J. Microbiol. Biotechnol. (2002), **12**(4), 679–682

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Inhibition of Aflatoxin B_1 Biosynthesis by Piperlongumine Isolated from *Piper longum* L.

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Received: November 19, 2001
Accepted: March 28, 2002

Abstract The alkaloids, piperlongumine, piperine, pipernonaline, and piperoctadecalidine, isolated from *Piper longum* L., were found to inhibit the biosynthesis of aflatoxin B_1 (AFB_1) in *Aspergillus flavus* WRRC 3-90-42-12. Piperlongumine was the most active among the compounds tested, with a 96% inhibition of AFB_1 biosynthesis at 0.2% (w/v) supplement in a potato dextrose agar (PDA) medium. The three other piperidine alkaloids, piperine, pipernonaline, and piperoctadecalidine, also inhibited the biosynthesis of AFB_1. Of these three alkaloids, piperoctadecalidine exhibited a potent inhibitory activity with a 100% inhibition of AFB_1 production at 0.7% (w/v) supplement in a PDA medium. Therefore, piperlongumine and piperoctadecalidine could be used as antiaflatoxigenic agents in agricultural industries. To determine the antiaflatoxigenic mode of action of piperlongumine, further studies are needed.

Key words: Aflatoxin B_1, *Aspergillus flavus*, *Piper longum*, piperlongumine

Aflatoxins are secondary metabolites produced by *Aspergillus flavus* Link. Ex Fries and *A. parasiticus* Speare [4]. Aflatoxin B_1 (AFB_1) and related difuranocoumarin compounds are major concerns to public health, mainly due to their being potential carcinogens for humans and animals with their proven toxicity [5, 6]. A number of agricultural commodities are prone to infection by aflatoxigenic *Aspergillus* and subsequent contamination with aflatoxins. The principal concern for U. S. crops include corn, peanuts, cotton seed, and tree nuts. In addition, aflatoxin M_1 (AFM_1), a metabolite of AFB_1 found in the milk of dairy cattle or lactating mothers exposed to aflatoxin, is of concern due to its potential hepatotoxic and immunotoxic effects in infants and children. Guideline threshold levels set by the U.S. Food and Drug Administration for aflatoxins in foods for domestic consumption are not to exceed 20 parts per billion (ppb). The European Union and Japan have recently set threshold levels at lower than four ppb. Moreover, current efforts to control aflatoxin-producing *Aspergillus* with fungicides or antibiotics are not environmentally or economically sound [12]. Therefore, it is essential to establish a new method to inhibit aflatoxin production during pre- or post-harvest processing of food commodities. Accordingly, the current study was attempted to find potent inhibitors of aflatoxin biosynthesis from natural and edible plant sources, and we present here several potent inhibitors of AFB_1 biosynthesis isolated from the dried fruits (infructescences) of *Piper longum* L. These compounds were previously shown to be nontoxic to mice [13].

A. flavus WRRC 3-90-42-12 was used in the bioassay. In addition to substantial amounts of AFB_1, this isolate also produces a trace amount of aflatoxin B_2 (AFB_2) [10]. Whole dried fruits and four isolated alkaloids isolated from *P. longum* were supplemented into a potato dextrose agar (PDA), and autoclaved, then 10 ml of the sterilized media was poured into 60-mm Petri dishes for each test. Each Petri dish was inoculated with 200 spores of *A. flavus* and incubated for seven days at 30°C. The control plates contained only the PDA agar inoculated with *A. flavus* without any test sample. After 5 days incubation, the total extracts from each Petri dish were subjected to quantitative aflatoxin analysis. Colony diameters were measured before extraction of plates for the aflatoxin assay [10]. Means of three replicates were compared and tested for any significant difference with the control using the Scheffe test at a P=0.05 level [11]. The active compounds were isolated from dried fruits of *P. longum* obtained from Kyung-Dong traditional market, Seoul, Korea. The dried fruits (2.5 kg) were crushed and extracted twice with hexane (10 liter) at

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room temperature and filtered (Toyo filter paper No. 2, Tokyo, Japan). The combined filtrate was concentrated *in vacuo* at 35°C. The total yield was about 2.3% (w/w) of the dried fruit. The extract was chromatographed on a silica gel column (Merck 70–230 mesh, 300 g, 4.5 i.d.×60 cm) eluated with a step gradient of hexane-ethyl acetate (3:1, v/v, 1 liter), ethyl acetate (1 liter), and ethyl acetate-methanol (1:9, v/v, 1 liter). The active fractions eluted with hexane-ethyl acetate (3:1) were separated by column chromatography on a silica gel eluted with hexane-ethyl acetate (4:1). Active eluates were collected and analyzed by TLC (hexane-ethyl acetate, 3:1). The fractions with a similar TLC profile were combined and further separated by HPLC (Water Delta Prep 4000) on a Bondapak C_{18} column (29 i.d.× 300 mm, Waters) using methanol-water (3:7) at a flow rate of 7 ml/min and detected at 260 nm. Four compounds were isolated: piperlongumine 1 (1.7%), piperine 2 (7.8%), pipernonaline 3 (0.05%), and piperoctadecalidine 4 (0.07%) (Fig. 1). The structural determination of the active isolates was based on a spectral analysis. The mass spectra were determined on a JEOLJMS-DX30 spectrometer and the structures compared to earlier papers [1, 3, 7, 15]. For an aflatoxin analysis, the contents of the plates were macerated using a pestle in 50 ml of methanol. A 1.0-ml aliquot was removed, dried with N_2 (40°C), and derivatized by the addition of 200 µl of trifluoroacetic acid (Pierce Co. Rockford, IL, U.S.A.) and 200 µl of hexane for 10 min at room temperature [10]. The derivatized product was dried with N_2 (40°C) and then redissolved to 1.0 ml with water-acetonitrile (9:1). The samples were quantitatively analyzed for aflatoxins by reverse-phase HPLC using a Hewlett Packard HPLC Work Station. The analysis consisted of isocratic elution with water-methanol-acetonitrile (60:20:20) at 1.0 ml/min through a C_{18} 5 µm Microsob column (4.6 by 250 mm) attached with a 50-mm guard column (Rainin Instrument Co., Woburn, MA, U.S.A.). The aflatoxin

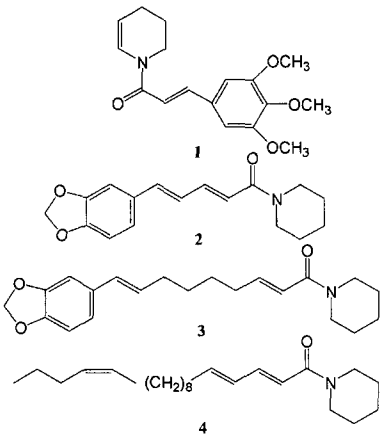

Fig. 1. Structures of alkaloids from *Piper longum*.
(**1**), Piperlongumine; (**2**), piperine; (**3**), pipernonaline; (**4**), piperoctadecalidine.

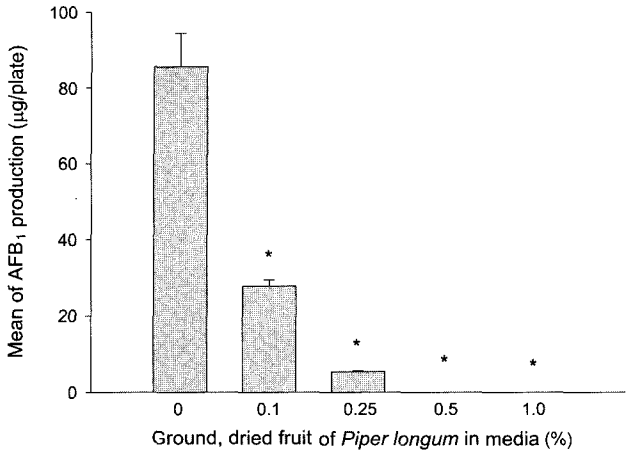

Fig. 2. Effects on production of aflatoxin B_1 by various amounts of ground dried-fruit of *Piper longum* incorporated into growth media of *Aspergillus flavus* (WRRC 3-90-42-12).
The means are significantly different from the control (*P<0.05), using the Sheffe test when n=3. '0' means control.

peaks were analyzed with a fluorescence detector with an excitation at 365 nm and emission at 455 nm. In this system, AFB_1 had a retention time of 6.3 min. Trace levels of AFB_2 were detected at approximately 0.1% of the AFB_1 concentration, but not quantified. The retention times of the aflatoxins were confirmed using authentic compounds.

During the initial experiments, it was observed that the ground dried fruit of *P. longum* possessed a potent inhibitory activity of AFB_1 biosynthesis (Fig. 2). Interestingly, fungal growth was only slightly affected by the addition of the fruit extracts into the medium (Table 1). However, the production of AFB_1 was inhibited by 64% in the media containing 0.1% (w/w) of the extract (Fig 2). The complete inhibition of AFB_1 production was observed with 0.5% of dried fruit in the medium, while the fungal growth was inhibited about 10% (Table 1). We next examined inhibitory effects of each alkaloid on aflatoxin biosynthesis. Among

Table 1. Inhibition of growth of *Aspergillus flavus* WRRC 3-90-42-12 by alkaloids isolated from *P. longum* L.

Compounds (%)	Mean colony diameter (mm)	Growth inhibition (%)
Control	53	-
[*P. longum* dried fruit, %]		
0.1	50	5.7
0.25	48	9.5
0.50	47	11.3
1.0	35	34.0
Piperlongumine (0.2)	45	15.1
Piperine (0.7)	32	39.6
Pipernonaline (0.7)	37	30.2
Piperoctadecalidine (0.7)	28	47.2

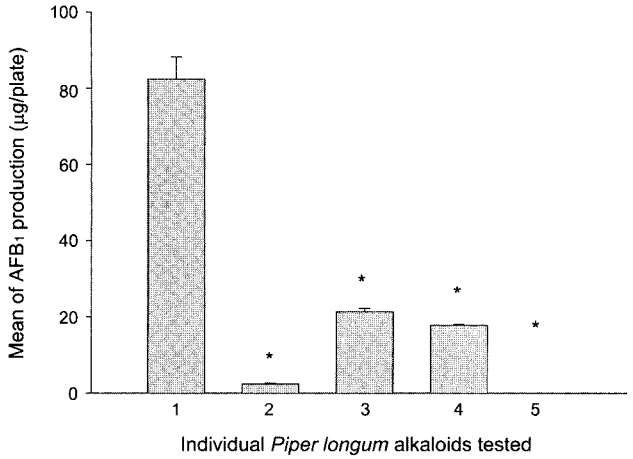

Fig. 3. Inhibition of aflatoxin B_1 production by alkaloids isolated from *Piper longum*.
1, Control; 2, piperlongumine (0.2%); 3, piperine (0.7%); 4, pipernonaline (0.7%); 5, piperoctadecalidine (0.7%). The means are significantly different from the control (*P<0.05), using Sheffe's test when n=3.

the compounds tested, piperlongumine was the most active with almost complete inhibition of AFB_1 production at 0.2% (w/w) in the medium (Fig. 3). Among the other three *Piper* alkaloids tested, piperoctadecalidine was the most active, and almost completely inhibited aflatoxin production at 0.7% (w/w) in the medium. However, this activity was weaker than that of the whole dried fruit (0.5%, w/w) and >3 times less active than piperlongumine. While both piperine and pipernonaline showed inhibitory activities against aflatoxin biosynthesis, these compounds were approximately 20 and 10 times less active than piperoctadecalidine, respectively. However, the most active compound, piperlongumine, was only 0.2% of the dried fruit by weight, therefore, it cannot be totally responsible for all the inhibitory activity in *P. longum*.

Piperaceae from the fruits are used as food-flavoring agents and some fruits also contain many potent insecticidal properties [9, 14, 16]. Among the natural products identified in these fruits, unsaturated amides constitute the major group. Dried black pepper, *P. nigrum* L., which is a common "table" black pepper available from supermarkets, has been reported to be toxic to the housefly, *Musca domestica* L. [16]. In addition, black pepper fruits are known to inhibit growth of *A. flavus* [2] and piperine has been shown to be active in inhibiting AFB_1 production [8]. The current results showed that piperine was not the most antiaflatoxigenic compound in *P. longum*, however, the amount of piperine in *P. longum* might be enough to significantly inhibit aflatoxin production in this study.

Piperlongumine, pipernonaline, and piperoctadecalidine have all been previously isolated from *P. longum* and *P. retrofractum* [1, 3, 15], yet their antiaflatoxigenic activity has not been determined. Recently, Lee *et al.* [7] reported that pipernonaline has a potent fungicidal activity, whereas piperlongumine and piperine have no fungicidal activities against the phytopathogenic fungi, *Puccinia recondita*. However, we found in the present study that the alkaloids isolated from *P. longum* exhibited a potent inhibitory activity towards AFB_1 biosynthesis. Further studies are underway to determine the mode of the piperlongumine action of antiaflatoxigenic activity.

Acknowledgment

This study was partially supported by the Brain Korea 21 project.

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