SUPPLEMENTARY MATERIAL

**Novel Regulation by Piperonal of Aflatoxin B1 to Aflatoxin G2 Biosynthesis in
*Aspergillus flavus***

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Piperonal is a methylenedioxy-containing compound that is abundantly present in *Piper* fruits. The present study investigated its inhibitory role in aflatoxin (AF) biosynthesis. Piperonal shows structural similarity to piperonyl butoxide (PBO) that has been used as a synergist with pyrethroid insecticides and acts by inhibiting cytochrome P450 monooxygenases in insect pests. Treating *Aspergillus flavus* with piperonal completely inhibited aflatoxin B1 (AFB1) production, inhibited fungal growth, and resulted in increased sclerotial formation. Addition of piperonal to the aspergilli growing medium resulted in 20 fold higher aflatoxin G2 (AFG2) production than AFB1 production. It is likely that piperonal inhibits AFB1 synthase and some cytochrome P450 activities involved in AFB1 biosynthesis because of its structural similarity to PBO. This novel regulation by piperonal was compared with benzodioxole, eugenol, methyleugenol, and piperine. Benzodioxole and eugenol suppressed the fungal growth and AFB1 production, while methyleugenol showed potent inhibition of AFB1 production with slight production of AFG1. At a concentration of 1 mg/ml, piperine increased AFB1, AFB2 and AFG1 production. This indicates that natural products may play an important role in aflatoxin biosynthesis, and highlights a novel regulation of AFG2 production by piperonal.

Keywords: Piperonal, *Aspergillus flavus*, aflatoxin B1 biosynthesis, aflatoxin G2

1. Experimental procedures

1.1. Chemicals and reagents

Aflatoxin B1, B2, G1, and G2, and the chemicals 1,3-benzodioxole, eugenol and methyleugenol were obtained from Sigma-Aldrich (St. Louis, MO). Piperonal and piperine were isolated from *Piper nigrum* fruits; it was analyzed and confirmed by a series of

spectrometric analyses including GC-MS, ¹H-, and ¹³C-NMR spectroscopy. Piperonal and piperine were also purchased from Sigma-Aldrich (St. Louis, MO) and compared with the corresponding compounds isolated in our laboratory. All other chemicals used in this study were of the highest analytical grade.

1.2. Aflatoxin analysis by high-performance liquid chromatography (HPLC)

Aspergillus flavus ATCC 22546 was used in this bioassay and the aspergilli produced a substantial amount of AFB1 with a trace amount of AFB2. It produced little AFG1 or AFG2 in our growth medium. Adequate concentrations of the test compounds were supplemented into potato dextrose broth (PDB) and autoclaved. Twenty five ml of the sterilized media were then poured into 250 mL flasks for each test. Each flask was inoculated with 10⁶ spores of *A. flavus* and incubated for five days at 30°C. The control flasks contained only the carrier solvent and the PDA agar inoculated with *A. flavus* without any test compound. Each concentration was tested at least in triplicates. After an incubation of 5 days, the total media was used to determine growth rates by measuring the mycelial and sclerotial dry weights and were subjected to analysis for AFB and AFG type mycotoxins using HPLC (Lee et al. 2002). Arithmetic means of three replicates of tests were calculated and analyzed for any significant difference in comparison to the control using one-way ANOVA test at a $p < 0.05$ level (SAS 2001).

References

- Lee SE, Mahoney NE, Campbell BC. 2002. Inhibition of aflatoxin B1 biosynthesis by piperlongumine isolated from *Piper longum* L. J Microbiol Biotechnol. 12:679-682.
- SAS. 2001. SAS User's Guide, 4th ed. SAS Institute, Cary, NC.

Supplementary Figure legends

Fig. S1. Chemical structures of the tested natural compounds used in this study

Fig. S2. Analysis of aflatoxin production by HPLC with a C18 column. (a), control; (b), piperonal treatment at a concentration of 1 mg/ml.

Fig. S3. Dry weight of *Aspergillus flavus* after 4 day incubation with or without treatment with natural compounds at a concentration of 1 mg/ml. 1, control aspergilli; 2, benzodioxole-treated aspergilli; 3, piperonal-treated aspergilli; 4, eugenol-treated aspergilli; 5, methyleugenol-treated aspergilli; 6, piperine-treated aspergilli.

Fig. S4. Pictures of *Aspergillus flavus* after 4 day incubation with or without treatment with natural products at a concentration of 1 mg/ml. 1, control aspergilli; 2, benzodioxole-treated aspergilli; 3, piperonal-treated aspergilli; 4, eugenol-treated aspergilli; 5, methyleugenol-treated aspergilli; 6, piperine-treated aspergilli.

Fig. S5. Schematic showing aflatoxin biosynthesis by *Aspergillus flavus*. “*” indicates the possible site of piperonal action and “***” indicates the possible site of methyleugenol action in the aflatoxin biosynthesis pathway.

Fig. S1

Fig. S2

Fig. S3

Fig. S4

Fig. S5