putative hydrolysis products formed by *Aspergillus flavus* tannase are shown.**

concentrated specifically in the pellicle which constitutes only about 6% of the overall weight (Mahoney *et al.*, 2003), so that in this tissue tellimagrandin I would amount to 0.12% by weight. It is known that some strains of *A. flavus* excrete an extracellular tannase (Yamada *et al.*, 1968) that is capable of cleaving ester linkages of hydrolysable tannins to yield glucose, together with gallic acid and ellagic acid, the latter formed by spontaneous lactonisation of the hexahydroxydiphenic acid (Figure 1). The ability of walnut pellicle to suppress aflatoxigenesis could therefore arise from the tannins themselves, their hydrolysis products, or a combination of both. Comparison experiments with tannic acid, a commercially available hydrolysable tannin, gallic acid and ellagic acid, showed that the tannin itself and gallic acid were much more potent than ellagic acid, even though all of these compounds are antioxidants.

In order to broaden the scope of our studies we selected a series of readily obtainable phenolic compounds (Figure 2) known to occur in almonds, pistachios and walnuts, and determined their ability to suppress aflatoxin formation

*in vitro*. Also included was lauryl gallate, a commercial food antioxidant with structural features similar to the anacardic acids found in pistachio, which were not available to us. Hydrolysable tannins were extracted from 'Kerman' pistachio hulls and the major component was isolated and purified by column chromatography and shown by  $^1\text{H}$  and  $^{13}\text{C}$  spectroscopy to be 3,4-di-*O*-galloylquinic acid. A previous report (Abdelwahed *et al.*, 2007) has claimed the isolation of pentagalloylglucose from *Pistacia lentiscus* fruits but no independent chemical or spectroscopic evidence was provided for this structural assignment, and a study of the tannins of pistachio leaves using HPLC-MS and NMR showed the presence of galloylquinic acid derivatives (Romani *et al.*, 2002). It therefore appears that in contrast to walnut tannins, which are glucose-based, the pistachio tannins are quinic acid based.

All compounds were tested at 12 mM, a concentration selected to be representative of the levels of these compounds in the nut kernels themselves. The *A. flavus* 4212 strain was a pistachio isolate and was grown on media



**Figure 2.** Structures of phenolic antioxidants present in tree nut seed coats. Lauryl gallate is a commercial antioxidant with structural affinities to anacardic acids in pistachio.

consisting of 5% raw ground pistachio kernel in agar, since we had previously found that aflatoxin levels produced on this media were much higher and more consistent than in PDA cultures. As shown in Figure 3, aflatoxigenesis inhibition ranged from 59.5-99.8%, relative to control. The most effective inhibitor was pentagalloyl glucose, the biosynthetic precursor of all hydrolysable tannins in walnuts. The major hydrolysable tannin from pistachios, 3,4-di-*O*-galloylquinic acid, also showed high activity, suppressing aflatoxin formation by 98.3%; surprisingly, the quinic acid core of the tannin was also an effective inhibitor (90.2%), even though it is not a phenolic compound. It should be noted that unlike the glucose-based walnut tannins, the stereochemistry of the quinic acid moiety of the pistachio tannins precludes dimerisation of galloyl substituents to a hexahydroxydiphenic ester, and therefore only gallic acid, but not ellagic acid, can be formed on hydrolysis. Of these two acids, gallic acid was much more anti-aflatoxigenic (83.9%) and ellagic acid (59.5%) was the least active of all compounds tested, as previously observed (Mahoney and Molyneux, 2004); nevertheless, the activity of the latter was still substantial.

Among the other simple phenolic acids tested, the potency of caffeic acid (99.5%) was particularly striking. In comparison, the activity of chlorogenic acid (88.5%), the quinic acid ester of caffeic acid, was less so. Since the activity of each of its hydrolysis products was greater than chlorogenic acid itself, this indicates that the hydrolysis due to the *A. flavus* tannase is minimal during the time-course of the experiment. The differences in activity between chlorogenic and caffeic acids may relate to the fact that the latter is an inhibitor of 5- and 12-lipoxygenases ( $IC_{50}$  3.7 and 5.1  $\mu$ M, respectively) whereas the former fails to inhibit 5-lipoxygenase at 100  $\mu$ M (Koshihara *et al.*, 1984;

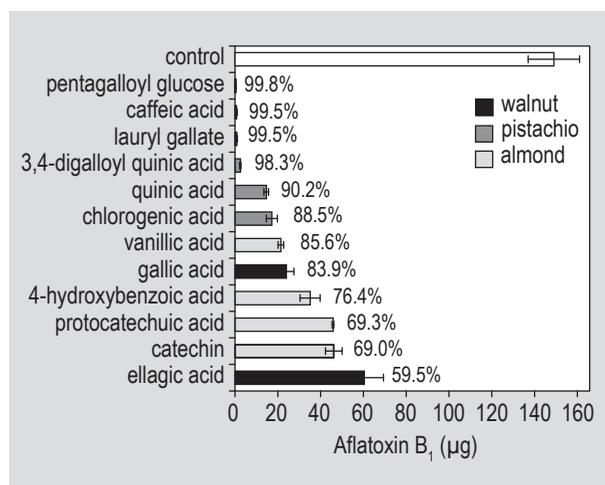
Nishizawa *et al.*, 1988). The commercial antioxidant, lauryl gallate, was equal in effectiveness to caffeic acid; whether the structurally similar anacardic acids in pistachio have similar activity will require their isolation and testing.

Although these compounds substantially suppressed biosynthesis of aflatoxins, there was no evidence that this was due to reduced growth of the fungus at the level tested. Experiments with the walnut and pistachio tannins, pentagalloyl glucose and 3,4-di-*O*-galloylquinic acid, respectively, when tested at 12 mM showed no change in radial growth, fungal weight, or appearance (data not shown).

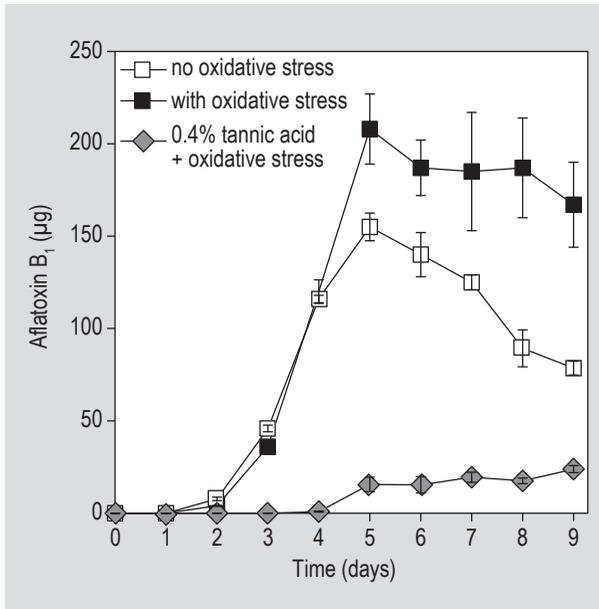
### Aflatoxin production under hydroperoxide-induced oxidative stress

The general, although variable, effect of the tree nut phenolics in reducing aflatoxin formation suggested that this activity was due to their antioxidant properties. We therefore hypothesised that growth of *A. flavus* on media incorporating an agent capable of inducing oxidative stress would stimulate aflatoxin production and that the addition of the phenolics would overcome this effect. *t*-BuOOH was chosen as the oxidative stress inducer because it is structurally more analogous to the lipid peroxides formed by fat oxidation, as opposed to an agent such as hydrogen peroxide. Initially, the hydroperoxide was evaluated at concentrations of 100 and 1000  $\mu$ M, but at the higher concentration, the normal growth of the fungus and aflatoxin formation was delayed (data not shown) and all subsequent experiments were conducted at the 100  $\mu$ M level. Figure 4 illustrates the appreciable stimulation of aflatoxin production over the time-course of the experiment, namely a 34% increase at day 5 and a 111% increase at day 9, relative to control. Furthermore, whereas the control experiment showed a decline in aflatoxin level to 51% of the maximum accumulation at day 5, the oxidatively stressed culture substantially maintained the level at 80% through day 9.

When the *t*-BuOOH stressed cultures were grown with the incorporation of 0.4% tannic acid in the media, aflatoxin levels were significantly reduced by 93% at day 5 and 86% at day 9 (Figure 4). Tannic acid is a commercially available hydrolysable tannin consisting of gallic acid moieties only (no hexahydroxydiphenic acid moieties) around a glucose core. The tannin is therefore analogous to pentagalloyl glucose but was used in these experiments because sufficient quantities of homogeneous material were not available for repeated experiments. The drastic reduction in aflatoxin production demonstrated that the antioxidative properties of the tannin were capable of over-riding the stimulative effect of the hydroperoxide on aflatoxigenesis in the fungal cultures. This effect parallels the suppression of aflatoxin produced by antioxidants in cultures unstressed by addition of peroxide.



**Figure 3.** Inhibition of aflatoxin formation by selected phenolic antioxidants incorporated into media at 12 mM. Each data point is the average of triplicate analyses ( $n=3$ ) and standard deviations are indicated by error bars.

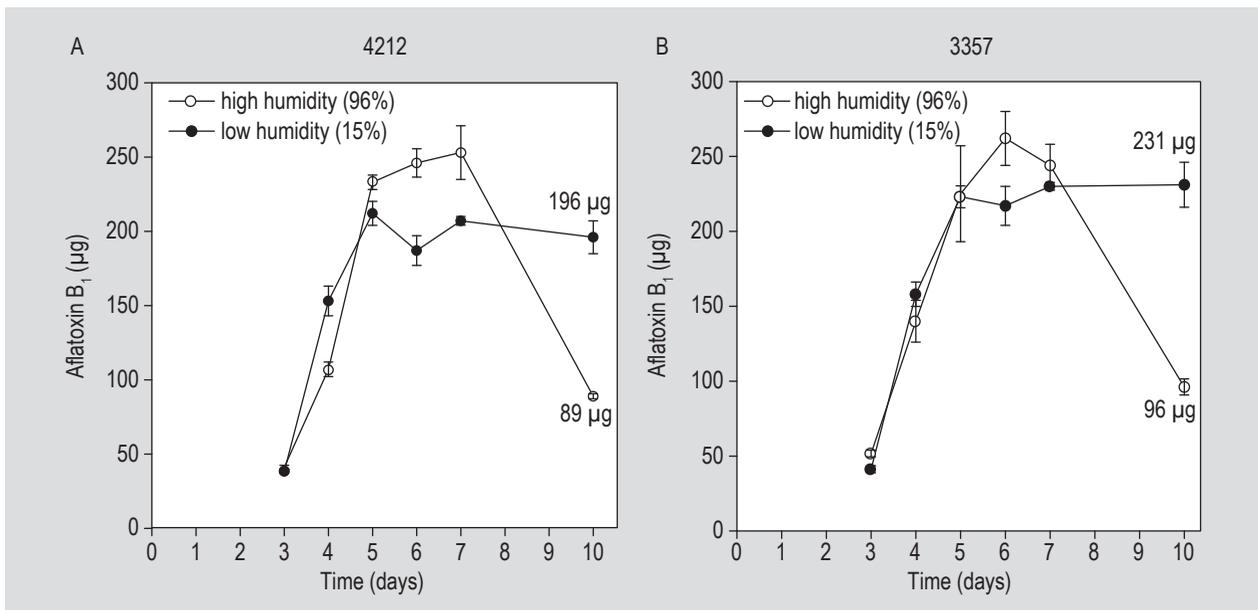


**Figure 4.** Time-course comparison of aflatoxin production by *Aspergillus flavus* on media without oxidative stress; with oxidative stress induced by incorporation of *t*-butyl hydroperoxide; and, with oxidative stress induced by incorporation of *t*-butyl hydroperoxide but suppressed by the presence of tannic acid. Each data point is the average of triplicate analyses (n=3) and standard deviations are indicated by error bars.

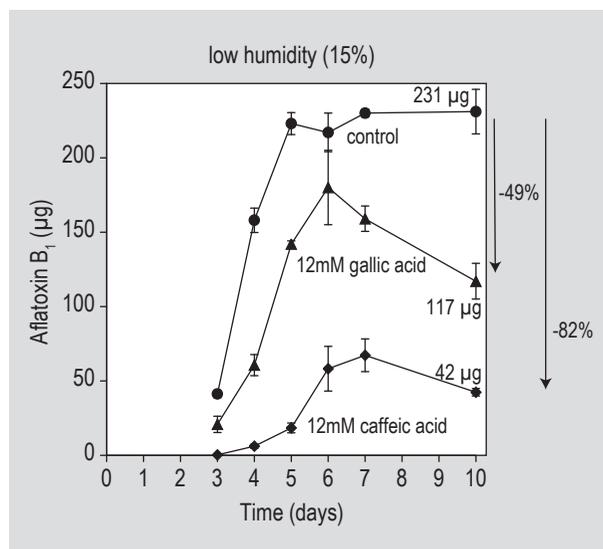
### Aflatoxin production under low humidity-induced oxidative stress

Oxidative stress can be induced by numerous factors, one of the most common of which is drought. *A. flavus* cultures were therefore grown under high (96%) and low (15%) humidity conditions to determine the effect on aflatoxin production. In addition to the pistachio isolate, *A. flavus* 4212, a peanut isolate, *A. flavus* 3357, was also evaluated to establish whether or not the effect was more general. In both cases, although there was no significant difference in aflatoxin levels between high- and low-humidity conditions at the period of maximum production on day 5, there was continued biosynthesis of aflatoxin through day 10 in drought-stressed cultures, with final levels 120% (4212) and 141% (3357), respectively, above those of the high-humidity cultures (Figures 5A and 5B). This absence of catabolic degradation of aflatoxin therefore parallels that seen in the peroxide-stressed cultures and indicates that similar mechanisms may be operating, whether the oxidative stress is induced by peroxide or drought.

The similarity persisted when the low-humidity cultures were grown with 12 mM caffeic acid or gallic acid incorporated into the media. Caffeic acid was particularly effective as an anti-aflatoxigenic agent with levels on day 10 reduced by 82% of control in strain 3357 (Figure 6); in strain 4212, the reduction was comparable at 88%. Gallic acid was less effective but still showed ca. 50% reduction in aflatoxin level in both strains at the end of the experiment. These results are consistent with our earlier experiments



**Figure 5.** Time-course comparison of aflatoxin production by *Aspergillus flavus* under high humidity and low humidity conditions. (A) *A. flavus* strain 4212; (B) *A. flavus* strain 3357. Each data point is the average of triplicate analyses (n=3) and standard deviations are indicated by error bars.



**Figure 6.** Time-course comparison of aflatoxin production by *A. flavus* strain 3357 under low humidity conditions, showing reduction in aflatoxigenesis with incorporation of gallic acid and caffeic acid. Each data point is the average of triplicate analyses (n=3) and standard deviations are indicated by error bars.

in non-stressed cultures, showing the superior potency of caffeic acid relative to gallic acid (Figure 3).

#### 4. Implications

The general ability of natural phenolic compounds in tree nuts to overcome aflatoxigenesis induced by oxidative stress provides tools with which to study the genes involved in aflatoxin biosynthesis. Kim *et al.* (2008) have shown by microarray analysis that expression of almost all the individual genes in the aflatoxin biosynthetic cluster were down-regulated in *A. flavus* NRRL 3357 cultured in the presence of caffeic acid. However, the aflatoxin pathway regulator gene, *aflJ*, and the secondary metabolism regulator gene, *laeA*, were barely affected. Particularly noteworthy was the up-regulation of genes that encode alkyl hydroperoxide reductases, suggesting that antioxidants trigger induction of these genes to detoxify peroxides. The fungus may thereby be protected from oxidising agents such as lipoperoxides, reactive oxygen species produced during host-plant infection or drought stress.

From a practical point of view, breeding strategies that incorporate increased levels of hydrolysable tannins and other phenolic antioxidants could create new tree nut varieties that conform to the requirements of domestic and export markets to minimise aflatoxin contamination. The finding that aflatoxigenesis is induced under low humidity also indicates that orchards should be provided with sufficient irrigation to prevent drought stress.

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