The Inhibitory Effect of Neem (*Azadirachta indica*) Leaf Extracts on Aflatoxin Synthesis in *Aspergillus parasiticus*[1]

Deepak Bhatnagar* and **Susan P. McCormick**[2]
ARS/USDA, Southern Regional Research Center, P.O. Box 19687, 1100 Robert E. Lee Blvd., New Orleans, LA 70179

The effect of neem (*Azadirachta indica*) leaf extracts on *Aspergillus parasiticus* growth and aflatoxin biosynthesis was investigated. The extracts were prepared by blending 50 g (wet weight) of fresh leaves in one l of 10 mM potassium phosphate (pH 7.0) or by boiling the leaves in the buffer. Extracts were added to fungal growth media at 1, 5, 10, 20 and 50% (vol/vol) concentrations prior to inoculation. The formulations did not affect fungal growth (i.e., mycelial dry weight) but essentially blocked (>98%) aflatoxin biosynthesis at concentrations greater than 10% (vol/vol). The inhibitory effect was somewhat diminished (60–70% inhibition) in heated leaf extracts. Volatile components of the extracts were analyzed using capillary gas chromatography/mass spectrometry; the major volatile component was 3-methyl 2-buten-1-ol. However, volatiles from blended leaf extracts did not affect either aflatoxin synthesis or fungal growth. The neem-mediated inhibition appears to involve regulation of secondary metabolism, because once secondary biosynthesis was initiated the inhibitory effect of the neem leaf constituents was lost.

Azadirachta indica Juss. (syn. *Melia azadirachta* L.) commonly known as "margosa," "neem" and "nim," is an ornamental tree of Asia and Africa. Neem components have reputed value for their medicinal, spermicidal, antiviral, antibacterial, antiprotozoal, insecticidal, insect repellent, antifungal and antinematode properties (1,2). Several active principles from different parts of the neem tree have been reported (3). The neem leaves are known to contain desactylimbin, quercetin and sitosterol (4,5). Neem oil yields various acids, sulphur, etc. (6, 8). The effects of these components on aflatoxin biosynthesis by either *Aspergillus parasiticus* or *Aspergillus flavus* have not been studied so far. Owing to the antimicrobial properties of the leaves of the neem plant, the current study assessed the fungicidal role of leaf formulations against aflatoxigenic strains of *Aspergillus parasiticus*.

EXPERIMENTAL PROCEDURES

Preparation of neem leaf extracts. Neem leaves were obtained from the ARS/USDA Subtropical Horticulture Research Station, Miami, Florida. After washing the leaves thoroughly with sterile distilled water, extracts were prepared by blending 50 g (wet weight) of fresh leaves in one l sterile 0.01 M potassium phosphate buffer, (pH 7.0) (blended). In another formulation (heat-extracted), leaves were boiled in sterile buffer for 30 min and the volume was adjusted to one l after cooling. Extracts were filtered through several layers of cheesecloth, and the filtrate was centrifuged for 15 min at 7,000 × *g*. Soluble extracts were sterilized by passing through a Millipore filter (0.22 μm pore size). In addition, one-half of both leaf formulations (blended and heat-extracted) were autoclaved to achieve sterilization.

Fungal growth conditions in submerged culture. The fungal strain used in this study was a wild-type aflatoxigenic isolate of *A. parasiticus* designated SU-1 (SRRC 143). The fungus was grown on growth medium (GM) (9) by inoculating with 0.1 ml of a spore suspension (10^6 spores/ml) to 100 ml of the medium containing 0 to 20% vol/vol neem leaf formulations in 250-ml flasks. The flasks were incubated for 2–4 days on a shaker incubator (Lab-Line Instrument Inc.) at 150 rpm and 28°C. Growth of the fungus was recorded after four days by harvesting the mycelial pellets through filtration on oven-dried Whatman No. 42 filter papers. The filter papers containing the fungal growth mass were oven-dried at 70°C for 24 hr, and the dry weight of the fungus was determined as an index of fungal growth. For the study of the conversion of secondary metabolites to aflatoxins, mycelia were washed thoroughly and transferred to low sugar replacement media (LSRM) (10).

Extraction and assay of metabolites and aflatoxins. After the desired incubation, mycelial pellets and media were extracted with aqueous acetone, followed by methylene chloride (10). Aflatoxins were separated on silica gel thin layer chromatographic (TLC) plates in ether:methanol:water (96:3:1). The toxins were quantitated by fluorometric scans (360 nm) of TLC plates containing the extracted samples and comparison with aflatoxin standards run on the same plate (10). Aflatoxin precursors from the mycelia were identified by the procedure described by McCormick et al. (11).

Determination of volatile components of neem leaves. Neem leaf extracts were placed in 1.2-l Kontes solvent storage bottles fitted with Teflon valves and an inlet tube that extended to two in. from the bottom of each bottle. Air-space above the extracts was purged with nitrogen for 30 min onto Tenax GC (60–80 mesh) tubes. Volatiles were analyzed with a Finnigan MAT GC/MS 4000 system interfaced with an external closed inlet device. The GC column was a 50-m capillary SE-54 column held at -30°C for loading. The temperature program used was -30°C (3 min) → 30°C (15°/min) → 150°C (2.5°/min) ∞ 250°C (10°/min). Data aquisition and analysis were accomplished with a Finnigan-Incos data system. Compounds were identified on the basis of computer-assisted library searches.

Effect of volatile components of neem leaves. Activity of volatiles from the blended neem leaf extracts on growth and aflatoxin production was determined by: (i) removal of two 10-mm diameter plugs from the outer edges of the PDA nutrient agar in Petri plates;

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*To whom correspondence should be addressed.

[2]Present address: Northern Regional Research Center, ARS/USDA, 1815 N. University St., Peoria, IL 61604.

TABLE 1

Effect of Neem Leaf Extracts on *A. parasiticus* Growth and Aflatoxin Synthesis in Submerged Cultures[a]

Treatment[b]	Mycelial dry Weight (g)	Aflatoxin (% of control)			
		B_1	B_2	G_1	G_2
Control	1.28 ± 0.19	100[c] ± 11	100 ± 14	100 ± 17	100 ± 9.0
Blended extract	1.32 ± 0.18	1.0 ± 0.1	1.4 ± 0.1	ND[d]	ND[d]
Blended extract (autoclaved)	1.34 ± 0.20	18.2 ± 2.7	13.5 ± 0.2	3.0 ± 0.4	3.8 ± 0.5
Heated extract	1.29 ± 0.13	7.6 ± 1.3	6.6 ± 0.7	ND[d]	ND[d]
Heated extract (autoclaved)	1.30 ± 0.08	30.1 ± 3.3	13.0 ± 0.7	15.8 ± 1.0	14.4 ± 0.2

[a]The results are the means of 4 experiments with 2 replicates each.
[b]The neem extracts were prepared as described in Materials and Methods.
[c]100% refers to 23.2 μg aflatoxin B_1, 5.2 μg aflatoxin B_2, 14.3 μg aflatoxin G_1 and 3.6 μg aflatoxin G_2 per g mycelial dry weight.
[d]ND = not detected.

an additional plug was removed from the center of the plate; and (ii) addition of a 0.5-ml *A. parasiticus* spore suspension (10^5 spores/ml) to the center opening and introduction of neem leaf extract in 1.5-ml beakers placed in the outer holes. The Petri plates were sealed and radial growth of the fungus was recorded each day over a four-day test period. At the end of the four days, Petri plate contents were extracted for aflatoxin.

RESULTS AND DISCUSSION

Effect of neem leaf formulations on A. parasiticus *growth and aflatoxin production in submerged culture.* The effect of 20% (vol/vol) neem leaf extracts on fungal growth and aflatoxin synthesis was determined after four days of fungal growth in the presence of the extract. Addition of extracts did not alter the pH profiles of the growth medium from that of controls during the four-day incubation. The results (Table 1) demonstrate that the neem leaf constituents do not affect fungal growth in submerged culture but do inhibit aflatoxin production by the mycelia. A gradual increase in inhibition of aflatoxin biosynthesis was observed with increasing concentration of neem leaf extracts (Table 2). Presence of 10% extract in the fungal growth medium was sufficient to obtain maximal (>98%) inhibition of toxin production. However, the inhibitory property was considerably reduced after heating the leaf extracts by either boiling the leaves in the buffer or autoclaving the leaf extracts (both blended and heat-extracted) (Tables 1 and 2). The observations suggested that the inhibitory factor(s) are unstable to heat and might be volatile. The differences in the volatile components of the blended and heat-extracted leaf formulations were, therefore, determined.

Effect of neem leaf volatiles on fungal growth and aflatoxin production on agar. The content of 10 of 13 major volatile components was significantly higher in the blended neem leaf extract than in the autoclaved heated extract. The major volatile component present, 3-methyl-2-buten-1-ol, was nearly 400-fold greater in the blended extract than in the heated extract. Observing these differences in the volatile components of the two extracts, the effect of neem leaf volatiles on fungal growth and aflatoxin production was investigated. The bioactivity of the neem leaf volatiles was assessed by measuring the fungal growth and aflatoxin production by the fungus grown on agar medium and exposed to an atmosphere containing volatiles from neem leaf extracts (Table 3). The ratio of neem extract to the agar medium was nearly 1:5, and the amount of inoculum added in the bioassay was in the same ratio as that added in submerged cultures. However, volatiles from blended neem leaf extracts did not affect either aflatoxin synthesis or fungal growth during a four-day incubation of the fungus on agar medium. The radial fungal growth was 3.2 ± 0.6 and 5.6 ± 1.5 cm in two and four days, respectively, for the controls and 3.4 ± 0.8 and 6.1 ± 1.9 cm for the same duration for the blended neem extract. The total aflatoxin content after four days of fungal growth was determined to be 21.3 ± 2.8 μg and 20.4 ± 4.0 μg for the control and neem extract treatment, respectively.

Localization of the inhibition of aflatoxin biosynthesis by neem leaf extracts. To elucidate the factor(s) responsible for the inhibition of aflatoxin synthesis

TABLE 2

Effect of Concentration of Neem Leaf Extract in the Incubation Medium on Aflatoxin B_1 Biosynthesis

Concentration of extract (vol/vol)	Aflatoxin B_1 (% of control)[a]			
	Blended extract	Blended extract (autoclaved)	Heat extracted	Heat extracted (autoclaved)
0	100[b]	100	100	100
1	15.1	48.9	52.3	60.2
5	6.5	36.2	35.2	43.4
10	2.6	24.6	18.7	35.2
20	2.0	17.8	9.2	29.8
50	1.8	16.3	6.4	31.2

[a]The pooled mean standard error in the results was ± 12.4% (n=3).
[b]100% refers to 20.6 μg aflatoxin B_1 produced/g mycelial dry weight.

TABLE 3

Ability of *A. parasiticus* Mycelia Grown in the Presence of Neem Extract to Utilize Aflatoxin Precursors

	Aflatoxin B_1 produced in LSRM (μg)[a]					
	Control mycelia			Neem-treated mycelia		
Incubation (hr)	No precursor	+20 μg AVN	+10 μg ST	No precursor	+20 μg AVN	+10 μg ST
0	0	0	0	0	0	0
24	4.8 ± 0.6	7.9 ± 1.0	9.6 ± 1.3	0.2 ± 0.06	3.5 ± 0.4	5.9 ± 0.3
48	6.3 ± 0.6	10.0 ± 0.8	12.6 ± 1.6	0.4 ± 0.10	4.2 ± 0.7	6.8 ± 0.4

[a]One-g fractions of wet 48-hr-old mycelia were obtained from fermentations carried out either in the absence (control mycelia) or presence (neem-treated mycelia) of 20% (vol/vol) of neem blended extract in GM. Mycelia were thoroughly washed in distilled water and transferred to 10 ml LSRM for assay. Secondary biosynthesis was determined by production of aflatoxin B_1 after addition of pathway precursors AVN (averantin) or ST (sterigmatocystin).

by neem leaf extracts, the effect of the aqueous leaf extracts on initiation/inhibition of secondary metabolism was investigated. The ability of resting mycelia to carry out secondary biosynthesis was used as a test system to monitor the effects of leaf extracts on the process. Mycelia were grown in GM containing 20% neem extract (Table 3) and the aflatoxin content measured after transferring the mycelia to a low sugar resting medium. The results demonstrated that aflatoxin biosynthesis was irreversibly inhibited in *A. parasiticus* mycelia by neem leaf constituents; removal of mycelia from exposure to leaf extracts did not restore aflatoxin synthesis. Enzymes required for aflatoxin biosynthesis were, however, apparently intact in the treated mycelia because the aflatoxin precursors [averantin and sterigmatocystin, (10)] fed to these mycelia were converted to aflatoxin B_2 at the same rate as control mycelia not exposed to leaf extracts (Table 3). Norsolorinic acid and averantin, both early precursors in aflatoxin biosynthesis (10), were not detected in mycelial extracts of treated mycelia, but the compounds were present in extracts of the control mycelia. Therefore, norsolorinic acid and averantin apparently were not synthesized in mycelia grown in the presence of neem extracts, whereas the enzymes required for the conversion of averantin and sterigmatocystin to aflatoxin B_1 were present in the mycelia (Table 3). Inhibition of aflatoxin biosynthesis by neem extracts in fungal cells appears to occur in the very early stages of the biosynthetic pathway because after the initiation of secondary metabolism, the inhibitory effect of the neem leaf consitutents was lost (Table 4). The enzymes involved in the latter stages of aflatoxin synthesis were not affected by the neem extracts.

In conclusion, nonvolatile neem leaf constituents irreversibly and almost totally inhibit aflatoxin biosynthesis in *A. parasiticus*, but they do not affect fungal growth. Inhibition of aflatoxin biosynthesis appeared to occur in the early stages of the biosynthetic pathway. If the inhibitory factor(s) could be effective in field studies, neem leaf extracts might be used in controlling the preharvest aflatoxin contamination of food and feed commodities.

TABLE 4

Direct Effect of Neem Leaf Extract on Aflatoxin B_1 Synthesis by Resting *A. parasiticus* Mycelia

	Aflatoxin B_1 produced (μg)[a]	
Incubation (hr)	Control mycelia	Neem-treated mycelia
2	2.7 ± 0.4	2.7 ± 0.4
24	5.8 ± 0.7	5.5 ± 0.6
48	7.1 ± 1.1	6.8 ± 1.0

[a]One-g fractions of wet 72-hr old mycelia were obtained from fermentations in GM and transferred to 10 ml LSRM containing either 0 (control mycelia) or 20% (neem-treated mycelia) vol/vol of neem-blended extract. Aflatoxin content was determined after 2, 24 and 48 hr of incubation.

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