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Original Research Article

Inhibition of Fungal Aflatoxin B1 Biosynthesis by Diverse Botanically-Derived Polyphenols

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Abstract

Purpose: *To identify and characterize the capacity of diverse botanically-derived polyphenols to inhibit aflatoxin B1 (AFB1) production by Aspergillus flavus.*
Methods: *A tea-derived polyphenol mixture and numerous individual polyphenols were tested for their effects on A. flavus growth and AFB1 production. Fungal spores were cultured for 60 h with polyphenols (range 0 – 1,000 µg/mL). The fungi were enumerated by hemocytometry, and AFB1 in culture supernatants was quantified by high-performance liquid chromatography (HPLC).*
Results: *Neither the tea-derived polyphenol mixture nor individual polyphenol compound, except quercetin, inhibited A. flavus growth. Quercetin detectably inhibited growth at 800 µg/mL; none of the remaining polyphenols inhibited fungal proliferation, even at 1,000 µg/mL. However, catechin mixture and all individual polyphenols differentially inhibited fungal AFB1 biosynthesis. Non-ester catechin derivatives revealed stronger inhibitory activity than ester derivatives.*
Conclusion: *Quercetin exhibits the strongest inhibitory effect on AFB1 production and is the only test compound that also inhibits fungal proliferation. Botanically-derived polyphenols are, therefore, promising reagents for controlling fungal contamination and associated toxic aflatoxin deposition in harvested crops and in food processing operations.*

Keywords: *Polyphenols, Quercetin, Aflatoxin B1, Inhibition, Antioxidation*

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INTRODUCTION

Aflatoxin B1 (AFB1) is a potently carcinogenic food contaminant produced by the filamentous fungus *Aspergillus flavus*, which constitutes a general food safety problem [1-3]. Extensive research has focused on identifying compounds that inhibit AFB1 biosynthesis. Numerous plant-derived compounds and extracts that inhibit AFB1 biosynthesis have been reported [4-7]. Of particular interest from both agronomic and human health perspectives are compounds that can bolster resistance of host plants to AFB1 contamination. Ideally, such a compound would be plant-derived, nutritionally positive or neutral, and synthesized through a well-described biosynthetic pathway [8].

As a globally-consumed beverage, tea contains many components with physiologic effect on the human body, including disease prevention and treatment [9]. Main ingredients in tea extracts are polyphenols, alkaloids, tea polysaccharide, tea pigment, and theanine [10]. Polyphenols play important inhibitory roles against aflatoxin production [8,11]. Quercetin can prevent the

conversion from AFB1 to the carcinogenic product AFB1-8, 9-epoxide [12].

In our previous study, tea extracts inhibited AFB1 production by *A. flavus*, and different types of tea extracts had dissimilar inhibitory effects [11]. The current study further elucidated the activity of tea extract components, by comparing the effects of tea-derived polyphenol mixture and 10 polyphenol monomers present in tea extracts on fungal growth and AFB1 production. By better understanding the inhibitory effects of diverse polyphenol components on fungal growth and AFB1 production, we can identify new compounds that are useful for controlling AFB1 contamination during food processing and storage.

EXPERIMENTAL

Chemicals

Aflatoxin B1 (Sigma-Aldrich Inc, St. Louis, MO) was used as a standard for HPLC analysis. A naturally-derived tea polyphenol mixture and polyphenol monomers present in tea extracts (shown in Table 1) were obtained from Nacalai Tesque Inc. (Kyoto, Japan; online catalog at http://www.nacalai.co.jp/global/download/pdf/Plant_Extract_Compounds.pdf). All the other chemicals were obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

Fungal cultures

The toxigenic *A. flavus* strain CGMCC 3.2890 was purchased from China General Microbial Culture Collection Centre (CGMCC, Bejing, China). This fungal strain was maintained on potato-dextrose agar medium at 30 °C. Sabouraud-dextrose medium was used for toxigenic culture of *A. flavus*. Fungal spores were suspended in 1 % Triton X-100 and enumerated using a hemocytometer.

Determination of polyphenol effects on fungal growth and AFB1 production

Antifungal activities of a tea-derived polyphenol mixture and various related individual polyphenols were tested in 96-well culture plates. Two-hundred-µL aliquots of *A. flavus* suspended in Sabouraud-dextrose medium at 1×10^6 CFU/mL were added to each well. Spore suspensions were then mixed with different concentrations of the polyphenol mixture or individual polyphenols (5, 10, 25, 50, 100, 200, 400, 800 or 1,000 µg/mL), and cultured at 28 °C for 60 h. The medium without the test polyphenols was used as a growth control and the blank control used contained only the medium. After incubation, OD600 nm was measured using a microplate reader and the minimum inhibitory concentration (MIC) was defined as the lowest concentration of polyphenol mixture or individual polyphenols that prevented fungal growth. AFB1 produced by *A. flavus* in each well was quantified by high-performance liquid chromatography (HPLC).

Determination of AFB1 concentration in fungal culture supernatants

AFB1 in *A. flavus* culture supernatants was extracted with three volumes of chloroform. Chloroform solubilized extracts were under blowing nitrogen and then re-dissolved in 1.0 mL methanol. After filtering through a 0.22 µm microporous membrane, samples were injected through an Aglient HPLC 1100 system equipped with an octadecylsilyl column (COSMOSIL 5C18-AR, column 250 x 4.6 mm; Nacalai Tesque Inc) kept at 22 °C. The mobile phase was acetonitrile:methanol:water (1:1:2, v/v/v) at a flow rate of 1 mL/min. UV detection was at 365 nm. For each injection, a volume of 20 µL of AFB1 standard or sample was used. Quantification was based on the chromatograms relative to the external standards.

Statistical analysis

All data presented are mean ± standard error of the mean (SEM) of three determinations. Student's t-test was used to determine significant differences between group means in all experiments. Differences were considered significant at $p < 0.05$.

RESULTS

Effect of tea-derived polyphenol mixture on fungal AFB1 production

The tea-derived polyphenol mixture, containing four tea catechin polyphenols, inhibited fungal AFB1 production, though the inhibitory effect was not stringently polyphenol concentration-dependent (Fig 1). Compared to the untreated control (shown as dotted line in Fig 1), all concentration of tea-derived polyphenol mixture treatments showed significantly decreased AFB1 production, and concentrations ≥ 800 µg/mL completely abrogated AFB1 production.

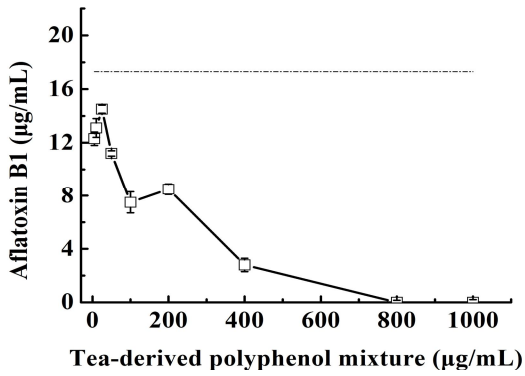

Figure 1: A tea-derived polyphenol mixture containing four catechins inhibits AFB1 production by *A. flavus*. Shown are results from untreated control fungi (dash-dot line) and fungi treated with a range of 5 – 1,000 µg/mL tea-derived polyphenol mixture. Each data point (□) represents the mean ± SEM (n = 3, per condition)

Effect of individual polyphenols on fungal AFB1 production

Three individually tested, botanically-derived polyphenols that occur in tea (gallic acid, part of hydrolyzable tannins; quercetin, a flavonoid; and catechin) inhibited AFB1 biosynthesis by *A. flavus*, with different concentration dependencies (range 5 – 1,000 µg/mL; Fig 2).

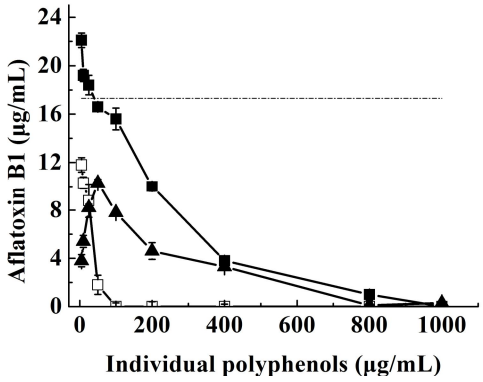

Figure 2: Effects of select individual polyphenols on AFB1 production by *A. flavus*. Shown are results from untreated control fungi (dash-dot line) and fungi treated with a range of 5–1,000 µg/mL of each individual polyphenol. Tested polyphenols included gallic acid (▲), quercetin (□), and catechin (■). Each data point represents the mean ± SEM (n=3, per condition)

Gallic acid and quercetin showed significant inhibitory activity at low concentrations. Whereas gallic acid required ≥ 800 µg/mL to completely inhibit fungal AFB1 production, quercetin completely abrogated toxin biosynthesis at all concentrations ≥ 100 µg/mL. However, purified catechin displayed a unique, biphasic, concentration-dependent inhibitory pattern on *A. flavus* AFB1 production. Very low catechin concentrations (i.e., 5 and 10 µg/mL) increased fungal AFB1 production versus untreated controls; however, catechin concentrations ≥100 µg/mL, inhibited AFB1 production. All three individual purified polyphenols completely inhibited AFB1 biosynthesis when their concentration reached 1,000 µg/mL.

Effect of individual non-ester and ester catechins on fungal AFB1 production

All tested non-ester derivatives of catechins, including epicatechin (EC), epigallocatechin (EGC), and gallocatechin (GC) inhibited *A. flavus* AFB1 biosynthesis (Fig 3), at even lower concentrations than the catechins depicted in Figure 2. EC's inhibitory activity and concentration were positively correlated. Of the three tested non-ester catechin derivatives, EGC had the best inhibitory activity, and completely inhibited AFB1 production at 400 µg/mL (Fig 3). Catechin ester derivatives such as catechin gallate (CG) epicatechin gallate (ECG), epigallocatechin gallate (EGCG) and gallocatechin gallate (GCG) also inhibited fungal AFB1 production (Fig 4). At lower concentrations, CG and EGCG promoted toxigenic activity. *A. flavus* was still able to produce a considerable amount of AFB1 in the presence of EGCG and GCG, even at treatment concentrations up to 1,000 µg/mL. ECG completely suppressed fungal AFB1 production at 800 µg/mL, which was consistent with catechin effects (Fig 2).

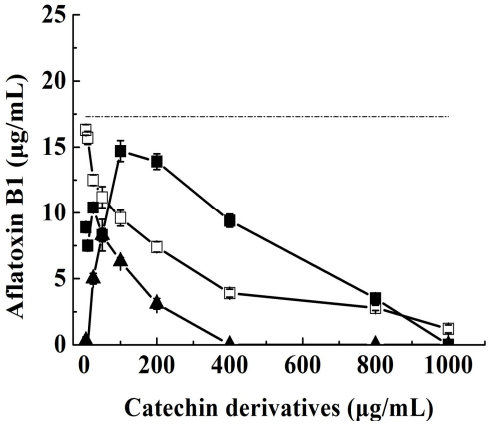

Fig. 3: Effect of three non-ester catechin derivatives on aflatoxin production by *A. flavus*. Tested polyphenols included epigallocatechin (EGC, ▲), epicatechin (EC, □), and gallocatechin (GC, ■). Shown are results from untreated control fungi (dash-dot line) and fungi treated with a range of 5–1,000 µg/mL of each individual polyphenol. Each data point represents the mean ± SEM (n = 3 determinations per condition)

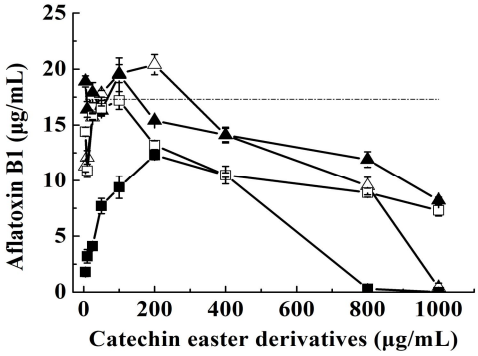

Fig. 4: Effect of some catechin ester derivatives on the aflatoxin production by *A. flavus*. Tested polyphenols included catechin gallate (CG, Δ), epicatechin gallate (ECG, ■), epigallocatechin gallate (EGCG, ▲) and gallocatechin gallate (GCG, □). Shown are results from untreated control fungi and fungi treated with a range of 5–1,000 μg/mL of each individual polyphenol. Each data point represents the mean ± SEM (n=3 determinations per condition)

Botanically-derived polyphenols have minimal effect on *A. flavus* proliferation

We evaluated the effect of a tea-derived polyphenol mixture and various individual polyphenols on *A. flavus* proliferation, after 60 h of treatment in culture. Except for quercetin, whose MIC was determined to be 800 μg/mL, no tested polyphenol mixture or individual compound affected *A. flavus* growth, even at concentrations up to 1,000 μg/mL (Table 1).

Table 1: Effect of botanically-derived polyphenols on the growth of *A. flavus*

Polyphenol	MIC against *A. flavus* (μg/ml)
Tea-derived polyphenol mixture	>1000
Gallic acid	>1000
Quercetin	800
Catechin	>1000
Epigallocatechin (EGC)	>1000
Epicatechin (EC)	>1000
Gallocatechin (GC)	>1000
Catechin gallate (CG)	>1000
Epicatechin gallate (ECG)	>1000
Epigallocatechin gallate (EGCG)	>1000
Gallocatechin gallate (GCG)	>1000

Note: *MIC = minimum inhibitory concentration*

DISCUSSION

Tea leaves contain diverse polyphenols, including flavonoids, epigallocatechin gallate and other catechins [13,14]. The tea-derived polyphenol mixture and all individual polyphenol compounds tested in this study exhibited inhibitory activity of aflatoxin synthesized by *A. flavus*, although with different concentration-dependent patterns. Among the individual compounds, quercetin and gallic acid displayed better inhibitory activities than catechins at the same treatment concentration. For catechin polyphenols, non-ester derivatives had better AFB1 inhibitory activity than the ester derivatives. Previously, we found that water extracts of tea had no significant effect on *A. flavus* growth, with the exception that Pu'er tea had in vitro antifungal properties against *A. flavus* [15]. In the current study, neither the tea-derived mixture nor any of the individually tested compounds, with the exception of quercetin inhibited the growth of *A. flavus*.

Reverberi *et al* [16] found that the *A. flavus* redox equilibrium played important roles in aflatoxin biosynthesis, and that antioxidants such as butylated hydroxytoluene inhibited aflatoxin production. In human, tea polyphenols demonstrate antioxidant activities and can effectively scavenge oxygen free radicals and lipid free radicals, thereby preventing lipid peroxidation [17]. Therefore, we suspected that the antioxidant activity of tea polyphenols might be a mechanism by which these polyphenol compounds inhibit fungal AFB1 production. However, the inhibitory activities of the tested polyphenol compounds on fungal aflatoxin production were not consistent with their antioxidant capacities. The capacity of polyphenol compounds for scavenging both oxygen and hydroxyl free radicals, sorted by strength from strongest to weakest, is polyphenols > quercetin > gallic acid. Conversely, the capacities of polyphenol compounds for scavenging DPPH (diphenyl picryl hydrazinyl) radicals were ranked as gallic acid > quercetin > polyphenols [18]. While quercetin only possesses moderate antioxidant activity compared to the many other polyphenols that we tested, it clearly had the strongest inhibitory effect on fungal AFB1 production. This suggests that other pathways unrelated to intracellular redox status are involved in regulating fungal toxin production. Quercetin is a flavonoid widely distributed in many fruits, vegetables, grains and tea leaves [19]. It can reportedly restrict the conversion of AFB1 to carcinogenic substances [12]. Our study indicated that quercetin was a highly effective aflatoxin synthesis inhibitor. Therefore, quercetin can be developed as anti-fungal agents used in animal feed or food processing areas. However, the mechanism of quercetin's inhibition of fungal aflatoxin production needs further study.

CONCLUSION

Polyphenol compounds in tea effectively inhibit aflatoxin production in A. flavus. Of these compounds, quercetin also hinders fungal proliferation. Thus, quercetin may be a promising candidate for controlling A. flavus contamination of post-harvest crops.

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