Available online at www.sciencedirect.com
SCIENCE DIRECT®
Industrial Crops and Products 22 (2005) 101–107
INDUSTRIAL CROPS AND PRODUCTS
AN INTERNATIONAL JOURNAL
www.elsevier.com/locate/indcrop

Antifungal lignans from the creosotebush (*Larrea tridentata*)

I. Vargas-Arispuro[a,*], R. Reyes-Báez[a], G. Rivera-Castañeda[a], M.A. Martínez-Téllez[a], I. Rivero-Espejel[b]

[a] *Centro de Investigación en Alimentación y Desarrollo, A.C. Carr. a la Victoria, km 0.6, Apdo. Postal 1735, 83000 Hermosillo, Sonora, México*
[b] *Instituto Tecnológico de Tijuana, Apdo. Postal 1166, Tijuana, B.C. 22000, México*

Received 4 March 2004; accepted 16 June 2004

Abstract

Aspergillus flavus and *Aspergillus parasiticus* are preharvest pathogens of several important food crops, including maize. Both fungi produce aflatoxins, which are a potent hepatotoxin and carcinogen. The growth of *A. flavus* and *A. parasiticus* and subsequent aflatoxins contamination are sometime unavoidable, when the grains are grown under stressful conditions such as drought. The ideal control approach is prevention of fungus growth in the field. The objectives of this study were: (a) to evaluate the antifungal properties of *Larrea tridentata* extract against *A. flavus* and *A. parasiticus*; (b) to isolate and to identify the antifungal constituents from *L. tridentata*. Assay-guided chromatography of the *Larrea tridentata* extracts resulted in the isolation of two naturally occurring lignans, methyl-nordihydroguaiaretic acid (methyl-NDGA) and nordihydroguaiaretic acid (NDGA). Their chemical structures were identified by comparison of their physical and spectroscopic data (mp, ^{1}H NMR, ^{13}C NMR, and MS). The antifungal activity of the lignans was evaluated by radial growth inhibition assay against *A. flavus* and *A. parasiticus*. Methyl-NDGA was very effective in inhibiting mycelial growth of both fungi at 300 μg/ml, whereas 500 μg/ml of NDGA was necessary to completely inhibit the growth of the fungi. These compounds may have a biopesticidal potential as control agents for the aflatoxin fungi.

Keywords: *Larrea tridentata*; Antifungal activity; Biopesticides; *Aspergillus flavus*; *Aspergillus parasiticus*

1. Introduction

Preharvest contamination of corn (*Zea mays* L.) with aflatoxin, which are carcinogenic fungal secondary metabolites produced by *A. flavus* Link and *A. parasiticus* Speare, is a recurrent problem occurring in northwest Mexico and worldwide (Cleveland and Bhatnagar,

* Corresponding author. Tel.: +52 662 289 2400; fax: +52 662 280 0058.
E-mail address: iris@cascabel.ciad.mx (I. Vargas-Arispuro).

0926-6690/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.indcrop.2004.06.003

1992). Aflatoxins are recognized as potential human health hazard (Goldblatt, 1969). These compounds can cause mortality or reduced productivity in farm animals (Smith, 1985) and have been associated with increased incidence of liver cancer in humans (Hsieh, 1989; Cleveland and Bhatnagar, 1992).

Maize is an important crop in the grain and livestock economy of northwest México. The actual loss of grain yield due to these fungi is minimal, but fungal toxin contamination reduces the value of grain in both national and international markets (Nichols, 1983). Aflatoxins contamination has been treated as a post-harvest storage problem until *A. flavus* and *A. parasiticus* were found growing and producing aflatoxins in developing corn ears in the field (Anderson et al., 1975). Postharvest elimination of aflatoxins from corn kernels is not economically feasible (Lillehoj and Wall, 1987; Huang et al., 1997), and thus, research efforts are focused on control strategies, that prevent preharvest contamination. Among these strategies one is to develop fungicides from natural bioactive compounds for the effective control of aflatoxins in the field (Duvick et al., 1992; Huynh et al., 1992).

The objectives of this study were to evaluate and to identify the antifungal properties of *Larrea tridentata* extract against *A. flavus* and *A. parasiticus* as part of our project focused on the search for biopesticides agents from plant extracts.

2. Materials and methods

2.1. Plant materials

Leaves and stems of *Larrea tridentata* were collected from native populations of the Sonoran Desert and sun-dried for several weeks. A voucher specimen was deposited at the Herbarium of DICTUS, Universidad de Sonora (Mexico).

2.2. Fungal cultures

A. flavus Link (NRRL 55210) and *A. parasiticus* Speare (NRRL 2999), were isolated from corn. They were grown on a potato dextrose agar (PDA, Difco, Laboratories, Detroit, MI) in the dark at 28 °C for 7 days and stored as stock cultures in sterile mineral oil at −4 °C.

2.3. Extraction and isolation of the antifungal compound

Five hundred grams of both leaves and stems of *L. tridentata* were extracted by maceration in 1 l 70% ethanol (EtOH) and the slurry stored at room temperature for 7 days in darkness. Plant crude extracts were evaporated to dryness under reduced pressure and weighed (23 g). Dried plant crude extract (13 g) was suspended in water and partitioned with ethyl ether (Etet), ethyl acetate (EtOAc) and *n*-butanol (Bt). Each extract was concentrated in vacuum and weighed. The three type of extracts were evaluated for antifungal activity. The EtOAc extract (6.5 g) of leaves of *L. tridentata* was chromatographed on silica gel 60 (300 g) [particle size 0.3–0.63 mm (Merck, Darmstories, Germany)] and eluted with increasing polarities of chloroform:acetone (90:10 to 50:50, v/v) mixtures. The elution was monitored by thin layer chromatography (TLC) and antifungal assay. The fraction eluting with the chloroform:acetone (90:10 between 900 and 1250 ml) gave a fraction of 3.6 g with antifungal activity. This fraction was purified by high-performance liquid chromatography (HPLC, Waters 600E; Milford, USA) at 254 nm with a photodiode array detector HP-1100 (Hewlett-Packard). The column used was Lichrosphere ODS-C_{18} (10 mm × 250 mm, 5 μm, Merck) at room temperature. HPLC separations were done with a linear polarity gradient of water:methanol:acetonitrile (80:20:0 to 5:15:80, v/v) at a flow rate of 2 ml/min, for 130 min. Peaks were collected using a Gilson (Gilson Medical Electronics, Middleton, WI) fraction collector, and then concentrated under reduced pressure and weighed. The dried compound was stored in the freezer (−20 °C) for antifungal assay and spectral analysis. The compound was identified by comparison of their spectral data (UV, MS, ^{1}H NMR, ^{13}C NMR) with those shown in the literature (Domínguez, 1975; Konno et al., 1989; Gnabre et al., 1996).

2.4. Structure elucidation of antifungal compound

Nuclear magnetic resonance (NMR) spectra of the pure lignans was recorded on a Varian Unity-400 MHz (Varian Instrument, Germany). ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$. Chemical shifts are given in δ values (ppm) relative to the internal standard,

trimethylsilane. The mass spectral analyses (MS) were carried out on a HP-5988A quadrupole spectrometer, with electron impact at 70 eV by direct introduction of a small amount of product. The melting point of NDGA was obtained on a Melt-Temp Electrothermal 1001 (Lab-Tech, Mexico).

2.5. Antifungal assay

The effects of plant crude extracts, separates generated by fractionation, and pure compounds on colony growth of *A. flavus* and *A. parasiticus* were evaluated according to the procedure described by Vargas-Arispuro et al. (1999). Plant crude extracts and portions obtained from chromatographic separation were dissolved in 50 μl of acetone-water (50:50, v/v), pure compounds were dissolved in 25 μl. These solutions were then incorporated into 5 ml of PDA media at final concentrations of 500 μg/ml for crude extracts, 100, 50 μg/ml or collected fractions and 25 μg/ml for pure compounds. Three plates of solid PDA media containing the fractions or pure compounds at the specified concentrations were centrally point-inoculated with spores from 7-day-old cultures of *A. flavus* and *A. parasiticus*. Two control plates were included as blanks that contained 25 or 50 μl of solvent without extracted the compounds. Cultures were incubated in the dark at 28 °C. The colony diameter was measured daily. The percentage of growth inhibition was obtained by comparing the experimental with the control, which was considered to be 100% growth. The mean colony diameter and standard deviation (S.D.) of the mean values were calculated. Experimental set was run in triplicate.

3. Results

3.1. Fungitoxic activity of crude extracts

Crude extracts from the leaves and stems of *L. tridentata* showed moderate (between 50 and 90%) antifungal activity at 500 μg/ml (Table 1). The crude extract from the leaf exhibited more fungitoxicity against both fungi than the crude stem extract. This result suggests that the antifungal compounds are more concentrated in the leaves than the stems or that antifungal compounds in leaves have a higher toxicity potential against *A. flavus* and *A. parasiticus*. Similar fungitoxic activity of *L. tridentata* crude extracts had been reported by Hurtado et al. (1979).

Table 1
Antifungal activity of stems and leaves crude extracts from *Larrea tridentata* at 500 μg/ml against *A. flavus* and *A. parasiticus*

	Mycelial growth inhibition (%)	
	Stems	Leaves
A. flavus	56.11	86.5
A. parasiticus	53.37	68.7

3.2. Assay-guided fractionation of crude extract of leaves

The EtOAc extract (6.5 g) of crude extract of leaves prepared by solvent partition retained antifungal activity that inhibited the growth of both test fungi. Etet, Bt, and water fractions showed no antifungal activity against the evaluated fungi (data not shown). Based on this result, EtOAc extract was fractionated on column chromatography. The antifungal effects of the eight fractions that were eluted using chloroform:acetone (90:10, v/v) against *A. flavus* and *A. parasiticus* are listed in Table 2. Fraction number 4 showed the highest antifungal activity against both test fungi, and thus, the HPLC was selected to purify antifungal compounds.

The results of the analytic reverse phase HPLC separation of antifungal fraction are presented in Fig. 1. Fraction (F4/3) eluted in water:methanol:acetonitrile (40:35:25, v/v) and 78–92 min of retention time, retained antifungal activity, which was further purified following the HPLC separation.

Two pure compounds were isolated from antifungal fraction (F4/3) using fractionated crystallization with methanol:water system. One of them was isolated as yellow needles with 183.5–186 °C melting point, whereas the other compound separated as brown needles.

3.3. Structural identification

The structural identification of compounds was carried out by MS, ^{1}H NMR, and ^{13}C NMR. Spectroscopic pattern of lignans has been reported by different authors (Domínguez, 1975; Konno et al., 1989; Gnabre et al., 1996), and these characteristic patterns provide a useful diagnostic tool to elucidate the structural specificity of lignans. By comparing our data with those reported

Table 2
Antifungal effect of fractions generated by fractionation of EtOAc extract on silica gel-60 against *A. flavus* and *A. parasiticus* at 300 μg/ml

Fraction (number)	Colony diameter[a] (cm)		Inhibition[b] (%)	
	A. flavus	*A. parasiticus*	*A. flavus*	*A. parasiticus*
1	5.0 ± 0.1	5.0 ± 0.4	0	0
2	3.75 ± 0.05	4.1 ± 1.1	28	19
3	4.0 ± 0.2	4.28 ± 0.6	22	14
4	0.71 ± 0.04	1.75 ± 0.1	86	65
5	3.76 ± 0.4	3.06 ± 0.09	25	36
6	3.90 ± 0.09	4.1 ± 0.1	22	19
7	4.25 ± 0.1	5.0 ± 0.9	16	0
8	5.0 ± 0.05	5.0 ± 0.7	0	0
Control	5.0 ± 0.02	5.0 ± 0.03	0	0

[a] Mean values of three replicated of colony diameter ± S.D.
[b] Percentage of colony growth inhibition respective to the control (100% of growth with no extract added).

in the literature, we identified two structurally related lignans. The compound isolated as yellow needles was identified as nordihydroguaiaretic acid (NDGA), 1,4-bis(3-4-dihydroxiphenyl)-2,3-dimethylbutane; and the compound isolated as brown needles was identified as 3′ methyl nordihydroguaiaretic acid (methyl-NDGA) (Perry et al., 1972; Hwu et al., 1998).

3.4. Antifungal activity

The in vitro antifungal effect of pure lignans against *A. flavus* and *A. parasiticus* was shown in Fig. 2. As the concentration of lignan increased, colony diameter decreased for NDGA. At 300 and 500 μg/ml, the NDGA gave 81.92 and 100% inhibition of growth against *A. flavus* and 82.17 and 100% against *A. parasiticus*. Because we had a low recovery of methyl-NDGA, it was evaluated at a concentration of 300 μg/ml. At this concentration, methyl-NDGA completely inhibited growth of both the test fungi. The NDGA and the methyl-NDGA had the same level of antifungal activity over the initial 48 h of incubation, but after that incubation time, methyl-NDGA had a major inhibition effect on fungal growth. Our data show that the most promising antifungal agent for the control of *A. flavus* and *A. parasiticus* was methyl-NDGA, as judged by its consistency in completely inhibiting the growth of fungi.

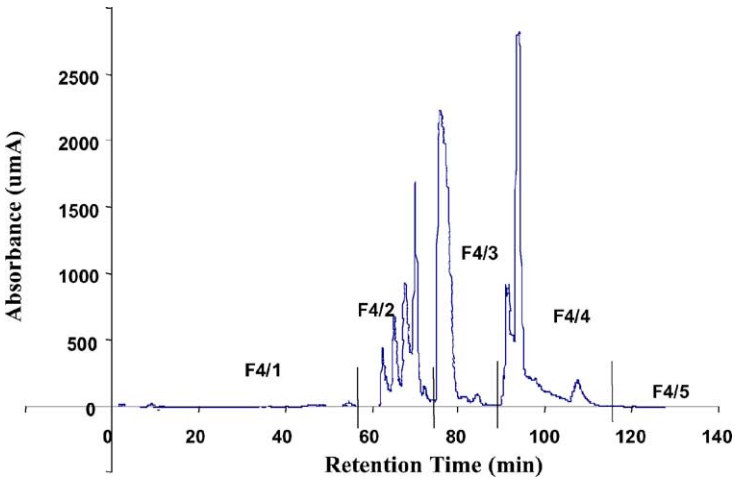

Fig. 1. Chromatogram of high-performance liquid chromatography (HPLC) of EtOAc antifungal fraction. Experimental conditions: semipreparative column Licosphere ODS-C_{18}; photodiode array detector; solvent system water:methanol:acetonitrile; flow rate 2 ml/min.

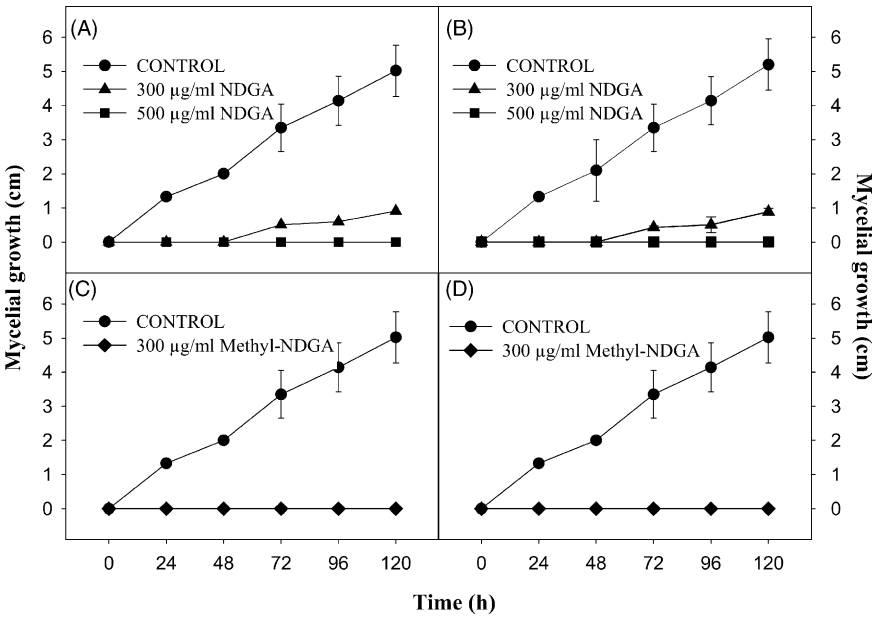

Fig. 2. Effects of NDGA and methyl-NDGA on in vitro mycelia growth of *A. flavus* (A and C) and *A. parasiticus* (B and D). Values shown are the means of three experiments ± S.D.

4. Discussion

Preharvest corn grain colonization by *A. flavus* and *A. parasiticus*, and subsequent aflatoxin contamination is a serious problem that negatively affects humans and animals health (Guo et al., 1998). Several attempts to identify inhibitory compounds have been made to control aflatoxins biosynthesis and fungal growth of aflatoxigenic fungi. The purpose of using these compounds in the field was to control aflatoxin contamination (Duvick et al., 1992; Neucere et al., 1995; Huang et al., 1997). Norton (1995) reported nonpolar compounds from corn kernels to be inhibitory to aflatoxin accumulation and fungal growth, and Duvick et al. (1992) identified small basic peptides from corn kernels with novel antimicrobial activities. Also, Nagarajan and Bhat (1972) found that the extract from opaque-2 corn line almost completely inhibited aflatoxins formation. However, no further results have been reported on the isolation and characterization of these active compounds.

We report here the isolation of NDGA and methyl-NDGA from leaf extract of *L. tridentata*. Both compounds inhibit fungal growth of *A. flavus* and *A. parasiticus*. Our data indicate that methyl-NDGA is a more potent antifungal agent than NDGA. Lignans exhibit a wide range of biological properties including antimicrobial, antitumorigenic, antiviral activities, and inhibition of many enzymes systems (Ayres and Loike, 1990; Gnabre et al., 1996). The antifungal mechanism of lignans has not been clearly demonstrated. However, some authors concluded that a biologically active compound such as a fungicide must first diffuse from the site of application to the site of action where it can exert its toxicity (Laks and Pruner, 1989; Hwu et al., 1998). Overton's rule indicates that the permeability of a compound is roughly proportional to its lipid solubility (Petty, 1993). A molecular mechanism of action of methyl-NDGA against HIV virus has been elucidated by Gnabre et al. (1995). The lignan interferes with the binding of the Sp1 protein to Sp1 site in HIV-LTR promoter that leads to a blockade of the HIV transcription mechanism, and thus, the suppression of HIV replication. Our results show that the methylated-NDGA, the most lipophilic compound, inhibited the fungal growth of both test fungi at lower concentrations than the hydrophilic NDGA. This result agrees with these reported by Craigo et al. (2000) and Chen et al. (1998). They found that the lipophilic-NDGA derivatives were much more active in inhibiting human papillomavirus

and herpes simplex virus than the hydrophilic structure. Results from some of toxicity studies in humans and mice support the nontoxicity nature of this class of compounds at 300 mg/kg (Chen et al., 1998; Heron and Yarnell, 2001). Lignans with methylated catecholic hydroxyl groups, such as methyl-NDGA, can be produced in large quantities at a low cost (Hwu et al., 1998). This indicates that this agent may well be used as an agricultural fungicide with a reasonable safety margin to inhibit *A. flavus* and *A. parasiticus*.

Acknowledgment

We are grateful to Dr. Susan Lurie of the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel, for the critical manuscript review. We also thank Socorro Vallejo for her excellent technical assistance. This work was supported by a grant from Consejo Nacional de Ciencia y Tecnología (CONACyT) of Mexico (grant no. 3931P-B9608).

References

Anderson, H., Nehring, E., Wichser, R., 1975. Aflatoxin contamination of corn in the field. Agric. Food Chem. 23, 775–782.

Ayres, D.C., Loike, D.J., 1990. Chemistry and pharmacology of natural products. In: Ayres, D.C., Loike, D.J. (Eds.), Lignans: Chemical, Biological and Clinical Properties. Cambridge University Press, New York, p. 85 (Chapter 3).

Cleveland, T.E., Bhatnagar, D., 1992. Molecular strategies for reducing aflatoxins levels in crops before harvest. In: Bhatnagar, D., Cleveland, T. (Eds.), Molecular Approaches to Improving Food Quality and Safety. Van Nostrand Reinhold, New York, pp. 205–222.

Craigo, J., Callahan, M., Huang, R., DeLucia, A., 2000. Inhibition of human papillomavirus type 16 gene expresión by nordihydroguaiaretic acid plant lignan derivatives. Antiviral Res. 47, 19–28.

Chen, H., Teng, L., Li, J., Park, R., Mold, D., Gnabre, J., Hwu, R., Tseng, W., Huang, R., 1998. Antiviral activities of methylated nordihydroguaiaretic acid. 2. Targeting herpes simplex virus replication by the mutation insensitive transcription inhibitor tetra *O*-methyl-NDGA. J. Med. Chem. 41 (16), 3001–3007.

Domínguez, X.A., 1975. Lignanos en Métodos de Investigación Fitoquímica. Editorial Limusa. México, pp. 127–137 (Chapter 9).

Duvick, J., Rood, T., Rao, A., Marshak, D., 1992. Purification and characterization of novel antimicrobial peptide from maize kernels. J. Biol. Chem. 267, 1814–1820.

Gnabre, J.N., Huang, R., Bates, R., Burns, J., Calder, S., Malcomson, M., McClure, K., 1995. Characterization of anti-HIV lignans from *Larrea tridentata*. Tetrahedron 51, 12203–12209.

Gnabre, J.N., Ito, Y., Ma, Y., Huang, R., 1996. Isolation of anti-HIV-1 lignans from *Larrea tridentata* by counter-current chromatography. J. Chromatogr. A 719, 353–364.

Goldblatt, L.A., 1969. Aflatoxin: scientific background. Control and Implications. Academic Press, NY, pp. 79–110.

Guo, Z., Brown, R., Lax, R., Cleveland, T., Russin, J., Widstrom, N., 1998. Protein profiles and antifungal activities of kernel extracts from corn genotypes resistant and susceptible to *Aspergillus flavus*. J. Food Protect. 61 (1), 98–102.

Heron, S., Yarnell, E., 2001. The safety of low-dose *Larrea tridentata* (DC) coville (Creosote Bush or Chaparral): a retrospective clinical study. J. Alternat. Complement. Med. 7 (2), 175–185.

Hsieh, D.P., 1989. Potential human health hazards of micotoxins. In: Natori, S., Hashimoto, K., Ueno, Y. (Eds.), Mycotoxins and Phycotoxins. Elsevier, Biomedical Press, Amsterdam, pp. 69–80.

Huang, Z., White, D., Payne, G., 1997. Corn seed proteins inhibitory to *Aspergillus flavus* and aflatoxins biosynthesis. Phytopathology 87 (6), 622–627.

Hurtado, L., Hernandez, R., Hernandez, F., Fernandez, S., 1979. Fungi-toxic compounds in the larrea resin. In: Campos, E., Mabry, T., Fernandez, T. (Eds.), Larrea. CIQA, Saltillo, México, pp. 327–341.

Huynh, K., Hironaka, M., Levine, C., Smith, C., Borgemeyer, R., Shah, D., 1992. Antifungal proteins from plants: purification, molecular cloning and antifungal properties of chitinases from maize seed. J. Biol. Chem. 267, 6635–6640.

Hwu, R., Tseng, W., Gnabre, J., Giza, P., Huang, R., 1998. Antiviral activities of methylated nordihydroguaiaretic acid. 1. Synthesis, structure identification, and inhibition of tat-regulated HIV transactivation. J. Med. Chem. 41 (16), 2994–3000.

Konno, C., Xue, H., Lu, Z., Ma, B., Erdelmeier, C., Che, C., Cordell, G., Soejarto, D., Walle, D., Fong, H., 1989. 1-Aryl tetralin lignans from *Larrea tridentata*. Nat. Prod. 52 (5), 1113–1117.

Laks, P., Pruner, M., 1989. Flavonoid biocides: structure/activity relations of flavonoid phytoalexin analogues. Phytochemistry 28 (1), 87–91.

Lillehoj, B., Wall, H., 1987. Decontamination of aflatoxins-contaminated maize grain. In: M.S. Zuber, E., Lillehoj, B., Renfro, L. (Eds.), Proceedings of the Workshop on Aflatoxin in Maize. CIMMYT, México, D.F., pp. 260–279.

Nagarajan, V., Bhat, R., 1972. Factors responsible for varietal differences in aflatoxins production in maize. J. Agric. Food Chem. 20, 911–914.

Neucere, J.N., Brown, R.L., Cleveland, T.E., 1995. Correlation of antifungal properties and β-1-3-glucanases in aqueous extracts of kernels from several varieties of corn. J. Agric. Food Chem. 43, 275–276.

Nichols, T.E., 1983. Economic impact of aflatoxins in corn. In: Diener, L., Asquith, R., Dickens, J. (Eds.), Aflatoxin and *Aspergillus flavus* in Corn. South. Coop. Serv. Bull., vol. 279. Alabama Agricultural Experimental Station, Auburn, AL, pp. 67–71.

Norton, R.A., 1995. Effect of selected corn metabolites on growth and aflatoxins production by *Aspergillus flavus*. In: Abstracts of

8th Aflatoxins Elimination Workshop. USDA Agric. Res. Serv., Atlanta, GA, pp. 25–26.

Perry, C., Kalnins, W., Deitcher, K., 1972. Synthesis of lignans. Nordihydroguaiaretic acid. J. Org. Chem. 37, 4371–4382.

Petty, H.R., 1993. Molecular Biology of Membranes. Plenum Press, NY (Chapter 6).

Smith, J.E., 1985. Mycotoxins: Formation Analyses and Significance. Wiley, Chichester, NY.

Vargas-Arispuro, I., Sanz, I., Moya, P., Primo-Yúfera, E., 1999. Antimicrobial compounds in the non-volatile fraction of expressed orange essential oil. J. Food Protect. 62 (8), 929–932.