SUPPLEMENTARY MATERIAL

GC-EIMS Analysis, Antifungal and Anti-aflatoxigenic Activity of *Capsicum chinense* and *Piper nigrum* Fruits and Their Bioactive Compounds Capsaicin and Piperine upon *Aspergillus parasiticus*

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Abstract

GC-EIMS analysis, antifungal- and anti-aflatoxigenic activities of the ethanolic extract of *Capsicum chinense* and *Piper nigrum* fruits and their main bioactive compounds were evaluated upon *Aspergillus parasiticus*. The GC-EIMS analysis showed capsaicin (50.49%) and piperine (95.94%) as the major constituents in *C. chinense* and *P. nigrum*, respectively. MIC_{50} values revealed that capsaicin (39 μg/mL) and piperine (67 μg/mL) required lower concentration unlike fruit extracts of *C. chinense* (381 μg/mL) and *P. nigrum* (68 μg/mL) to inhibit the 50% of *A. parasiticus* growth. Extracts and compounds showed anti-aflatoxigenic activity as aflatoxin biosynthesis inhibition. Maximum aflatoxin inhibition occurred at 150 μg/mL of extracts and compounds. The present study showed satisfactory results concerning the effects of ethanolic

extract of *C. chinense* and *P. nigrum* fruits upon *A. parasiticus*, showing the capabilities of inhibiting fungal growth development and altering aflatoxins production.

Keywords: Antifungal activity; anti-aflatoxigenic activity; *Aspergillus parasiticus;* capsaicin; *Capsicum chinense* fruits*; Piper nigrum* fruits; piperine.

Experimental

Plant material and standards

Capsicum chinense and *Piper nigrum* fruits were purchased in a local supermarket. Identification of the plant species was carried out at the Herbarium of Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional (CIIDIR), of the Instituto Politécnico Nacional de Durango in Durango, Durango (México), and a voucher specimen of each plant was deposited there (*Capsicum chinense* 51645, *Piper nigrum* 51646). Capsaicin (8-methyl-N-vanillyl-6-noneamide cat. M-8147) and piperine (1-piperoylpiperidine cat. P-49007) were purchased in Sigma-Aldrich (St. Louis, MO). All other chemicals used were of analytical grade.

Preparation of ethanolic fruit extracts

C. chinense fruits were oven-dried at 45 ºC for 24 h. The dried fruits from *C. chinense* and *P. nigrum* were mill ground until to pass through an 80-mesh sieve. Ethanolic fruit extracts were obtained following the method described by Molina-Torres et al. (2004) with some modifications. The extracts were prepared at a ratio of 1:5 (powder/solvent) with absolute ethanol. The mixtures were stirred for 1 h and stored at room temperature for 1 week under dark conditions. After that, the extracts were filtered through Whatman filter paper No. 1; the filtrates obtained were concentrated to complete dryness under vacuum at 45 °C and 175 mBar. Finally, the solids were re-suspended in absolute ethanol at concentration of 1 g/mL.

GC-EIMS analysis of the ethanolic fruit extracts

C. chinense and *P. nigrum* extracts were phytochemical analyzed by gas chromatography-electron impact mass spectrometry (GC-EIMS) following the method described by Molina-Torres et al. (2004). The data obtained was collected with the software MassHunter Qualitative Analysis Version B.08.00 (Agilent Technologies, Inc., La Jolla, CA). Retention indices (RI) and mass spectrum were determined with the Automated Mass Spectral Deconvolution and Identification System (AMDIS) software. Compounds identification was

based on the comparison of the RI and mass spectra fragmentation patterns with library software and database NIST MS Search (National Institute of Standards and Technology Database, 2011 Mass Spectral Search Program) version 2.0 (Mell and Grance 2011).

Determination of capsaicin and piperine content

Capsaicin and piperine contents were determined by GC-EIMS analysis under identical conditions as above (Molina-Torres et al. 2004). A stock solution of each compounds was prepared at a concentration of 0.05 g/mL with absolute ethanol. From this stock, four 1 mL solutions were prepared at 0.1, 0.3, 1.0 and 3.0 mg/mL as a standard curve. Quantification was performed from integrated peak area measurements observed in the chromatograms. Finally, the standard curves were produced using linear regressions (R^2=0.9903 for capsaicin and R^2=0.9925 to piperine).

Antifungal activity

The antifungal activity of both extracts and their main bioactive compounds against *Aspergillus parasiticus* (ATCC 16992) strain (1×10^5 spores mL^{-1}) was evaluated by determination of the radial growth inhibition (Rosas-Burgos et al. 2009; 2011). Petri dishes of PDA medium containing the extracts or bioactive compounds at 50, 75, 150 or 200 µg/mL were employed. The results were expressed as percentage (%) calculated following the equation 1 (Plascencia-Jatomea et al. 2003).

$$\text{Radial growth inhibition } (\%) = [(Rc - Ri)/Rc] \text{ x } 100 \qquad (1)$$

Where: *Rc*, is the value of colony radius in the control and *Ri*, the colony radius value of colonies grown in presence of the antifungal extract/compound.

The percentage of radial growth inhibition was determined to each extract and compound with the aim to establish the minimal concentration that inhibited 50% (MIC_{50}) of the fungus growth using probit analysis.

Kinetics of spore germination

Fruit extracts and their bioactive compounds at 200 µg/mL was employed to evaluate the effect on spore germination rate following the method reported by Paul et al. (1993) and Larralde et al. (1997). At this concentration, the higher antifungal activities of the extracts and compounds against *A. parasiticus* were obtained. The inhibition of spore germination was calculated

following the equation 2 (Rosas-Burgos et al. 2011). Spore counting was carried out using the Image-Pro Plus version 6.3 software (Media Cybernetics, Inc., Bethesda, MD).

$$\text{Germination inhibition } (\%) = [(Sc - Si)/Sc] \text{ x } 100 \qquad (2)$$

Where: *Sc:* is the number of germinated spores in the control and *Si,* the number of germinated spores presence of the antifungal extract/compound.

Total aflatoxins production

The antifungal extracts and their main bioactive compounds at 150 and 200 µg/mL, were used to evaluate their effects on total aflatoxins production in white maize grains inoculated with *A. parasiticus*. Mycotoxins production was induced according to method reported by Castellá et al. (1999) and most recently by Rosas-Burgos et al. (2011). Separation, purification and quantification of total aflatoxin was carried out by the VICAM procedure (Cota-Arriola et al., 2011).

Statistical analysis

Results were presented as mean value ± standard error of three replicates. Analysis was carried out by one-way ANOVA and Tukey test using $p \leq 0.05$. IBM SPSS Statistic 21 program was employed.

References

Castellá G, Bragulat MR, Cabañes FJ. 1999. Fumonisin production by *Fusarium* species isolated from cereals and feeds in Spain. J Food Prot. 62(7):811-813.

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Larralde CC, López LF, Viniegra GG. 1997. Morphometry evaluation of the specific growth rate of *Aspergillus niger* grown on agar plates at high glucose levels. Biotechnol Bioeng. 56(3):287-294.

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Table S1 GC-EIMS analysis of capsaicinoids and piperamides in the ethanolic extracts of *Capsicum chinense* and *Piper nigrum.*

Molina-Torres J, Salazar-Cabrera CJ, Armenta-Salinas C, Ramírez-Chávez E. 2004. Fungistatic and bacteriostatic activity of alkamides from *Heliopsis longipes* roots: affinin and reduced amides. J Agric Food Chem. 52(15):4700-4704.

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Rosas-Burgos EC, Cortez-Rocha MO, Plascencia-Jatomea M, Cinco-Moroyoqui FJ, Robles-Zepeda RE, López-Cervantes J, Sánchez-Machado DI, Lares-Villa F. 2011. The effect of *Baccharis glutinosa* extract on the growth of mycotoxigenic fungi and fumonisin B_1 and aflatoxin B_1 production. World J Microbiol Biotechnol. 27(5):1025-1033.

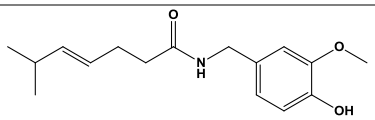
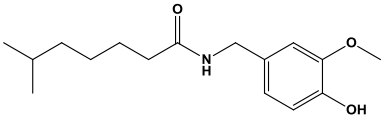
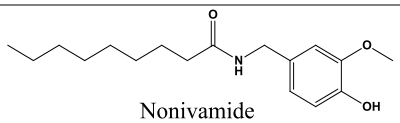
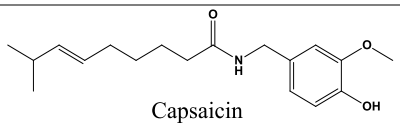
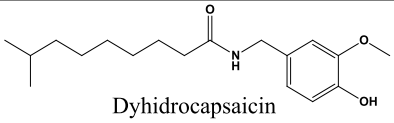
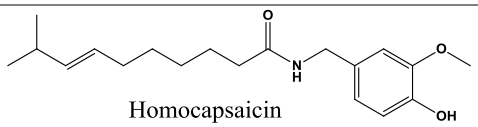
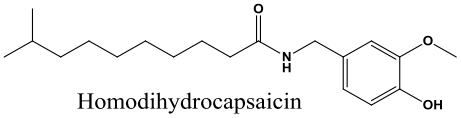
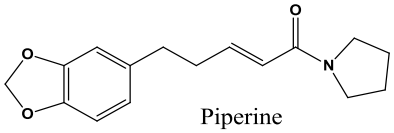
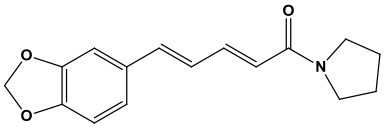
Compound structure	Molecular Ion [M+]	Molecular formula	Fragmentation patters m/z
***Capsicum chinense* fruits**			
	277	$C_{16}H_{23}NO_3$	137(999), 138(94), 277(79), 122(60), 59(51), 152(45), 109(28), 135(27), 58(22), 195(22).
	279	$C_{16}H_{25}NO_3$	137(999), 279(255), 138(137), 151(135), 152(132), 195(107), 122(86), 136(63), 57(55), 280(46).
 Nonivamide	293	$C_{17}H_{27}NO_3$.	137(999), 293(203), 138(142), 151(133), 152(123), 195(109), 122(87), 136(59), 55(42), 94(39).
 Capsaicin	305	$C_{18}H_{27}NO_3$	137(999), 138(138), 152 (137), 151 (93), 122(85), 305(54), 55(58), 109(53), 41(52), 94(50).
 Dyhidrocapsaicin	307	$C_{18}H_{29}NO_3$	137(999), 307(997), 151(649), 195(616), 138(570), 152(529), 122(333), 308(204), 153(163), 94(131).
 Homocapsaicin	319	$C_{19}H_{29}NO_3$	137(999), 138(127), 152(101), 55(94), 319(81), 122(66), 151(50), 195(39), 94(38), 67(32).
 Homodihydrocapsaicin	321	$C_{19}H_{27}NO_3$	137(999), 321(182), 138(134), 151(120), 152(110), 195(105), 122(73), 57(64), 136(60), 55(51).
***Piper nigrum* fruits**			
 Piperine	273	$C_{16}H_{19}NO_3$	115(999), 201(963) 173(691), 273(646), 143(334), 172(296) 171(260), 174(241), 216(221), 116(189).
 Trichostachin	271	$C_{16}H_{17}NO_3$	201(999), 115(938), 271(565), 173(370), 143(285), 202(248), 171(229), 200(220), 172(210), 116(168).

Table S2 Effect of *Capsicum chinense* and *Piper nigrum* extracts and capsaicin and piperine, upon *Aspergillus parasiticus* growth [a, b]

Treatment	Mean colony diameter (cm)	Growth (%)	Radial growth inhibition (%)
PDA	1.80 ± 0.41 [a]	100.00 ± 0.41 [a]	0 .00 ± 0.41 [l]

PDA+ Solvent	1.75 ± 0.56 [a]	97.22 ± 0.56 [a]	2.78 ± 0.06 [l]
Capsicum chinense			
50 µg/mL	1.37 ± 0.03 [b]	76.11 ± 0.03 [b]	23.89 ± 0.03 [k]
75 µg/mL	1.30 ± 0.10 [c]	72.22 ± 0.10 [c]	27.78 ± 0.10 [j]
150 µg/mL	1.20 ± 0.60 [d]	66.67 ± 0.60 [d]	33.33 ± 0.60 [i]
200 µg/mL	1.00 ± 0.90 [e]	55.56 ± 0.90 [e]	44.44 ± 0.90 [h]
Capsaicin			
50 µg/mL	0.90 ± 0.00 [f]	51.43 ± 0.0 [f]	51.00 ± 0.00 [g]
75 µg/mL	0.87 ± 0.03 [g]	49.53 ± 0.03 [g]	56.00 ± 0.03 [f]
150 µg/mL	0.77 ± 0.03 [h, i]	43.81 ± 0.03 [h, g]	59.00 ± 0.03 [d, e]
200 µg/mL	0.67 ± 0.03 [j]	38.10 ± 0.03 [j]	61.90 ± 0.03 [c]
Piper nigrum			
50 µg/mL	1.03 ± 0.03 [e]	57.22 ± 0.03 [e]	42.78 ± 0.03 [h]
75 µg/mL	0.90 ± 0.85 [f]	50.00 ± 0.85 [f]	50.00 ± 0.85 [g]
150 µg/mL	0.50 ± 0.02 [k]	27.78 ± 0.02 [k]	72.72 ± 0.02 [b]
200 µg/mL	0.40 ± 0.70 [l]	22.22 ± 0.70 [l]	77.78 ± 0.70 [a]
Piperine			
50 µg/mL	0.90 ± 0.08 [f]	51.50 ± 0.08 [f]	48.57 ± 0.08
75 µg/mL	0.83 ± 0.08 [h, g]	47.62 ± 0.08 [h, g]	49.00 ± 0.08 [e, f]
150 µg/mL	0.80 ± 0.04 [h, g]	45.71 ± 0.04 [h, g]	58.00 ± 0.04 [e, f]
200 µg/mL	0.73 ± 0.03 [i]	41.90 ± 0.03 [i]	59.00 ± 0.03 [d]

[a] Values are the average of three replicates ± standard error.

[b] Values with different letter are significantly different ($p < 0.05$).

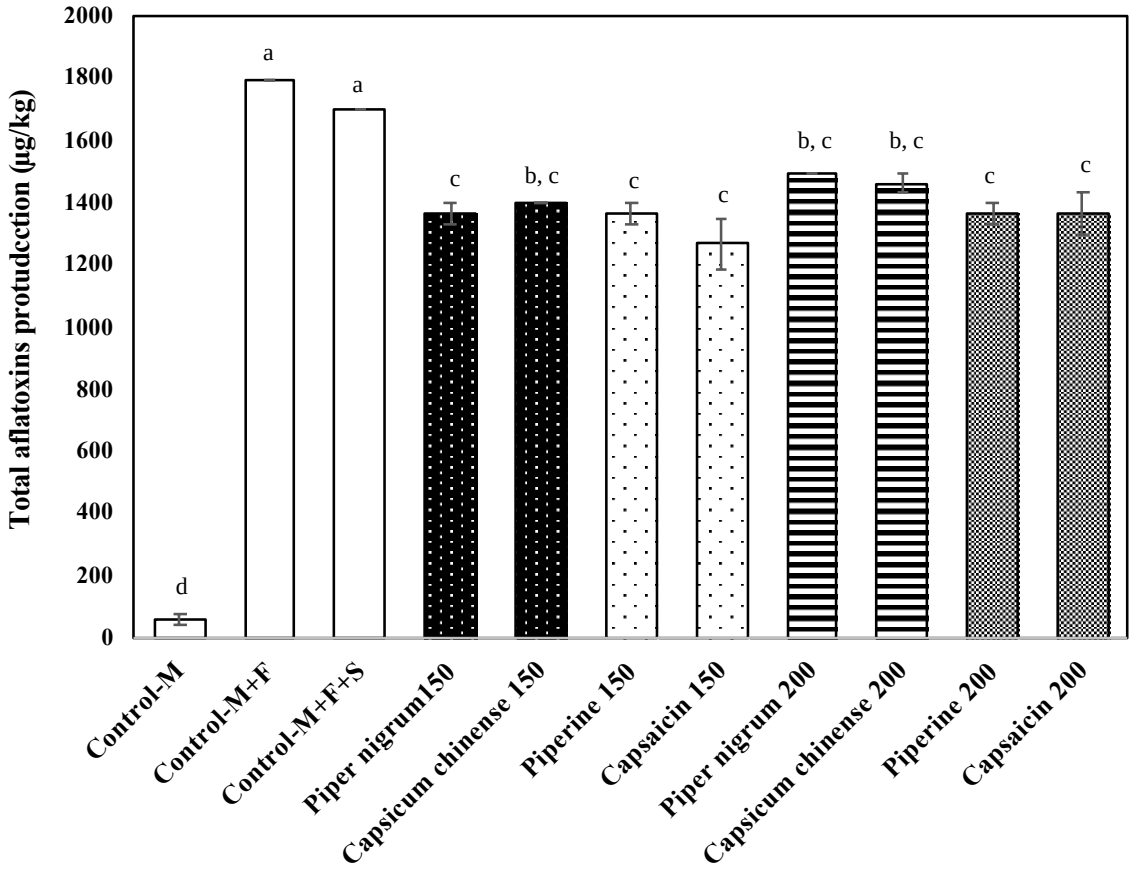


Figure S1 Effect of *Capsicum chinense* and *Piper nigrum* extract and capsaicin and piperine at 150 and 200 µg/mL on total aflatoxins production by *Aspergillus parasiticus*.

*Controls: M= maize grains, M+F= maize grains inoculated with fungus, M+S+F= maize grains plus solvent and inoculated with fungus. Error bars represent standard error for n= 3. Bars followed with different letter are significantly different ($p \leq 0.05$).