Short note [Nota corta]

GARLIC (*Allium sativum* L.) ESSENTIAL OIL AGAINST GROWTH AND AFLATOXIN PRODUCTION OF *Aspergillus parasiticus* †

[ACEITE ESENCIAL DE AJO CONTRA EL CRECIMIENTO Y PRODUCCIÓN DE AFLATOXINA DE *Aspergillus parasiticus*]

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SUMMARY

Background: *Aspergillus parasiticus* is a mold to produce aflatoxins, and growth in maize; the used synthetic fungicides for their inhibition are risk to health and the environment. However, garlic essential oil to have antifungal activity and can be alternative for control *A. parasiticus*. **Objective/hypothesis:** Essential garlic oil can inhibit the *in vitro* growth of *A. parasiticus* and its aflatoxins. **Methodology:** The *in vitro* antifungal activity of garlic (*Allium sativum* L.) essential oil was evaluated against *A. parasiticus* (radial growth and spore germination). In addition, the mean inhibitory concentrations (IC_{50}) and minimum inhibitory (MIC) were calculated. Aflatoxins were tested on maize with High-Performance Thin-Layer Chromatography (HPTLC). **Results:** The mean inhibitory concentration (IC_{50}) and minimum inhibitory (MIC) obtained by Probit analyses with radial growth were 971 and 2875 ppm of garlic essential oil, respectively, in *A. parasiticus*. These concentrations inhibited spore germination until 24 h. Starting from 100 ppm of garlic essential oil, mycelial inhibition was shown. Evidence of inhibition of aflatoxin production by *A. parasiticus* in maize was not possible at concentrations of 971 and 2875 ppm garlic essential oil. **Implications:** The concentrations 971 and 2875 ppm of garlic essential oil inhibited the growth *in vitro* of *A. parasiticus*. However, they did not inhibit aflatoxin production by *A. parasiticus* into maize. **Conclusion:** Garlic essential oil inhibit the *in vitro* growth of *A. parasiticus* on growth radial and germination spores; but not inhibit aflatoxins production on maize to concentrations 971 and 2875 ppm of garlic essential oil.

Key words: *Aspergillus parasiticus*; *Allium sativum* L.; radial growth; spore germination; mycotoxins.

RESUMEN

Antecedentes: *Aspergillus parasiticus* es un hongo que produce aflatoxinas y crece en el maíz; los fungicidas sintéticos utilizados para su inhibición son un riesgo para la salud y el medio ambiente. Sin embargo, el aceite esencial de ajo tiene actividad antifúngica y puede ser una alternativa para el control del *A. parasiticus.* **Objetivo / hipótesis:** El aceite esencial de ajo puede inhibir el crecimiento *in vitro* de *A. parasiticus* y sus aflatoxinas. **Metodología:** La actividad antifúngica *in vitro* del aceite esencial de ajo (*Allium sativum* L.) fue evaluada contra *Aspergillus parasiticus* (crecimiento radial y germinación de esporas). Adicionalmente, la producción de aflatoxinas fue calculada en maíz con cromatografía en capa fina de alta resolución (HPTLC). **Resultados:** Las concentraciones inhibitoria media (IC_{50}) y mínima inhibitoria (MIC) obtenidas mediante análisis Probit con crecimiento radial fueron 971 y 2875 ppm de aceite esencial de ajo, respectivamente en *A. parasiticus*. Estas concentraciones inhibieron la germinación de esporas hasta las 24 h. A partir de 100 ppm de aceite esencial de ajo, se observó inhibición del micelio. No se encontró evidencia de inhibición de la producción de aflatoxinas por *A. parasiticus* en el maíz a concentraciones de 971 y 2875 ppm de aceite esencial de ajo. **Implicaciones:** Las concentraciones de 971 y 2875 ppm de aceite esencial de ajo inhibieron el crecimiento *in vitro* de *A. parasiticus*. Sin embargo, no inhibieron la producción de aflatoxinas de *A. parasiticus* en el maíz. **Conclusión:** El aceite esencial de ajo inhibe el crecimiento *in vitro* del *A. parasiticus* en crecimiento radial y germinación de esporas; pero no inhibe la producción de aflatoxinas en el maíz a concentraciones de 971 y 2875 ppm de aceite esencial de ajo.

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Palabras clave: *Aspergillus parasiticus*; *Allium sativum* L.; crecimiento radial; germinación de espora; micotoxinas.

INTRODUCTION

One of the factors that affects maize (*Zea mays* L.) quality is fungi contamination. Some fungal species produce aflatoxins, biologically active compounds toxic for animals and humans; the most relevant are *Aspergillus flavus* and *A. parasiticus* (Paster et al., 1995; Klich, 2007; Baker and Bennett, 2008; Chulze, 2010). The control methods based on synthetic fungicides have been insufficient, due to the risks associated with health and the environment (Campbell and López-Ortiz, 2014). However, some volatile compounds of plants can be an alternative to synthetic fungicides because they have antifungal activity (Kedia et al., 2016) and inhibit production aflatoxin (El-Habib, 2012). For example, garlic (*Allium sativa* L.) is one plant used word-wide; it has volatile compounds with a broad antimicrobial spectrum (Benkeblia, 2004; Bayan et al., 2014). The antifungal properties of garlic essential oil have been showed in several studies against *A. pasasiticus*, *A. niger*, *Penicillium cyclopium*, *Fusarium oxysporum*, *Penicillium funiculosum* and *Alternaria tenuissima* (Gowda et al., 2004; Li et al., 2014; Muy-Rangel et al., 2017). In addition, Gowda et al. (2004) previously reported that garlic essential oil reduced aflatoxin production in mixed feed. For that reason, essential garlic oil can inhibit the *in vitro* growth of *A. parasiticus* and its aflatoxins production on maize. The goal of this study is to evaluate the effect of garlic essential oil on the *in vitro* growth of *A. parasiticus* and its aflatoxins production on maize.

MATERIAL AND METHODS

Aspergillus parasiticus (ATCC 16992) culture was grown on potato dextrose agar (PDA, BD Bioxon®) medium for seven days at 27 °C. Afterwards, spores were suspended in sterile Tween 20 (0.1 % v v^{-1}), and spore concentration was determined using a cell-counting hematocytometer.

Radial growth

Antifungal activity of garlic (*Allium sativum* L.) essential oil (Sigma-Aldrich®, lot MKBB8390V; characterized by Muy-Rangel et al., 2017) was determined by the poisoned food technique (Balouiri et al., 2016). Essential oil was dissolved in sterile PDA-Tween® 80 (1 % v v^{-1}) at ≈ 45 °C to obtain different concentrations (10, 100, 500, 1000 and 10,000 ppm). The culture medium was poured into Petri plates (5 cm ø) and cooled to solidify. Next, a hole in the center of the solidified cultured media was prepared using a sterile Pasteur pipette. The hole without medium was inoculated with 10^5 spores of *A. parasiticus* (25 µL). Also, PDA and PDA-Tween® 80 without essential oil (controls) was prepared. Inoculated plates were place in incubation at 27 °C. Radial growth technique was used to determinate the minimum inhibitory concentration (MIC) and the mean inhibitory concentration (IC_{50}). Briefly, the radial growth of each colony was obtained by measuring manually every 24 hours until 96 h. The test Probit analysis was used to calculate MIC and IC_{50} with the NCSS Data Analysis vs. 12 software. (Number Cruncher Statistical Systems, Utah, USA). For this study, MIC and IC_{50} were calculated from radial growth technique because inoculum was of spores, and the lag period of colony growth coincided with start and completion of the spore germination process (Dantigny et al., 2002).

Spore germination

Sterile PDA-Tween® 80 and garlic essential oil (MIC or IC_{50}) were poured into a Petri plate (9 cm ø) by poisoned food technique (Balouiri et al., 2016). Then, 10^5 spores (25µL) were spread uniformly over the medium surface into the plate and incubated at 27 °C. Next, 200 randomly selected spores were counted every 4 hours until 24 h (90-100 % spores germinated in the PDA-Tween® 80 without essential oil) (Plascencia et al., 2003).

Aflatoxins

Maize grains sampled at local stores from Culiacán (Mexico) were sterilized (fifty grams of grain) at 15 psi by 15 min, cooled, and inoculated with 10^5 *A. parasiticus* spores (5 mL). Afterwards, grains inoculate were mixed with 10 mL of a solution with essential oil (MIC or IC_{50}). In addition, were assayed grains inoculated without essential oil and not inoculated grains with 10 mL distiller water. Treatments (maize without inoculum, maize with inoculum, maize-Tween® 80 with inoculum, maize-971 ppm garlic oil with inoculum and maize-2875 ppm garlic oil with inoculum) were incubated 18 d at 27 °C. Later, they were milled and homogenized in the Osterizer Blender®, and were mixed (20 g) with 60 mL of water-methanol (80:20) and NaCl (1 g); the mixed was homogenized for 2 min. The mixture was centrifuged at 4500 rpm for 15 min, and supernatant was used as an aflatoxin extract.

Aflatoxin purified was obtained of aflatoxin extract with immunoaffinity column (Easi-Extract, R-Biopharm AG, Germany) and used according to the manufacturer's instructions.

The analysis of aflatoxin was conducted under conditions similar to those reported by Mishra and Aeri (2017) and Broszat et al. (2010). Briefly, standard solutions of aflatoxin B_1, B_2, G_1 and G_2 (Sigma-Aldrich®, Louis MO, USA) mixed in

methanol were assayed to 0.5, 1, 2, 5, 10, 20, 40, and 60 ng mL^{-1}. Chromatography was done with High-Performance Thin-Layer Chromatography (HPTLC) (CAMAG, Switzerland, Linomat 5, Software vision CATS v. 2.3) on aluminum plates precoated with silica gel 60 RP-18 F254s (10 cm × 20 cm, 0.2 mm thickness, 2–3 particle size, Merck®, Germany). Standard and aflatoxins solutions were spotted as bands of 8 mm width by using the auto sampler fitted with a syringe (Hamilton, Switzerland) with capacity for 100 µL. Silica gel plates were deposit at 75 mm of a horizontal developing chamber with methanol–aqueous $ZnSO_4$ (4 %) solution–ethyl methyl ketone a 15:15:3 (v v^{-1}) as mobile phase. The developed plates were air dried and scanned at 366 nm.

Experimental design and statistical analysis

One-way analysis of variance (ANOVA) was conducted, and treatments were PDA, PDA-Tween® 80 (Zero concentration), 10, 100, 500, 1000 and 10, 000 to assay of radial growth; PDA-Tween® 80, IC_{50} and MIC in spore germination; maize without inoculum, maize with inoculum, maize-Tween® 80, maize-971 ppm garlic oil with inoculum and maize-2875 ppm garlic oil with inoculum to assay of aflatoxins. The experimental unit was the plate Petri in growth radial (mm) and spore germination (%). The flask Erlenmeyer was used to assay of aflatoxins (µg kg^{-1}). All experiments were assayed out in triplicates (n = 3, α = 0.05) and means were analyzed with Tukey test ($p \leq 0.05$) using the statistical program JMP version 5.0 (Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

The garlic essential oil inhibited the radial growth of *A. parasiticus* at 96 h (Table 1). The IC_{50} and MIC of essential oil obtained by Probit were 971 and 2875 ppm, respectively. In spore germination, IC_{50} and MIC of essential oil garlic showed significant differences with respect to controls (PDA and PDA-Tween® 80) (Figure 1).

This study supports evidence from previous observations, that the growth for hypha treated with garlic oil is affected. Li et al. (2014) report the cytoplasmic loss and hyphal collapsed in *Penicillium funiculosum* by effect of garlic oil. The inhibitory effect of garlic essential oil against *A. parasiticus* may be due to sulfur compounds. The essential oil used previously was analyzed by Muy-Rangel et al. (2017) and report diallyl disulphide (23.64 %) and diallyl sulphide (20.33 %) in garlic essential oil (Sigma-Aldrich®, lot MKBB8390V). These compounds of sulfur may interact with sulfhydryl groups of proteins (El-Sayed et al., 2017) in the cell membrane. In *A. flavus* the volatile compounds of dill oil (*Anethum graveolens* L.) altered the permeability of the cell membrane (Tian et al., 2012); it possible that essential oil garlic causes the same effect on *A. parasiticus* growth.

All treatments showed aflatoxins B1 and G1 in maize (Table 2), plus an absence of B_2 and G_2. Inoculated maize with *A. parasiticus* showed a significant increase of aflatoxin B1 with respect to the uninoculated maize (Table 2). However, inoculated maize with *A. parasiticus* not showed a significant difference of aflatoxin G_1 in all treatments (Table 2). These results showing the aflatoxin production capacity of the fungus. Figure 2 shows an example the chromatography peak got for HPTLC analysis of aflatoxins.

Table 1. Effect of garlic essential oil at different concentration on the radial growth of *Aspergillus parasiticus* at 96 hours of incubation (27 ° C).

Concentration (ppm)	Growth radial (mm) †
PDA	17.17 ± 0.14^{B}
PDA-Tween® 80	21.33 ± 0.76^{A}
10	20.50 ± 0.50^{A}
100	18.50 ± 0.87^{B}
500	15.17 ± 0.38^{C}
1000	14.33 ± 0.29^{C}
10,000	11.42 ± 1.01^{D}

†Average of three replicates and SD. Letters in superscripts show statistical groups in column (Tukey, $p < 0.05$).

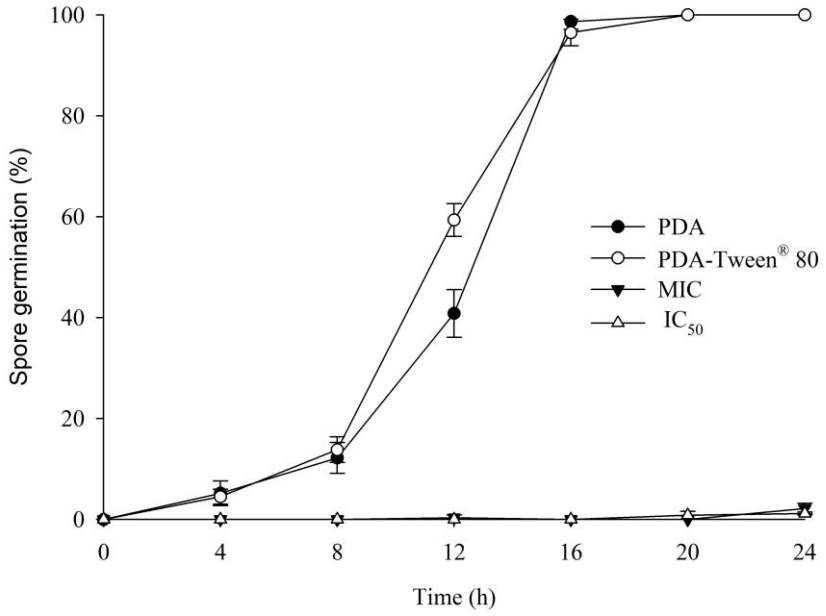

Figure 1. Percentage of spore germination of *Aspergillus parasiticus* in the presence of garlic essential oil.

In this study, the garlic essential oil did not inhibit the production of aflatoxin B_1 and G_1 in *A. parasiticus* in maize. Chalfoun et al. (2004) no evidenced of a correlation between concentrations of garlic oil and inhibition aflatoxin B_1. However, Deabes et al. (2011) mentioned that increased inhibition in fungal development and aflatoxin production is proportional to the increase in essential oil concentration. Also, Gowda et al. (2004) reports reduction of aflatoxin production in feed; however, their incubation time was seven days, and essential oil concentration evaluated was 10,000 ppm. Our study had an incubation time of 18 days and essential oil concentrations of 971 and 2875 ppm. It is possible that the garlic oil concentration was enough to inhibit development of *A. parasiticus*, once that the evaporation of the essential oil decreases its concentration, the fungus normalizes its metabolism (Muy-Rangel et al., 2017) and does not generate stress or inhibit the production of aflatoxin in maize.

CONCLUSIONS

The concentrations 971 and 2875 ppm of garlic essential oil inhibited the growth *in vitro* of *A. parasiticus*. However, they did not inhibit aflatoxin production by *A. parasiticus* in maize. It is necessary to explain with other studies the mechanisms of action of essential oil of garlic and decide if the effect is detoxifying or inhibiting.

Table 2. Effect of minimum inhibitory (MIC) and mean inhibitory (IC_{50}) concentrations of garlic essential oil on aflatoxins production of *Aspergillus parasiticus.*

Treatment	Aflatoxins† (μg kg^{-1})	
	B_1	G_1
Maize without inoculum (control)	19.82 ± 6.70^{B}	13.88 ± 2.35^{A}
Maize with inoculum (control)	81.64 ± 13.81^{A}	24.65 ± 2.44^{A}
Maize-Tween® 80 with inoculum	82.67 ± 9.97^{A}	23.95 ± 4.61^{A}
Maize-971 ppm garlic oil with inoculum **(IC_{50})**	96.93 ± 6.13^{A}	13.67 ± 2.35^{A}
Maize-2875 ppm garlic oil with inoculum **(MIC)**	95.455 ± 0.83^{A}	23.62 ± 5.35^{A}

†Average of three replicates and SD. Letters in superscripts show statistical groups in column (Tukey, $p < 0.05$).

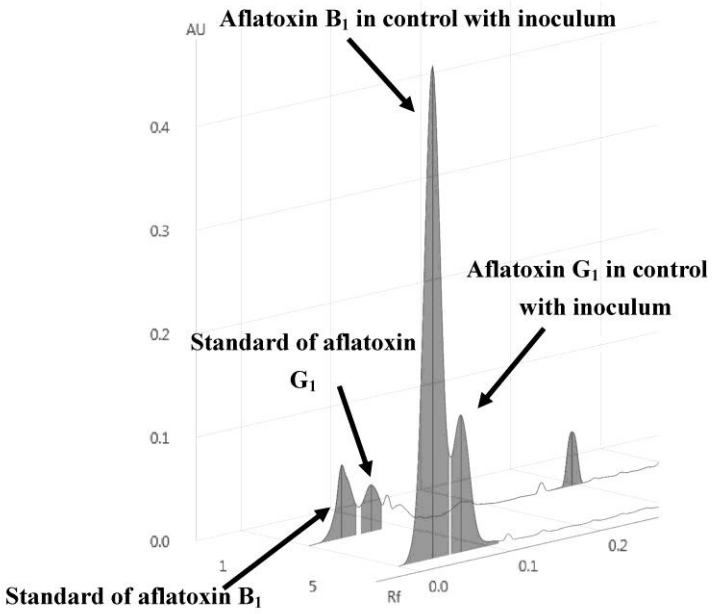

Figure 2. Aflatoxin chromatograms obtained by High-Performance Thin-Layer Chromatography (HPTLC).

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Compliance with ethical standards. The study complies with the ethical standards of the participating institutions.

Data availability. Data available if necessary, you can contact with the author correspondence.

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