Metarhizium robertsii with insecticidal activity for the control of the fruit fly (Anastrepha obliqua), the main pest in mango crop in Colombia

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Research Article

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Abstract

The aim of this study was to evaluate and select entomopathogenic fungi that produces insecticidal compounds for the control of adults of *Anastrepha obliqua* Macquart (Diptera: tephritidae), that are the main pest of mango (*Mangifera indica* L. Bark) in Colombia. One strain of the species *Metarhizium robertsii* with insecticidal activity was selected. By column fractionation, an active fraction was obtained, this compound caused mortalities higher than 90% after 48 hours of exposure. By HPLC it was determined that the active fraction is composed of more than 22 metabolites. Identification of the extracts by UHPLC MS/MS reveal the presence of destruxin in the groups E, D, A and B (destruxin E-diol, destruxin D, destruxin D1, destruxin D2, destruxin A2, destruxin A, destruxin A3, dihydrodestruxin A, desmB, destruxin B2, destruxin B, and destruxin B1). The evaluation of the insecticidal capacity of the organic fractions obtained by HPLC indicated that the extract obtained from the isolate *Metarhizium robertsii* had a compound with high activity on adults of *A. obliqua* (destruxin A) causing massive mortality of up to 100%, 48 hours after treatment administration. Furthermore, two other compounds with medium activity were found (destruxin A2 and destruxin B), showing mortalities between 60.0 and 81.3% respectively. The extract of the isolate MT008 of *M. robertsii* showed higher insecticidal activity and is a potential source for the control of *A. obliqua.*

Key Points

- *Anastrepha obliqua* is the main pest of mango (*Mangifera indica)* in Colombia.
- *Metarhizium robertsii* releases secondary metabolites for the control of *A. obliqua*
- The fruit fly (*A. obliqua*) is susceptible to destruxins A, A2 and B
- Extracts from *M. robertsii* are a potential source for the control of *A. obliqua*

1. Introduction

Fruit flies (Diptera: Tephritidae) are an important pest for fruit-producing countries due to their high incidence and severity which conduces to quarantine restrictions and economic losses (Ekesi et al. 2016; Pérez et al. 2008). *A. obliqua* (the West Indian fruit fly) is the main pest in mango and guava (*Psidium guajava* L.), it also infests species of the botanical families Anacardiaceae, Annonaceae, Bignoniaceae, Fabaceae, Myrtaceae and Rosaceae (Oroño et al. 2006). *A. obliqua* has an extremely wide distribution with records ranging from the south of United States to the northern region of Argentina, extending from Mexico to Panama, Colombia, Venezuela, Ecuador, and Brazil with farther distribution throughout the Caribbean in Jamaica and Trinidad (Norrbom and Korytkowski 2007). In Colombia, the genus *Anastrepha* includes 47 species, five of them have quarantine importance: *A. fraterculus* (Wiedemann), *A. grandis* (Macquart), *A. obliqua* (Macquart), *A. serpentina* (Wiedemann) and *A. striata* (chinner) (Canal 2010). The distribution of this genus in Colombia ranges from the sea level to 2000 m a.s.l. (Martínez 2007). It is estimated that more than 40% of the fruit production in Colombia is already affected by several species of the Tephritidae family. According to information provided by the Instituto Colombiano Agropecuario

[Colombian Institute of Agriculture] (ICA 2015), "Colombia, does not have areas free of fruit flies; however, there are areas with low prevalence of this pest. Nonetheless, this limits the domestic market as well as exports" (Giraldo et al. 2015). Furthermore, the excessive use of chemical insecticides for fruit fly control, for more than 50 years, has caused important effects on ecosystems as well as on human health (Haniotakis 2005). The repeated use of a very limited number of active ingredients in these insecticides (organophosphates, carbamates, pyrethroids and spinosads) allows the generation of resistant populations of fruit fly, resulting in a loss of effectiveness (Margaritopoulos et al. 2008; Kakani et al. 2008, 2010).

On the other hand, entomopathogenic fungi are the most predominant controllers for arthropod populations in nature, thus the great potential of these organisms in the regulation of crop pests (Lacey et al. 2015). Extracts obtained from entomopathogenic fungi have shown high insecticidal activity in several studies. For instance, extracts obtained from *Metarhizium* isolates have proved to be toxic for adults of the Mediterranean fruit fly (*Ceratitis capitata* Wiedemann), with mortalities close to 90% (Castillo et al. 2000). Likewise, extracts obtained from isolates of the fungus *Beauveria* spp. and *Metarhizium* spp. produce toxic secondary metabolites that affect adults of *C. capitata* and *Bactrocera olea* (Rossi) with mortalities above 80% after 48 hours of application (Yousef et al. 2013; Lozano-Tovar et al. 2015). Other compounds with high molecular weight (soluble proteins), from *Metarhizium brunneum* have shown chronic insecticidal activity after being fed to adults of *C. capitata* (Ortiz-Urquiza et al. 2009). Perhaps destruxins (dtx) are one the most researched secondary metabolites of entomopathogenic fungi. Insect susceptibility to dtxs is variable, but in general, dtxs A and E seem to be more toxic compared to dtxs D and B (Hu et al. 2009; Lozano-Tovar et al. 2015).

Taking this background into account, the aim of this study was to select the entomopathogenic fungi with the insecticidal capacity and identify its bioactive compounds for the generation of biological management alternatives for the control of the fruit fly (*Anastrepha obliqua*), the major mango pest in Colombia.

2. Materials And Methods

2.1. Biological material

2.1.1. Microorganisms: nine entomopathogenic fungi isolates supplied by the work collections of AGROSAVIA were used. Five belonging to the genus *Metarhizium* (MT005, MT007, MT008, MT009, MT040), and four belonging to the genus *Beauveria* (BV002, BV009, BV012, BV016) (Table 1).

Table 1
Isolates used in this study.

Isolate Code	Genus	Origen* and host
MT005	*Metarhizium* spp	Melolonthidae larvae. Rionegro (Antioquia)
MT007	*Metarhizium* spp	Nematode.Cajamarca (Tolima)
MT008	*Metarhizium* spp	*Rhammatocerus schistocercoides* Villavicencio (Meta).
MT009	*Metarhizium* spp	*Rhammatocerus schistocercoides*. Puerto Gaitan (Meta)
MT040	*Metarhizium* spp	Melolonthidae larvae. Rionegro (Antioquia)
BV002	*Beauveria* spp	*Premnotrypes vorax*. Savanna of Bogotá (Cundinamarca).
BV009	*Beauveria* spp	Coleoptera. Puerto Gaitan (Meta)
BV012	*Beauveria* spp	Coleoptera. Rionegro (Antioquia)
BV016	*Beauveria* spp	Melolonthidae adults. Cajamarca (Tolima)
* Colombian regions		

2.1.2. Insects: Adults of *A. obliqua* were obtained from standardized breeds from the Instituto Colombiano Agropecuario (ICA), Regional Office of Ibagué, Tolima, Colombia. Insects were maintained at 25 ± 2°C and humidity of 65 ± 5% with a photoperiod consists of 12 hours of light and 12 hours of darkness.

2.2. Location: The process of obtaining active compounds and their evaluation were executed in the microbiology laboratory of C.I. Nataima, Corporación Colombiana de Investigación Agropecuaria (AGROSAVIA). The research station is located at an altitude of 418 m a.s.l., at 4° 11' 31.65" of Latitude N and 74° 57' 41.49" of Longitude W in the department of Tolima, municipality of El Espinal, Colombia.

2.3. Selection of microorganisms with insecticidal capacity

2.3.1. Production of crude extracts: Isolates were placed in sabouraud dextrose agar culture medium (Merck KGaA, Germany). Incubation was carried out for 7 days (d) at 28 ± 2°C. Then the conidia were collected, and a suspension of them was prepared (1 x 10^7 conidia/ml), then 2 ml of this suspension were transferred to 250 ml of liquid medium G20P20 (20 g of glucose, Merck KGaA, Germany and 20 g of peptone Merck KGaA, Germany, per liter). The liquid culture was incubated for 20 d under the specified conditions. Then the cells were filtered with paper (Whatman No.3) and centrifuged (Heal Force Model Neofuge 23R) at 10000 g for 20 min and then supernatants were preserved at -20^{o}C until their use.

2.3.2. Evaluation of crude extracts: The supernatants were evaluated both concentrated and non-concentrated. Supernatants were concentrated through drying using an air current at a temperature of 28 ± 2°C, then resuspended in a volume 15 times less than the initial. Subsequently supernatants were mixed

with hydrolyzed protein and sugar at a 4: 1 ratio, this was given daily to *A. obliqua* adults in dosis of 100 µL of the treatment per repetition, placed in plastic wells of 150 µl. Adult mortality was recorded periodically after feeding adults with different treatments. Concentrated supernatants mortality was monitored for 66 hours meanwhile non-concentrated extracts mortality was monitored for 5 d.

2.4. Molecular identification of selected isolates

The strains that showed the highest insecticidal activity in this work (MT005 and MT008) were molecularly characterized. The fungal DNA was extracted from conidia using a Zymo research Quick-DNA fungal/bacterial miniprep kit, according to the manufacturer's protocol.

Characterization was based on the sequencing of the internal transcribed spacer (ITS) region (White et al., 1990) and two additional genes were sequenced: the partial beta-tubulin (Btub) (Tartar et al., 2002) and the partial Elongation Factor EF-1α (Rehner & Buckley, 2005). PCR reactions were carried out in 25 µl, using 5 µl reaction buffer, 6.25 mM dNTPs, 2.5 mM $MgCl_2$, 1 µL of each primer (10 µM), 0.2 µL DNA taq polymerase (Promega), and approximately 100–300 ng of genomic DNA. The general thermal conditions were: 94 ^{o}C for 4 min, followed by 35 cycles of 10s at 92 ^{o}C, 20s at 55 ^{o}C, and 60s at 72 ^{o}C, and a final extension of 5 min at 72 ^{o}C. The products were visualised on a 1.5% agarose gel in TBE buffer, using SYBR® Safe (Thermo Fisher Scientific). The sequences, which were generated by the AGROSAVIA laboratory, were aligned, edited, and analyzed by MEGA X (Kumar et al., 2018), and BLASTn was carried out on the GenBank database of the National Centre for Biodiversity Information (NCBI) for identification. Phylogenetic analyses were conducted, using concatenate sequences of ITS, Btub, and EF-1α genes using representative sequences of different *Metarhizium* species from the database of the National Center for Biotechnology Information (NCBI) (Table 2) and *Beauveria* species as outgroup. There was a total of 2515 positions in the final dataset for concatenated sequences. The phylogenetic tree was inferred using the Neighbor-Joining method (Saitou & Nei, 1987) and the bootstrap consensus tree was inferred from 1000 replicates (Felsenstein, 1985).

Table 2
Sequences used in phylogenetic analysis.

Specie and strain code	Analyzed region	GenBank accession number
Metarhizium anisopliae	ITS - EF-1α - β-tubulin	PRJNA530366
Metarhizium acridum CQMa 102	ITS - EF-1α - β-tubulin	PRJNA245139
Metarhizium rileyi RCEF 4871	ITS - EF-1α - β-tubulin	PRJNA72739
Metarhizium majus ARSEF297	ITS - EF-1α - β-tubulin	PRJNA302308
Metarhizium brunneum ARSEF 3297	ITS - EF-1α - β-tubulin	PRJNA608152
Metarhizium guizhouense ARSEF 977	ITS - EF-1α - β-tubulin	PRJNA184755
Metarhizium robertsii ARSEF23	ITS - EF-1α - β-tubulin	PRJNA245140
Beauveria bassiana ARSEF 2860	ITS - EF-1α - β-tubulin	PRJNA225503
Beauveria pseudobassiana	ITS - EF-1α - β-tubulin	PRJNA314175

2.5. Evaluation of the effect of environmental factors on the insecticidal activity of crude extracts obtained from Metarhizium robertsii.

A variety of environmental factors like temperature and light have been shown to have dramatic effects on the efficacy of entomopathogens against insect pests (Inglis et al. 2001). Mango crops in the country are developed under conditions of high temperatures (32ºC) and high solar radiation (700–890 Watt/m^2), the effects of these two factors on the insecticidal activity of concentrated supernatants obtained from the selected *Metarhizium robertsii* MT008 and MT005 were evaluated. Concentrated supernants were exposed to three different temperatures for a different time, as follows: room temperature (30°C ± 2°C) for 3 hours; 50°C for 3 hours; and 120°C for 20 minutes). Concentrated supernatants were subjected to 4

hours of direct solar radiation (from 10:00 hours to 14:00 hours) with irradiance of 784 W/m^2 and average temperature of 32.08ºC (Davis Vantage Pro2 station, C.I. Nataima-Agrosavia). Then the supernatants were exposed to four hours of ultraviolet light in a flow chamber. The concentrated crude extracts remained stored under environmental conditions (30ºC ± 2°C) in the shade for 0, 24, 48 and 72 h. These extracts were evaluated subsequently on adults of *A. obliqua*. Concentrated supernatants with no exposure to environmental conditions were used as controls.

2.6. Evaluation of insecticidal activity in fractions obtained from MT008 crude extracts

2.6.1. Fractionation: The concentrated supernatant from isolate MT008 was fractionated by dialysis, against distilled water (1:20 fungal extract: water) using a dialysis membrane with a molecular weight cut-off of 3500 Da (Spectra/Por®). The process was carried out with constant agitation on magnetic stirrer for 48 h at 4ºC. The two fractions obtained (dialyzed and adialyzed) were evaluated. The dialyzed fraction was subjected to normal phase column fractionation with high-purity grade silica gel, with a pore size of 60 A, and a 70–230 mesh (Sigma-Aldrich) under two elution conditions, 2-propanol-EtOAc (Merck KGaA, Germany) at a ratio of 8:2, and H_2O-MeOH (Merck KGaA, Germany) at a 7:3 ratio (Lozano-Tovar et al., 2015). Two fractions were obtained, 2-propanol-EtOAc and H_2O-MeOH. The 2-propanol-EtOAc fraction was subjected to HPLC by a reverse phase chromatography (1260 Infinity System, Agilent Technologies) using a RP-C-18 column (Ascentis, Supelco, of 10 cm × 10 mm × 10 µm). The mobile phase was H_2O-MeCN: MeCN (in an 80 – 20: 70 ratio), it was applied in gradient for 70 minutes, with a flow of 1.5–2.5 ml/min. The injection volume was 300 µL.

2.6.2. Evaluation of fractions. To evaluate the effect of the fractions on fruit fly adults, all fractions were mixed with their food (hydrolyzed protein and sucrose in a 4:1 ratio), applying 100 µl of the treatment per repetition. The treatments were placed in plastic wells of 150 µl of capacity to feed the adults. Each treatment was constituted by one of 22 peaks obtained by HPLC fractionation; 0.6 mg of the fractions were resuspended in 300 µl of water. The experimental units were constituted by one-liter containers with tulle fabric as lid. Insects were kept at room temperature (28 ± 2ºC), at a relative humidity of 60% and 12 hours photoperiod.

2.7. Statistical analysis

Treatments were placed in a randomized block design, with three or four repetitions. newly emerged adults were distributed in each experimental unit (ten per unit). Data analysis was performed using ANOVA and the difference in means was established with Tukey's multiple range test, with a Honestly Significant Difference (HDS 0.5%). Homogeneity of variances and normality of the data were established as well. (Lozano-Tovar et al. 2015; Resquín-Romero et al. 2016; Khanday et al. 2018).

2.8. Identification of compounds

The chromatographic profiles of the compounds obtained by HPLC were collected. Each fraction was analyzed by a UHPLC system connected to an Ultimate™ 3000 masses (Thermo Fisher Scientific, U.S.A.), equipped with a C18 reverse phase analytical column (2.1 mm x 150 mm, 1.7 µm) (Kinetex Phenomenex, U.S.A.) and maintained at 40°C. The mobile phases used were water with 0.01% formic acid (A) and methanol with 0.01% formic acid (B). At a flow rate of 0.300 ml/min, the elution gradient was 10–90% of B during 14 min, with an equilibrium of 90% per 2 min, using an injection volume of 20 µl. Samples were resuspended in 1.0 ml of a mobile phase of a mix of A and B (50:50). Mass detection was performed in a Q exactive high-resolution mass spectrometer (Thermo Fisher Scientific, U.S.A.). The first experiment was performed in full MS mode at a resolution of 70000, with a 67 − 1,000 m/z range. The orbitrap was equipped with an electrospray ionization source (ESI), operated in a positive mode (ESI^+,) with a spray voltage of 3.5 kV, a capillary temperature of 280 ℃ and a heating temperature of 460℃. In a first run, the retention times and the target ions were established, and the ions corresponding to both the hydrogen and the sodium adducts were chosen. This was carried out to establish the molecular ion of the compound. A second run

was made, in which the mass spectrometer was set in the mass/mass mode; for this procedure, hydrogen was chosen as adduct using the selective ion mode (SIM); afterwards, the higher-energy C-trap dissociation (HCD) was applied, where energies of 10, 30, 50, 70 and 100 arbitrary units were set to generate the fragmentation of the hydrogen adducts. The compounds were tentatively identified by analyzing the data obtained from the orbitrap and using the exact masses and fragmentation patterns of the compounds.

3. Results

3.1. Selection of microorganisms due to their insecticidal capacity

Statistical differences between isolates were found ($F_{9,39}$ = 31.74, P = 0.0000). The concentrated supernatants obtained from the *Metarhizium* spp. isolates showed higher insecticidal activity than those obtained from the *Beauveria* spp. isolates. 66 hours after the treatment was distributed, the average mortality produced by *Metarhizium* spp. was 76.4 ± 3.2%, while the average mortality recorded by the *Beauveria* spp. isolates was 25.2 ± 3.7%.. From all this isolates, MT008 showed the highest mortality (95.5 ± 2.5%) (Fig. 1a). In the same way, statistical differences were found among isolates ($F_{9,39}$ = 4.77, P = 0.0007), when the non-concentrated extracts were evaluated. The highest mortalities were obtained with the extracts from *Metarhizium* MT005 (89.4 ± 6.1%) and MT008 (82.5 ± 13.1%); these were different compared to the control, that showed mortality values of just 4.5 ± 2.6%, five days after treatment was distributed (Fig. 1b).

3.2. Molecular identification MT005 and MT008 isolates.

The sequences generated for the MT005 and MT008 corresponded to *Metarhizium robertsii*. No differences were observed between sequences of both isolates. Using the BLASTn function, the closest match of the ITS region was found with the strain of *M. anisopliae* (ARSEF 488/ FJ609303.1: 99%). However, the EF-1α (with the closest match being *M. robertsii* ARSEF 9779/ MK156068.1: 99.85%) and the Btub gene sequences (with the closest match being *M. robertsii* ARSEF 23/ XM_007820079.1: 100%) confirmed the species to be *M. robertsii*. The sequences obtained were deposited in the GenBank (ITS: Mt005 - MW820167, MT008 - MW820168; EF-1α: MT005 - MW831678, MT008 – MW831679; Btub: MT005 – MW831676, MT008 - MW831677). The neighbour-joining phylogeny of the concatenated

sequences of the three regions, grouped MT005 and MT008 with *M. robertsii* in the same clade with a high bootstrap value (99%) (Fig. 2). *M. anisopliae* was found in a closely clade with *M. brunneum* and *M. robertsii* with high percentages of bootstrap (78%) (Fig. 2).

3.3. Evaluation of the effect of environmental factors on the insecticidal activity of crude extracts obtained from Metarhizium robertsii MT005 and MT008 isolates

The effect of temperature on insecticidal activity of the crude extracts obtained from MT005 and MT008 was evaluated and statistical differences were found ($F_{8,35}$ =18.20 P < 0.0000). The insecticidal activity of the MT008 isolate was not affected by temperature. The crude extract obtained from MT008 subjected to 30°C ± 2ºC for 3 hours, 50°C for 3 hours and 120ºC for 20 minutes maintained its insecticidal activity above 85%. On the other hand, the insecticidal activity of the extract obtained from MT005 was affected when it was exposed to 120ºC for 20 minutes, reducing its activity to 67%, with no significant differences between this treatment and the control. Furthermore, no negative effects were observed on the insecticidal activity of the extracts when these were exposed to solar radiation for four hours. Mortality of *A. obliqua* adults treated with MT008 and MT005 extracts, was higher when exposed to solar radiation, with values of 95.0 ± 2.9% and 82.5 ± 11.8%, respectively. The effect of storage time of the crude extract was evaluated as well. No statistical differences among treatments were found at environmental conditions (30ºC in the shade during 0, 24, 48 and 72 hours) in the MT008 extract. The insecticidal activity of the extract obtained

from MT008 registered the highest mortality values ranging between 86.7 ± 8.8% and 96.7 ± 3.3% (Fig. 3).

3.4. Fractionation and evaluation of the insecticidal activity of fractions obtained from crude extracts of Metarhizium robertsii MT008

The evaluation of dialyzed and adialyzed fractions indicated that the insecticidal activity was retained in the dialyzed fraction, which caused a mortality 90% when evaluated, while the adialyzed fraction showed a mortality of just 24.7% (Fig. 4). The evaluation of organic fractions in normal phase indicated that the highest activity was retained in the fractions obtained with 2-propanol-EtOAc (8: 2 ratio), causing mortalities of 80%,48 hours after treatment administration (Fig. 4).

HPLC fractionation of the active fraction 2-propanol-EtOAc (8: 2 ratio) obtained from the MT008 isolate extract, displayed 11 defined peaks and 11 fractions. The evaluation of the insecticidal capacity of the organic fractions obtained by HPLC indicated that the extract obtained from the isolate MT008 had a compound with high activity on adults of *A. obliqua* (P13, destruxin A) causing massive mortality of up to 100%, 48 hours after treatment administration. Furthermore, two other compounds with medium activity were found (P11, destruxin A2 and P18, destruxin B), showing mortalities between 60.0 and 81.3% after 48 hours(Figs. 5A and 5B).

3.5. Identification of the compounds obtained from the MT008 chromatographic profile.

From the chromatographic profile of the MT008 extract, 12 destruxins belonging to groups E, D, A and B were determined (Table 3). The mass/mass spectra obtained with collision energies identified these as destruxin E-diol, destruxin D, destruxin D1, destruxin D2, destruxin A2, destruxin A, destruxin A3, dihydrodestruxin A, desmB, destruxin B2, destruxin B, and destruxin B1. The mass/mass spectra of active destruxin (A, A2 and B) is show in Fig. 6.

Table 3
Destruxins found in the extract obtained from *Metarhizium robertsii* (MT008).

Peak	molecular mass		Fragmentation ions	Molecular formula	Compound name
	$[M + H]^+$	$[M + Na]^+$			
P6	612.35715	634.33850	594, 499, 471	C29H49N5O9	Destruxin E-diol
	624.35767	646.33905	511, 483, 370	C30H49N5O9	Destruxin D
	638.37347	660.35492	525, 497, 384	C31H51N5O9	Destruxin D1
P9	610.37823	632.35962	592, 497, 469	C30H51N508	Destruxin D2
P11	564.33710	586.31848	451, 423, 324	C28H45N5O7	Destruxin A2
P13	578.35223	600.33374	550, 465, 437	C29H47N5O7	Destruxin A
P14	566.35303	588.33441	467,439,340	C28H47N5O7	Destruxin A3
P15	580.36841	602.34967	552, 467, 439	C29H49N5O7	Dihydrodestruxin A
P16	580.36835	602.34973	552, 467, 439	C29H49N5O7	DesmB
P17	580.36792	602.34930	552,467,439, 368	C29H49N5O7	Destruxin B2
P18	594.38318	616.36383	566, 481, 453	C30H51N5O7	Destruxin B
P19	608.39996	630.38177	495,467	C31H53N5O7	Destruxin B1

4. Discussion

Metarhizium species are well known entomopathogens for their role in biological pest control. For the development of biopesticides based on these species, it is necessary to have a correct identification of the isolates. Phylogenetic studies with molecular tools have revealed the existence of closely related species in the *Metarhizium* group, corresponding to cryptic species of *M. anisopliae* (Mayerhofer et al., 2019). Four species are known as part of the clade PARB (*M. pingshaens, M. anisopliae*, *M. robertsii* and *M. brunneum*) (Rehner & Kepler, 2017, Mayerhofer et al., 2019) but is not possible to distinguish between this species using the marker (ITS), consequently, several assignments of *Metarhizium* species as

biocontrol agents (BCA) have required correction (Mayerhofer et al., 2019). In this work, the use of concatenated markers EF-1alpha and beta-tubulin made it possible to identify the isolates Mt005 and Mt008 as *M. robertsii*.

Of all the extracts evaluated, the one from the isolate MT008 showed high effect as insecticidal compound. Therefore, this strain, characterized as *M. robertsii* is a potential source to generate bioproducts for the control of adults of *A. obliqua* in an efficient way, due to the rapid action of its extract and its tolerance to stress factors such as sunlight, temperature, and storage time. The concentrated extract of MT008 caused 95.5% of mortality, 48 hours after treatment. These results suggest a reduction in populations due to the rapid action of the compounds once they are ingested by the insects; these results agree with those obtained by Lozano-Tovar et al (2015), who found that crude extracts of the strain *Metarhizium brunneum*, were efficient in the control of *C. capitata* under laboratory conditions, obtaining mortalities above 90%,48 hours after exposure. In addition, the species of *Metarhizium* are characterized by the production of secondary metabolites such as destruxins (Pedras et al. 2002). Some destruxins have been referenced with high insecticidal activity on fruit flies like *Ceratitis capitata* and *Bactrocera olea* (Lozano-Tovar et al. 2015; Yousef et al. 2013).

In general, the extract of the isolate MT008 showed higher insecticidal activity when it was subjected to 50°C for 3 hours and 120^{o}C for 20 minutes. According to Skrobek et al (2008), the temperature is a factor that strongly influences the decomposition of some destruxins, particularly dtxE. Lozano-Tovar et al (2015), showed that destruxin A2 is susceptible to high temperatures, meanwhile the insecticidal activity of destruxin A is maintained above 90% at 120°C for 20 minutes. Yousef et al (2014), showed thermostability of the insecticidal activity of extracts obtained from a *Metarhizium brunneum* when this extract was exposed to 100°C for 3 hours. In this work, solar exposure increased the concentration of the extract obtained from MT008 due to the effect of evaporation, without an adverse impact on its effectiveness as insecticide. The extract of MT005 however, showed a negative effect due to the sensitivity of its compounds at a high temperature. On the other hand, the effect of ultraviolet light on insecticidal compounds depends on its chemical structure, exposure time and wavelength (Soliman 2012). The insecticidal activity of the extracts in this study, was not affected by direct solar exposure. The insecticidal activity is influenced by the exposure time as this increase the concentration of the compounds, thus increasing mortality. In general, the insecticidal properties of the crude extracts were maintained during the 72 hours of storage under normal environmental conditions at 30^{o}C ± 2^{o}C. It is important to highlight this result, since this indicate that these compounds maintain their insecticidal activity in the field for a period longer than 72 hours.

Chromatographic profile and HPLC fractions revealed 22 compounds, twelve of these compounds were identified as destruxins as follows: Destruxin E-diol, destruxin D, destruxin D1, destruxin D2, destruxin A2, destruxin A, destruxin A3, dihydrodestruxin A, desmB destruxin, destruxin B2, destruxin B, and destruxin B1. Three of these destruxins: (destruxin A, destruxin A2, destruxin B) showed insecticidal activity against *A. obliqua* 48 hours after ingestion, with mortalities of 100%, 60% and 81.3% respectively.

Destruxins have a strong insecticidal activity against a wide range of insects that are considered pests. The toxicity of this group of metabolites is attributed mainly to its activity of forming ionophoric and lipophilic complexes with the cell membrane of insects, compromising its structural integrity. Furthermore, as part of the mechanism of mycosis in insects, destruxins destroy the mitochondria, inhibit fluid secretion of the Malpighi tubes, and cancel the immune response in the hemolymph, (Mustafa & Kaur 2013). Insect susceptibility to destruxins is variable, but in general, dtxs A and E seem to be more toxic to *Galleria* larvae than dtxs D and B. Similarly, studies have shown that larvae of *Musca domestica* (Diptera: Muscidae), are more susceptible to dtx E than to dtxs A or B (Vey et al. 2001); and research on adults of the Mediterranean fly (*C. capitata*) indicated that this insect is susceptible to dtxs A and A2 produced by *M. brunneum* (Lozano Tovar et al. 2015).

Abbreviations

Destruxin (dtx)

Declarations

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Author Contribution Statement

MDLT Conceived, designed, and wrote the manuscript. KLBA and GMPM conducted experiment. LASLT and GPBC contribute to write methodology and análisis of molecular results. All authors read and approved the manuscript.

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Ethical Statement

Authors confirm that this manuscript has not been published elsewhere and is not under consideration by another journal. No data have been fabricated or manipulated (including images) to support your

conclusions. No data, text, or theories by others are presented as if they were the author's own.

Authors whose names appear on the submission have contributed sufficiently to the scientific work and therefore share collective responsibility and accountability for the results.

All coauthors have approved this manuscript and agree with its submission to your journal.

The authors confirm that they have the authorization of the intellectual property department of the Corporación Colombiana de Investigación Agropecuaria AGROSAVIA to publish the information contained in the manuscript.

Moreover, all coauthors state that there are no conflicts of interest to declare.

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Figures

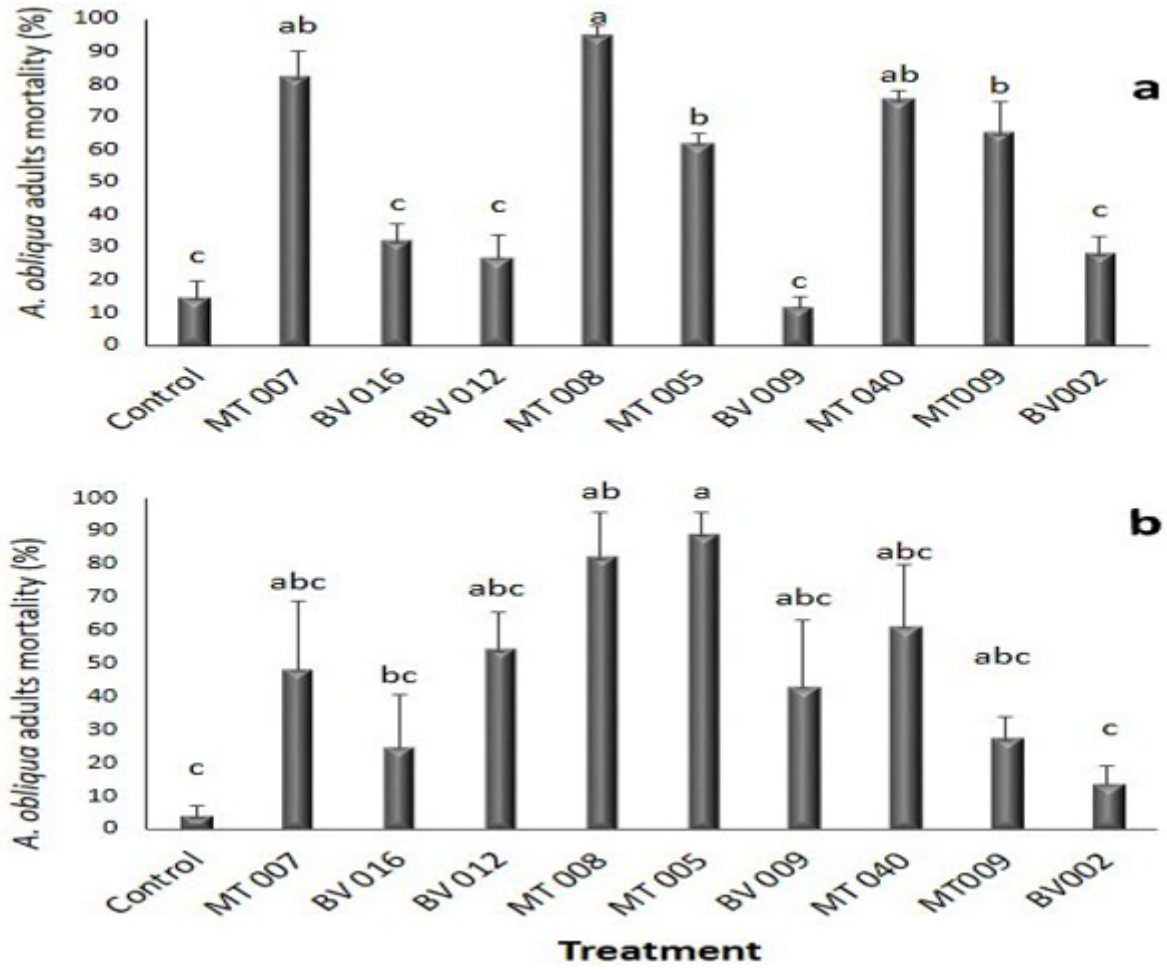

Figure 1

Evaluation of crude extracts obtained from *Metarhizium* spp. and *Beauveria* spp. on adults of *Anastrepha obliqua*. a) adult mortality (Mortalty % ± SE Mean), with concentrated supernatants at 66 hours after treatment. b) adult mortality (Mortalty % ± SE Mean), with non-concentrated supernatants at five days after treatment. Treatments with the same letter do not differ statistically. A multiple range test was run, using Tukey's Honestly Significant Difference (HDS 0.5%).

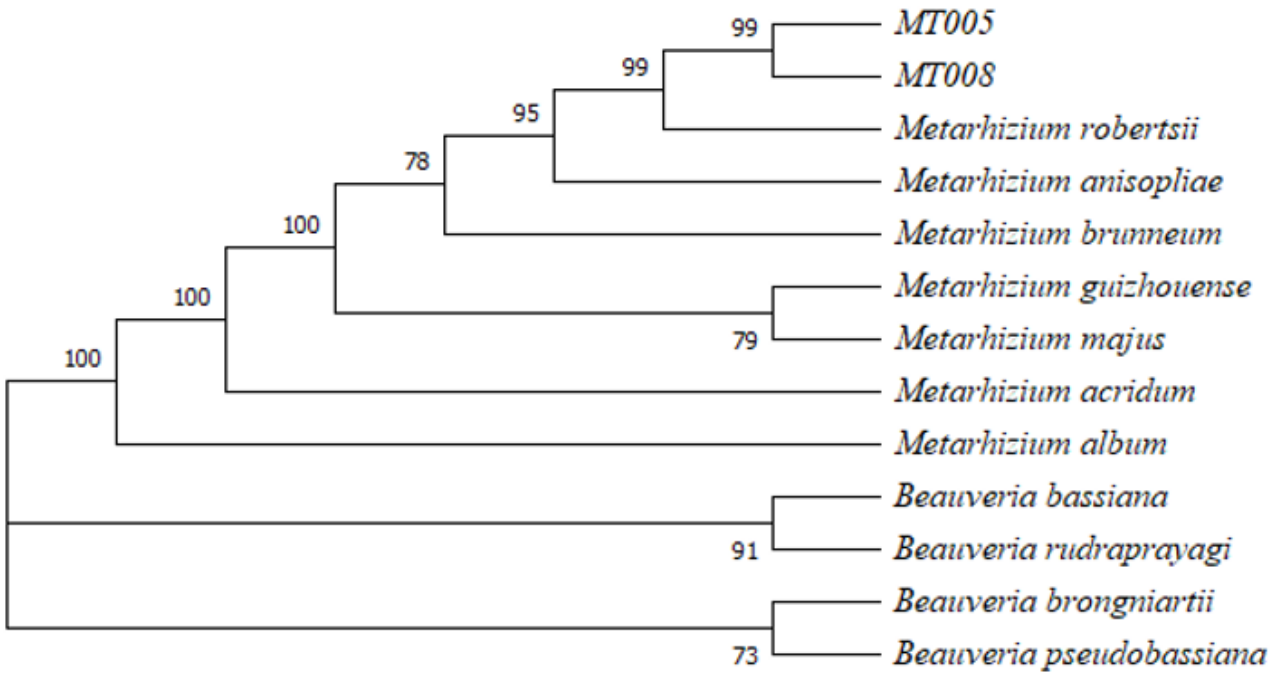

Figure 2

Phylogenetic analysis of concatenated partial sequences of ITS, Btub and EF-1α. Numbers at branches represent bootstrap values. Tree was inferred using the Neighbor-Joining method.

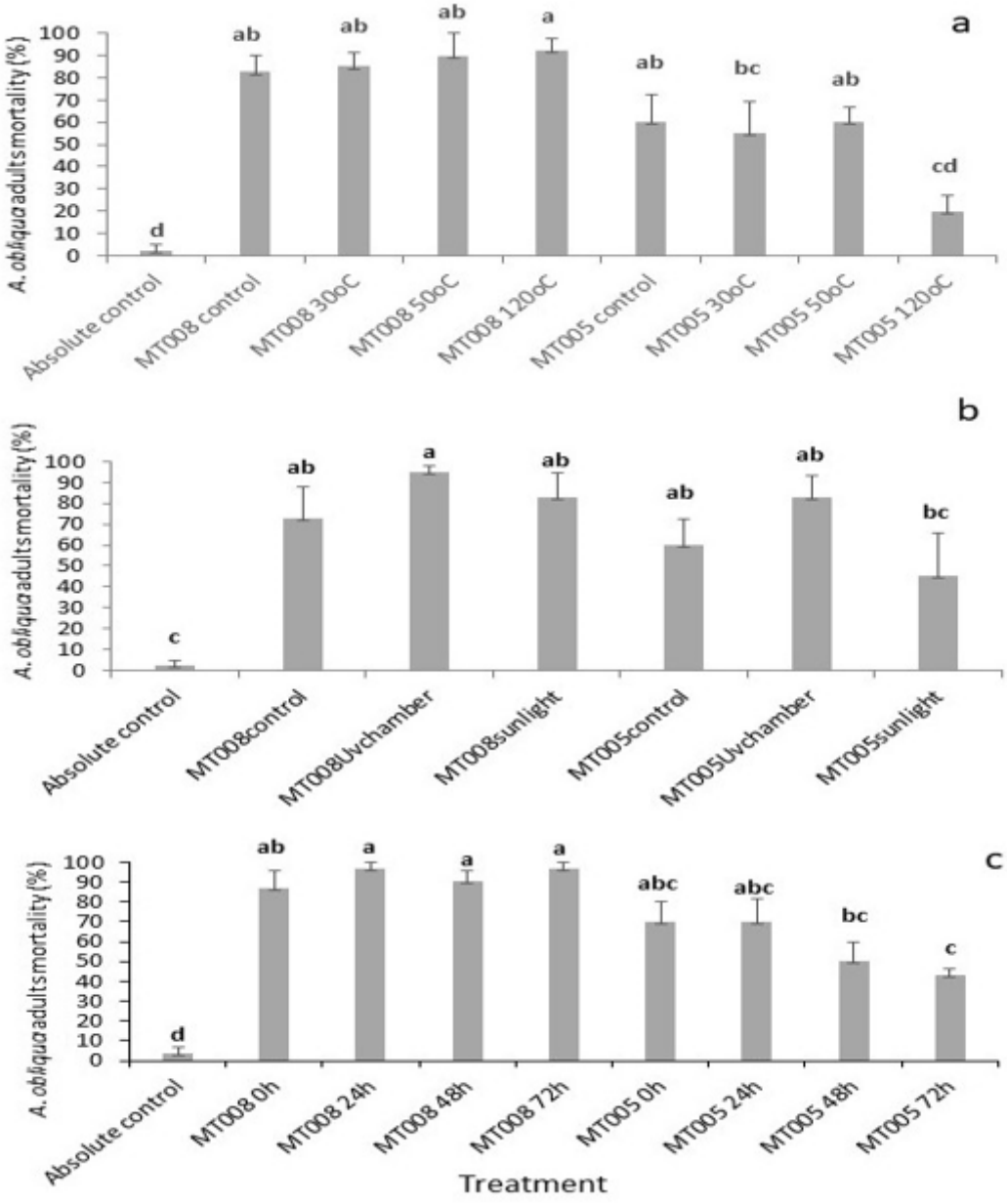

Figure 3

Effect of environmental factors on the insecticidal activity of crude extracts obtained from *Metarhizium robertsii* MT008 and MT005. a) Temperature, b) Sun light and ultraviolet light. c) Storage under environmental conditions (30oC). All the graphs show the mortality of *A. obliqua* 72 hours after treatment administration (Mortality % ± SE Mean). Treatments with the same letter do not differ statistically. A multiple range test was run, using Tukey's Honestly Significant Difference (HDS 0.5%).

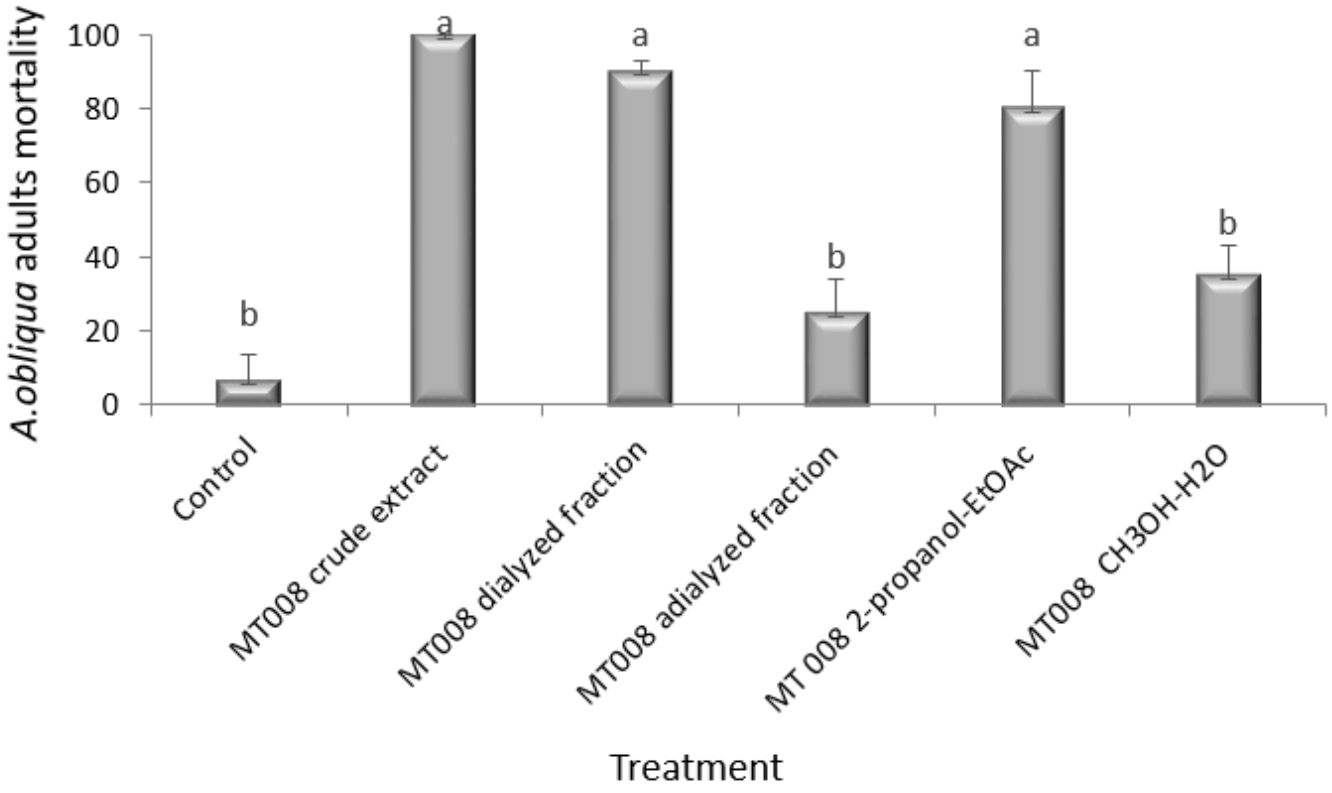

Figure 4

Evaluation of dialyzed, adialyzed and the organic fractions in normal phase with 2-propanol-EtOAc (8: 2 ratio) and H2O-MeOH (7:3 ratio). The graph shows the mortality of *A. obliqua* 48 hours after treatment administration (Mortality % ± SE Mean). A multiple range test was run, using Tukey's Honestly Significant Difference (HDS 0.5%).

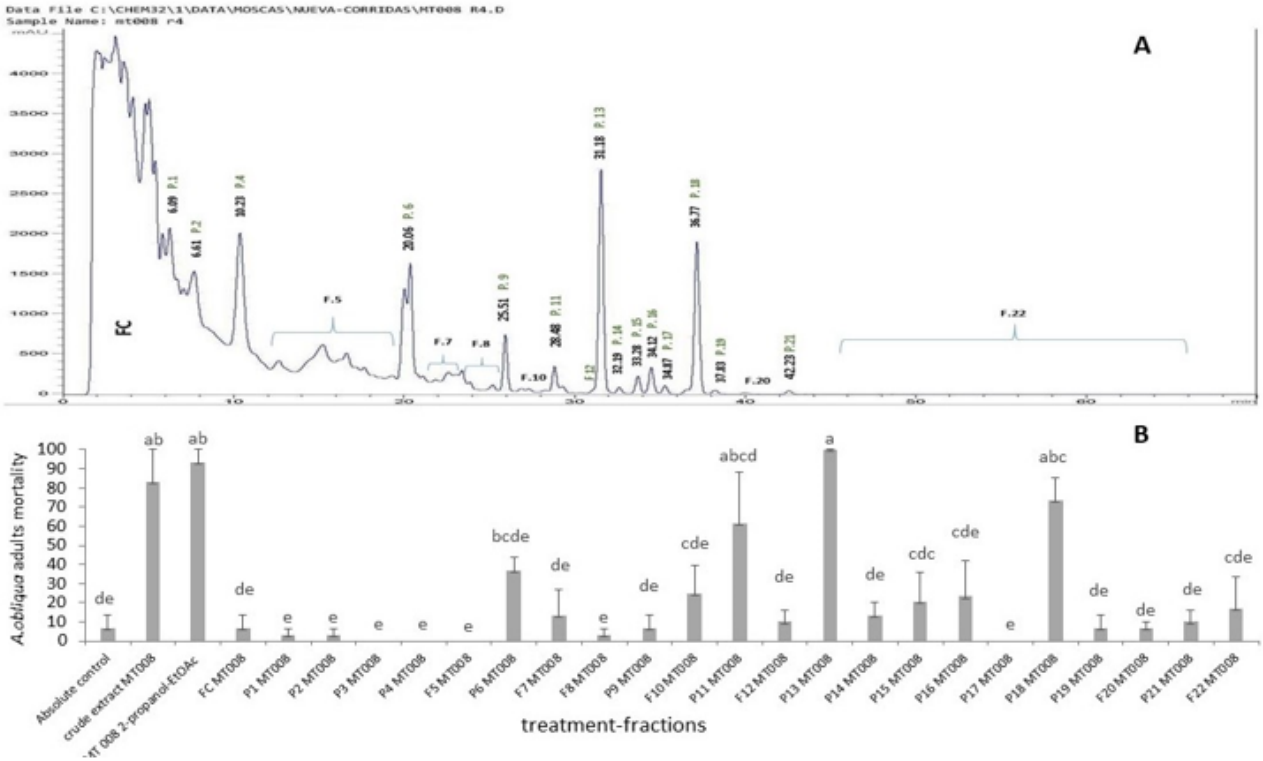

Figure 5

Chromatographic profile and evaluation of the HPLC fractions obtained from the extract of *M. robertsii* isolate MT008 by reverse phase on fruit fly adults, using H2O-CN as mobile phase: CN (80-20: 70), 1.5-2.5 ml/min and a run time of 70 minutes. (a) Chromatographic profile of MT008. (b) Insecticidal activity of the compounds obtained by HPLC from MT008 extract. The graph displays the average mortality of fruit fly adults on three repetitions 48 hours after treatment administration. (P: defined peak, F: fraction, non-defined peak)

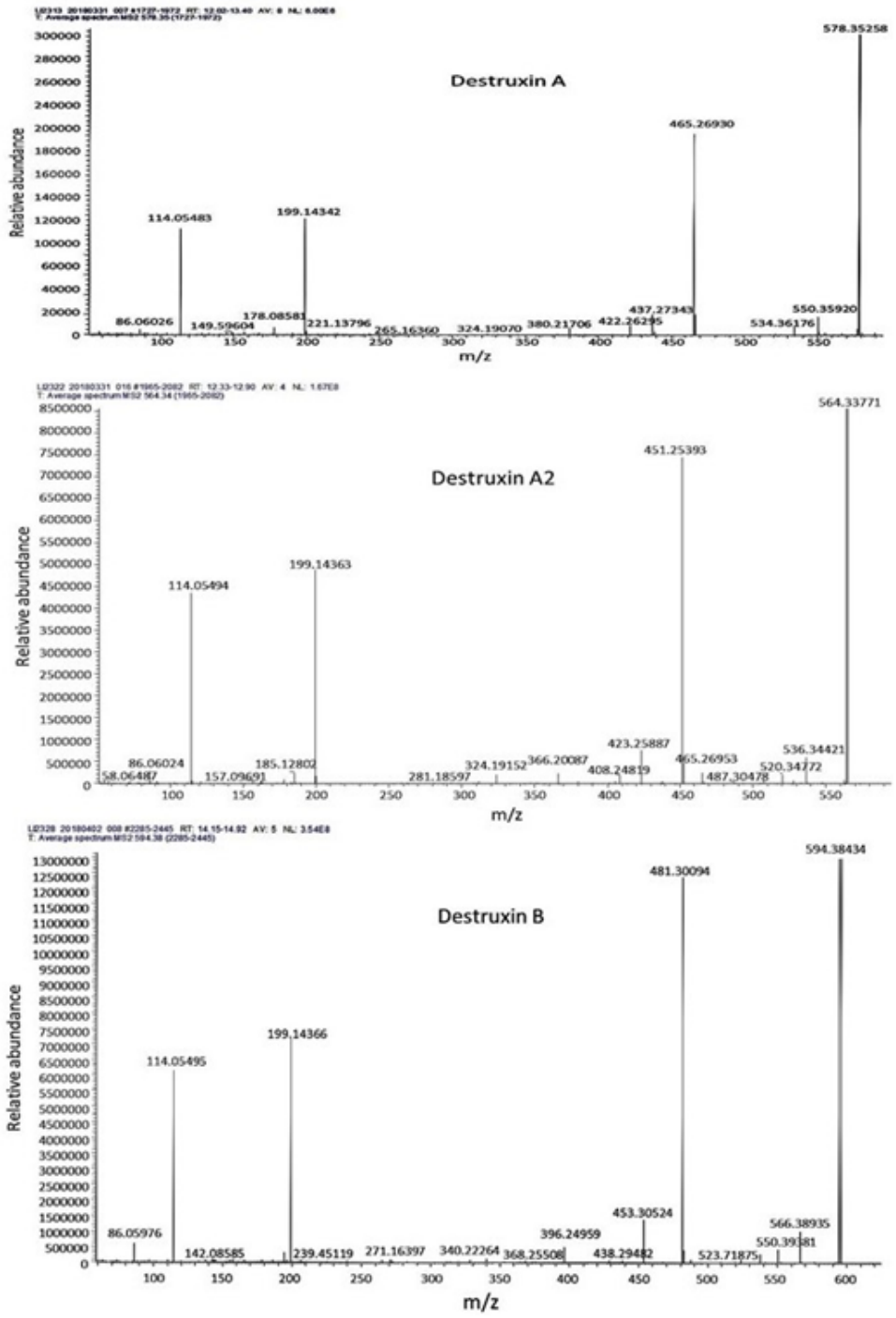

Figure 6

Mass/mass spectra profiles of active destruxins (A, A2 and B), obtained with several collision energies (10, 30, 50, 70 and 100) for the hydrogen adduct of the peaks analyzed