SUPPLEMENTARY MATERIAL

Antifungal and antimycotoxigenic metabolites from native plants of northwest Argentina: isolation, identification and potential for control of Aspergillus species

Analía A. Gomez¹, Zareath P. Terán Baptista¹, Mandova Tsvetelina²,³, Angeliki Barouti², Marina Kritsanida², Raphaël Grougne², Marta A. Vattuone¹, Diego A. Sampietro¹,*

¹LABIFITO, Facultad de Bioquímica, Química y Farmacia. Universidad Nacional de Tucumán, Ayacucho 471 (4000). San Miguel de Tucumán, Argentina. ²Laboratoire de Pharmacognosie, Université de Paris Descartes, Sorbonne Paris Cité, Faculté de Pharmacie de Paris, UMR-CNRS 8638 COMETE, 4 avenue de l’Observatoire, 75006 Paris, France. ³Gilson Purification SAS, 22 rue Bourseul, ZA du Poteau, 56890 Saint-Avé, France

* Corresponding author: Tel.: +54 381 4247752 Ext. 7007
E-mail address: dasampietro@hotmail.com (D. A. Sampietro).
ABSTRACT
Extracts from aerial parts of Prosopis ruscifolia, Bidens pilosa, Cercidium praecox and Phoradendron liga were assayed against toxigenic Aspergillus species. They were obtained by sequential extraction of the aerial parts with hexane (fHex), dichloromethane (fDCM), ethyl acetate (fEtOAc) and methanol (fMeOH). The fMeOH from P. ruscifolia showed the highest antifungal spectrum (MIC=750-1500 µg mL⁻¹; MID=50-200 µg; DI=1.7-3.0 mm). Indolizidine alkaloids (juliflorine and juliprosine) and tryptamine were identified with strong (MIC=188 µg mL⁻¹) and moderate antifungal activities (MIC=750 µg mL⁻¹), respectively, towards A. parasiticus and A. flavus. The fMeOH, the indolizidine alkaloids and tryptamine synergized the fungitoxic effect of potassium sorbate and propiconazole. They completely suppressed the biosynthesis of aflatoxins at concentrations of 47, 94 and 375 µg mL⁻¹, respectively. Our results indicate that fMeOH and its identified alkaloids are promisory additives of commercial antifungals and are antiaflatoxigenic agents at concentrations below of those required for complete suppression of fungal growth.

Keywords: alkaloids, antifungal, antiaflatoxigenic, Aspergillus, Prosopis.
Experimental

Plant materials
Leaves and twigs of *Prosopis ruscifolia* Griseb (8 kg) were collected on March 2016 in Icaño (Santiago del Estero province, Argentina); of *Cercidium praecox* (Ruiz & Pavon. Ex hook) Harms subsp *glaucum* (Cav.) Burkart & Carter (6 kg) on El Cadillal (Tucumán province) on March 2016; of *Phoradendron liga* (5 kg) (Gillies ex Hook. & Arn.) Eichler in the surroundings of San Miguel de Tucumán (Tucumán province). The aerial part of *Bidens pilosa* L. (6 kg) was collected in Tafí del Valle (Tucumán province, Argentina) on April 2016. The plant materials were identified by Dr. Nora Muruaga and the voucher specimens LIL 15832 (*P. ruscifolia*), LIL 15984 (*B. pilosa*), LIL 16410 (*C. praecox*), LIL 16428 (*P. liga*) were deposited at the Herbarium of Miguel Lillo Foundation (Tucumán, Argentina). The plant materials were carried to the laboratory into paper bags.

Microorganisms
The following strains isolated from foodstuffs were used: VSC23 (*A. nomius*), NRRL 13137 (*A. nomius*), NRRL 2999 (*A. parasiticus*), LABI 208 (*A. niger*), LABI 212 (*A. fumigatus*) and LABI 216 (*A. flavus*). They were provided by the mycology collection of the Laboratory of Bioactive Agents and Phytopathogens (LABIFITO - FBQF - UNT). The microbial strains were preserved in SNA medium (Spezieller Nahrstoffarmer agar: 0.1% K₂HPO₄, 0.1% NaNO₃, 0.05% MgSO₄. 7H₂O, 0.05% KCl, 0.02% glucose, 0.2% sucrose and 2% agar) at 4°C. The stored strains were transferred and cultured at 30°C on MPA medium (solid malt peptone agar medium: 1.5% malt extract, 0.5% peptone, 0.1% glucose and 1.8 % agar) for 7 days prior to use in bioassays.

Plant extracts
The aerial parts of the plant species were dried in a stove at 50°C, ground to a coarse powder and stored in sealed flasks at -20°C until use. Each dried material (10 g) was sequentially extracted with hexane, dichloromethane, ethyl acetate and methanol, which polarity indexes are 0.1 < 3.1 < 4.4 < 5.1, respectively (Wells, 2003). The powdered plant material was exposed 48 h to each organic solvent (1: 10, w/v), subsequently trapped in a Whatman 4 filter paper and dried at 50°C till complete dryness before immersion in the next organic solvent. This sequential extraction allowed a first
fractionation of the components of the aerial parts. Each filtered organic fraction was evaporated under reduced pressure at 40°C, the dry residues were weighed and weights were used to calculate the yields of dry matter (DM) as:

$$\text{DM (\%)} = \frac{\text{mg of dry residue}}{\text{mg of dry plant material}} \times 100$$

The dry residues of the filtered organic fractions were stored at -15 °C in sealed flasks until use.

**Antifungal assays**

The antifungal activity of the organic fractions obtained from the aerial parts of the plant species was assessed by the dot blot and the broth microdilution assays. In the dot blot assay, thin layer chromatography (TLC) plates (silica gel G60 F254, Merck, 6 x 8 cm x 0.2 mm) were submerged in 96% ethanol and dried in sterile conditions. Then, the organic plant extracts were punctually loaded on the plate at equidistant positions in doses of 200 µg to 2000 µg of dry matter. Organic solvents (10 µL) or propiconazole (20 µg) were also spotwise applied on the plates as negative and positive controls, respectively. Then, each plate was covered with 3 mL of semisolid MPA medium containing $1 \times 10^3$ spores mL$^{-1}$. Plates were incubated at 30 °C for 48-72 h. Then, they were covered with 1.5 mL of a solution of 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium (MTT) and incubated at 30 °C for 24 h. Dehydrogenases of the living fungal cells convert MTT into blue (Bluma et al., 2008). Hence, dead cells were visualized on the plates as yellow zones under a blue background. The minimal dose of an organic plant extract required to suppress fungal growth (MID) was determined. Two perpendicular diameters were measured in the each yellow zone observed at the MID with the software Fiji win 32 (Image J 1.44c, Wayne Rasband, NIH, Bethesda MD).

The organic extracts recovered from the aerial plant parts were also subjected to the broth microdilution assay in 96 flat well microplates. The protocol was developed according to M38-A document from the National Committee for Clinical Laboratory Standards with some modifications (NCCLS, 2002). Fungal colonies were grown in Petri dishes for 7 to 15 days in solid MPA medium in the darkness at 30 °C. Then, the fungal colonies were washed with 2 mL of physiological solution (0.9% of NaCl in distilled water) to obtain microconidial suspensions. The asexual spores were counted in a Neubauer chamber, and the suspension was diluted in semiliquid YES medium
(Yeast-malt extract-sucrose: 2% yeast extract, 15% sucrose, 0.05 % magnesium sulphate, 0.125 % agar) to obtain a density of $1 \times 10^5$ spores mL$^{-1}$. The stock solutions of each organic extract were prepared in DMSO and diluted with culture medium to prepare two fold dilution series comprised between 3000-87.5 μg mL$^{-1}$, in semiliquid YES medium. The final volume in each well was 200 μL containing 2% DMSO. This volume corresponded to 100 μL of fungal spore suspension and 100 μL of a dilution of an organic plant extract. Controls of growth consisted in 100 μL of YES medium plus 100 μL of spore suspension in each well. Controls of sterility were 200 μL of YES medium per well. Each treatment (organic extracts or controls) had three repetitions per microplate. Each microplate was prepared twice. The microplates were incubated 72 h at 30°C. Then, the minimum concentration of each organic fraction required to inhibit 100% of the microbial growth (MIC) was visually determined. The MIC values presented in tables are means of three replicates obtained from two experiments.

**Isolation and identification of the antifungal constituents from the methanolic extract of Prosopis ruscifolia**

The constituents of the methanolic extract of *P. ruscifolia* were separated following a bioassay guided isolation. The antifungal activity was evaluated by the dot blot assays on *A. flavus* and *A. parasiticus* at each step of the separation process. The dry residue of the methanolic extract (4 g) of *P. ruscifolia*, dissolved in a mixture of chloroform-methanol (8:2, v/v), was loaded on a column (123 g of silica gel, pore size 60 Å, 230-400 mesh particle size). Then, the column was eluted with a gradient of chloroform-methanol (200 mL, 8:2; 150 mL, 7:3; 150 mL, 6:4, v/v). The composition of the eluted fractions (20 mL each) was monitored by TLC with ethyl acetate-formic acid-water (65:15:10, v/v/v) as mobile phase. The TLC chromatograms were visualized under UV light at 254 nm and 365 nm, and then revealed with chromogenic reagents (vanillin/sulfuric acid, p-anisaldehyde or dragendorff) according to Wagner and Bladt (1996). Fractions were combined in eight groups (F1-F8). The constituents of F6 were loaded in a Sephadex LH 20 column (250 x 17 mm, Amersham Pharmacia Biotech) and recovered in three pools (P1-P3). The P1 and P2 were subjected to preparative thin layer chromatography (PTLC) on silica gel 60 F$_{254}$ (2 mm thickness). PTLC bands 1 (B1) from P2 and 2 (B2) and 3 (B3) from P1 were scrapped from the plate and eluted with methanol. After 24 h, the methanolic suspensions were centrifuged and the supernatants were recovered. Each supernatant was passed through 280 mg of silica gel packaged in
a Pasteur pipette. The $^1$H NMR spectra of B1, B2 and B3 recovered from the Pasteur pipettes (hereafter referred as Pp1, Pp2 and Pp3, respectively) were recorded using a Bruker Avance-III 400 MHz spectrometer in deuterated methanol. NMR Fourier transform, peak picking and integration were done with Bruker TopSpin software. These spectra allowed the identification of the compounds based on their matching with those obtained for standard compounds (i.e. tryptamine) or previously published in the literature (Barry et al., 2005).

**Brine shrimp assay**

The cytotoxicity of the antifungal constituents was evaluated by the brine shrimp assay (Meyer et al., 1982). Eggs of *Artemia salina* were placed in a plastic cage with seawater (3.8% NaCl). Half of the cage was exposed to light provided by a 60-watt lamp and was incubated for 48 h at 27 °C. The dried residue of the methanolic extract from *P. ruscifolia*, juliflorine, juliprosine and tryptamine were dissolved in the sea water and tested at concentrations of 3000-87.5 μg mL$^{-1}$. Positive controls were K$_2$Cr$_2$O$_7$ (González Pérez and Aportela Gilling, 2001) and propiconazole. Brine shrimps placed in sea water served as the viability control. Ten brine shrimp larvae were placed in each well of 24-well microplates and incubated for 18 h at 27 °C. The surviving nauplii were counted under a magnifying glass at a 3x magnification and percentages of inhibition were determined. Percentages of inhibition were calculated and then the concentration required to kill 50% of the nauplii (LC$_{50}$) was interpolated by probit analysis. Each assay was performed in triplicate. The LC$_{50}$ values were interpreted as follows: <1.0 μg mL$^{-1}$, highly toxic; 1.0-10.0 μg mL$^{-1}$, toxic; 10.0-30.0 μg mL$^{-1}$, moderately toxic; 31-100 μg mL$^{-1}$, mildly toxic, and >100 μg mL$^{-1}$, as non-toxic (Meyer et al., 1982). Selective index (LC$_{50}$/MIC) was also calculated.

**Joint action of the methanolic extract of Prosopis ruscifolia and its alkaloids with commercial antifungals**

The methanolic extract of *P. ruscifolia*, juliflorine and juliprosine were assayed in combination with propiconazole and with potassium sorbate on *A. parasiticus* and *A. flavus* by the chessboard technique and the inhibitory fractionated concentration (FICI) was calculated as: FICI = (Concentration of A in MICA+B/Concentration of A in MICA) + (Concentration of B in MICA+B/Concentration of B). Interpretation of FICI: ≤ 0.5, synergy; 0.5-4.0, no interaction; > 4.0, antagonism (Vitale et al., 2005).
**Antiaflatoxigenic activity of fMeOH and its alkaloids**

The inhibitory effect of fMeOH, tryptamine, juliflorine and juliprosine on aflatoxin accumulation of NRRL2999 (*A. parasiticus*) and LABI 216 (*A. flavus*) was tested in YES medium. Aliquots of 8 mL of the medium supplemented with fMeOH, tryptamine, juliflorine or juliprosine were placed in 125 mL Erlenmeyer flasks. Each flask was inoculated with $3 \times 10^4$ conidia of *A. parasiticus* or *A. flavus* suspended in 1 mL of medium in triplicate. Concentrations assayed ranged from 47 to 1500 µg mL$^{-1}$ of liquid medium. The flasks were then incubated in the darkness at 30 °C in an orbital shaker at 150 rev min$^{-1}$ for 7 days. Following incubation, cultures were centrifuged at 3000 g for 10 min. Supernatants were stored at -20°C before quantification of aflatoxins. Fungal biomass was measured by weighing the mycelial pellet after 48 h of freeze-drying. The initial pH of the culture medium was not affected by the supplementation, and the final pH conditions did not vary between treatments. Contents of mycotoxins were measured in the thawed supernatants with ELISA kits (Ridascreen Fast Aflatoxin, Biopharm, Germany), and expressed as µg mg$^{-1}$ of dry mycelial biomass. The obtained data were subjected to ANOVA and a subsequent post hoc Dunnet T3 test, with $P = 0.05$. 

Table S1. Yields (g dry matter/g of aerial part) recovered with the organic solvents after extraction of the aerial parts from the investigated native plants of northwest Argentina.

| Plant Type                | Hexane  | Dichloromethane | Ethyl acetate | Methanol |
|---------------------------|---------|-----------------|---------------|----------|
| *Prosopis ruscifolia*     | 2.0±0.5d | 4.1±0.2c        | 6.4±0.1b      | 11.3±0.1a |
| *Bidens pilosa*           | 1.0±0.3g | 1.1±0.1g        | 0.7±0.2f      | 8.1±0.2d  |
| *Cercidium praecox*       | 1.0±0.2g | 1.3±0.1g        | 0.4±0.2f      | 8.4±0.3d  |
| *Phoradendron liga*       | 1.3±0.3g | 1.2±0.2g        | 0.5±0.1f      | 2.1±0.2e  |
Figure S1. Silica gel chromatograms of the methanolic extract (fMeOH) obtained from aerial parts of *Prosopis ruscifolia*. From left to right, they were revealed with the Dragendorff reagent, vanillin-sulfuric acid and under ultraviolet light of 254 nm. Examples of bioautographies for B3 on *A. parasiticus* (NRRL2999) and *A. flavus* (LABI 216) are also presented.
Figure S2. $^1$H- NMR spectra at 400 MHz of (A) Pp1, (B) Pp2 and (C) Pp3 in basic medium. The NMR were performed in deuterated methanol. Chemical shifts (\(\delta\)) are in ppm, and J in Hz. Residual CH$_3$OH was recorded at 3.31 ppm.
Figure S3. Dot blot bioautographies of Pp1 (tryptamine), Pp2 (juliflorine) and Pp3 (juliprosine) when tested on *A. flavus* (LABI 216) and *A. parasiticus* (NRRL2999). C (+) = propiconazole (20 µg). C (-) = maximum volume of solvent used to seed the alkaloids in the TLC plate.
Table S2. Minimum inhibitory concentration (MIC), minimum inhibitory dose (MID) and diameter of inhibition (ID) at the MID obtained for the bioactive extracts from native plants of northwest Argentina against several Aspergillus strains.

| Plant species/extract | Aspergillus | Prosopis ruscifolia | Bidens pilosa | Bidens pilosa |
|-----------------------|-------------|---------------------|--------------|--------------|
|                       | Species     | /methanol           | /dichloromethane | /ethyl acetate |
|                       |             | MIC (µg mL\(^{-1}\)) | MID (µg) | ID (mm) | MIC (µg mL\(^{-1}\)) | MID (µg) | ID (mm) | MIC (µg mL\(^{-1}\)) | MID (µg) | ID (mm) |
| LABI 208\(^i\)        | LABI 208    | 1500                | 50          | 2.1±0.1a | >2000         | 200      | 1.4±0.2a | >2000         | 200      | 2.6±0.3a |
|                       | VSC23       | 750                 | 200         | 2.3±0.2a | >2000         | 1000     | 4.5±0.3b | >2000         | 1000     | 0.9±0.2b |
|                       | LABI 216    | 750                 | 200         | 1.7±0.1b | >2000         | 2000     | 5.7±0.3c | >2000         | >2000    | ND      |
|                       | NRRL 2999   | 1500                | 200         | 3.0±0.1c | >2000         | 2000     | 2.0±0.2d | >2000         | >2000    | ND      |
|                       | LABI 212    | 1500                | 200         | 2.3±0.1a | >2000         | 2000     | ND       | >2000         | >2000    | ND      |

| Plant species/extract | Aspergillus | Cercidium praecox | Cercidium praecox |
|-----------------------|-------------|-------------------|-------------------|
|                       | Species     | /hexane           | /dichloromethane  |
|                       |             | MIC (µg mL\(^{-1}\)) | MID (µg) | ID (mm) | MIC (µg mL\(^{-1}\)) | MID (µg) | ID (mm) |
| LABI 208\(^i\)        | LABI 208    | >2000             | 200       | 0.8±0.1a | >2000         | 200      | 0.3±0.1a |
|                       | VSC23       | >2000             | >2000     | ND       | >2000         | >2000    | ND      |
|                       | LABI 216    | >2000             | 500       | 1.4±0.1b | >2000         | 2000     | 2.0±0.1b |
|                       | NRRL 2999   | >2000             | >2000     | ND       | >2000         | >2000    | ND      |
|                       | LABI 212    | >2000             | 200       | 1.2±0.2b | 1500          | 1000     | 1.7±0.3b |

ND = Not detected. \(^i\)LABI 208 (A. niger), VSC23 (A. nomius), LABI 216 (A. flavus), NRRL 2999 (A. parasiticus) and LABI 212 (A. fumigatus). \(^a\)Data are reported as mean values, based on two experiments where each treatment had three replications. \(^b\)Data are reported as mean values±standard deviation, based on two experiments where each treatment had three replications. Different letters in a same column indicate differences among means (test of Dunnet T3, \(p = 0.05\).
Table S3. Minimum inhibitory concentration 100% (MIC), minimum inhibitory dose (MID) and diameter of inhibited zone (ID) at the MID generated by the methanolic extract of *Prosopis ruscifolia* and its alkaloids on NRRL 2999 (*A. parasiticus*) and LABI 126 (*A. flavus*).

|                  | NRRL 2999 (*A. parasiticus*) | LABI 216 (*A. flavus*) |
|------------------|-------------------------------|------------------------|
|                  | MIC¹ (µg mL⁻¹) | MID¹ (µg) | ID¹ (mm) | MIC (µg mL⁻¹) | MID (µg) | ID (mm) |
| Methanolic extract | 1500             | 50        | 2.1±0.1a | 1500             | 250        | 1.7±0.1a |
| Pp2 (juliflorine)  | 188              | 100       | 1.0±0.2b | 188              | 100        | 1.0±0.1b |
| Pp3 (juliprosine)  | 188              | 500       | 0.9±0.1b | 188              | 250        | 0.8±0.1b |
| Pp1 (tryptamine)   | 750              | 250       | 0.9±0.1b | 750              | 50         | 0.8±0.1b |
| Potassium Sorbate  | 1500            | >2000     | ND      | 1500            | >2000     | ND      |
| Propiconazole      | 6.25             | 1.5       | 0.5     | 6.25             | 1.5        | 0.5     |

ND = Not detected. ¹Data are reported as mean values, based on two experiments where each treatment had three replications. ²Means of ID in the same column with the same letter are not significantly different (Dunnet T3 test, p = 0.05).
Table S4. Lethal concentration 50% (LC$_{50}$) on *Artemia salina* nauplii and the selective index (LC$_{50}$/MIC) obtained for the methanolic extract of *Prosopis ruscifolia* and its alkaloids on NRRL 2999 (*A. parasiticus*) and LABI 216 (*A. flavus*).

|                | Interpretation of LC$_{50}$ | Selectivity Index (LC$_{50}$/MIC) | Interpretation of Selectivity index |
|----------------|-----------------------------|-----------------------------------|-------------------------------------|
| **Artemia salina** |                             | NRRL 2999 (A. parasiticus)        | LABI 216 (A. flavus)                |
| **LC$_{50}$ (µg mL$^{-1}$)** | Non toxic                  | 1.22                              | 1.22                                |
| Methanolic extract | 1829 (1798-1844)            | Fungitoxic                        |                                     |
| Pp2 (juliflorine)  | 83 (71-96)                  | Midly toxic                       | 0.44                                |
| Pp3 (juliprosine)  | 80 (75-89)                  | Midly toxic                       | 0.43                                |
| Pp1 (tryptamine)   | 289 (231-382)               | Non toxic                         | 0.39                                |
| Potassium Sorbate   | 1716 (1675-1781)            | Non toxic                         | 1.14                                |
| Propiconazole      | 9 (7-12)                    | Toxic                             | 1.44                                |
| K$_2$Cr$_2$O$_7$    | 6 (5-10)                    | Toxic                             | ND                                  |

ND = Not determined
**Table S5.** Joint action of the methanolic extract of *Prosopis ruscifolia* and its alkaloids with commercial antifungals determined by the chessboard technique on growth of NRRL 2999 (*A. parasiticus*) and LABI 216 (*A. flavus*).

| Compound assayed | NRRL 2999 (A. parasiticus) | LABI 216 (A. flavus) | Interpretation of the joint effect¹ |
|------------------|-----------------------------|----------------------|-----------------------------------|
| Propiconazole    |                             |                      |                                   |
| + fMeOH          | 0.28                        | 0.17                 | synergism                         |
| + Pp2 (juliflorine) | 0.31                      | 0.37                 | synergism                         |
| + Pp3 (juliprosine) | 0.38                      | 0.35                 | synergism                         |
| + Pp1 (tryptamine) | 0.28                      | 0.31                 | synergism                         |
| Potassium sorbate|                             |                      |                                   |
| + fMeOH          | 0.41                        | 0.40                 | synergism                         |
| + Pp2 (juliflorine) | 0.31                      | 0.31                 | synergism                         |
| + Pp3 (juliprosine) | 0.33                      | 0.35                 | synergism                         |
| + Pp1 (tryptamine) | 0.35                      | 0.40                 | synergism                         |

¹Interpretation of FICI: ≤ 0.5, synergy; 0.5-4.0, no interaction; > 4.0, antagonism
### Table S6. Antimycotoxigenic activity of the methanolic extract (fMeOH), Pp2 (juliflorine), Pp3 (juliprosine) and Pp1 (tryptamine) on NRRL 2999 (A. parasiticus) and LABI 216 (A. flavus). Propiconazole was included as control.

|                  | NRRL 2999 (A. parasiticus) | LABI 216 (A. flavus) |
|------------------|-----------------------------|----------------------|
| **Concentration** | **Aflatoxin** (µg mg⁻¹)** | **Biomass** (ref control) | **Aflatoxin** (µg mg⁻¹)** | **Biomass** (ref control) |
| 0                 | 1.91±0.03**                 | ND                   | 1.00±0.02**                 | 1.00±0.01**              |
| fMeOH             | 47                          | ND                   | 1.05±0.01                   | ND                       |
|                  | 94                          | ND                   | 1.00±0.01                   | ND                       |
|                  | 188                         | ND                   | 0.71±0.02                   | ND                       |
|                  | 375                         | ND                   | 0.35±0.01                   | ND                       |
|                  | 750                         | ND                   | 0.12±0.03                   | ND                       |
| Pp2 (juliflorine)| 6                           | 1.30±0.01d           | 1.00±0.01                   | 1.40±0.01b               | 1.00±0.01                |
|                  | 12                          | 1.23±0.02d           | 0.85±0.01                   | 1.36±0.01b               | 0.78±0.01                |
|                  | 24                          | 1.13±0.01d           | 0.65±0.01                   | 1.14±0.02c               | 0.61±0.01                |
|                  | 47                          | 0.66±0.01e           | 0.40±0.01                   | 1.00±0.01c               | 0.50±0.01                |
|                  | 94                          | ND                   | 0.10±0.01                   | ND                       |
| Pp3 (juliprosine)| 6                           | 1.40±0.01d           | 1.00±0.01                   | 1.45±0.01b               | 1.00±0.01                |
|                  | 12                          | 1.33±0.02d           | 0.85±0.01                   | 1.31±0.01b               | 0.75±0.01                |
|                  | 24                          | 1.00±0.01d           | 0.60±0.01                   | 1.24±0.02c               | 0.51±0.01                |
|                  | 47                          | 0.36±0.01b           | 0.25±0.01                   | 0.45±0.01d               | 0.45±0.01                |
|                  | 94                          | ND                   | 0.09±0.01                   | ND                       |
| Pp1 (tryptamine) | 12                          | 1.50±0.01b           | 1.00±0.01                   | 1.30±0.02b               | 1.00±0.01                |
|                  | 24                          | 1.00±0.01b           | 1.00±0.02                   | 0.90±0.01c               | 1.00±0.03                |
|                  | 47                          | 0.45±0.01b           | 1.00±0.01                   | 0.39±0.01d               | 1.00±0.01                |
|                  | 94                          | 0.39±0.04b           | 0.89±0.01                   | 0.28±0.02e               | 0.90±0.02                |
|                  | 188                         | 0.27±0.02b           | 0.76±0.02                   | 0.12±0.02e               | 0.75±0.02                |
|                  | 375                         | ND                   | 0.25±0.03                   | ND                       |
| Propiconazole    | 0.39                        | 1.20±0.01d           | 1.00±0.01                   | 1.60±0.01a               | 1.00±0.01                |
|                  | 0.78                        | 1.47±0.02g           | 0.88±0.01                   | 1.55±0.01a               | 0.75±0.01                |
|                  | 1.56                        | 1.94±0.01h           | 0.71±0.02                   | 1.51±0.01a               | 0.51±0.02                |
|                  | 3.13                        | 2.89±0.01i           | 0.38±0.01                   | 2.47±0.01g               | 0.23±0.01                |

*µg of mycotoxin per mg of dry fungal biomass**70 mg of dry weight/Petri dish.

ND: Not detected.

Means of aflatoxin accumulation in the same column with the same letter are not significantly different (Dunnet T3 test, p = 0.05)
References

González Pérez Y, Aportela Gilling P. 2001. Determinación de la toxicidad aguda del dicromato de potasio en larvas de *Artemia salina*. *Anuario Toxicología 1*, 104–108.

Meyer B, Ferrigni N, Putnam J, Jacobsen L, Nichols D, McLaughlin J. 1982. Brine shrimp: a convenient general bioassay for active plant constituents. *Planta Med. 45*, 31–34.

NCCLS. 2002. Performance standards for antimicrobial susceptibility testing. Twelfth Informational Supplement. NCCLS Document M100-S12. Wayne, PA, USA: NCCLS.

Vitale RG, Afeltra J, Dannaoui E. 2005. Antifungal combinations. *Methods Mol. Med. 118*, 143-152.

Wells, D. (2003). Liquid-liquid extraction: Strategies for method development and optimization. *Progress in Pharmaceutical and Biomedical Analysis 5*, 307-326.