ANTIOXIDANT ACTIVITY AND INHIBITION OF ACETYLCHOLINESTERASE OF INSECT-ASSOCIATED FUNGI FROM THE CLOUD FOREST OF CENTRAL VERACRUZ, MEXICO†

[ACTIVIDAD ANTIOXIDANTE E INHIBICIÓN DE LA ACETILCOLINESTERASA DE HONGOS ASOCIADOS A INSECTOS DEL BOSQUE DE NIEBLA DEL CENTRO DE VERACRUZ, MÉXICO]

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SUMMARY

Background. The central zone of Veracruz state in Mexico, includes areas of cloud forest in which some microbial interactions such as insect-associated fungi can be found, like *Isaria*, *Paraisaria*, *Ophiocordyceps*, *Paecilomyces* and *Cordyceps* genera. **Objective**. To isolate entomopathogenic fungi from the cloud forest of Veracruz and determine their antioxidant and acetylcholinesterase inhibitor potential. **Methodology**. Mycelium covered pupae and insects and those that present growth of fruiting bodies were collected. From these, six entomopathogenic fungi were purified and identified by morphology and sequencing of the ITS region of rDNA and maximum likelihood analysis and Bayesian inference. These strains were propagated by batch cultures using liquid fermentation. The culture broth and biomass were extracted to evaluate the antioxidant capacity, acetylcholinesterase inhibition and quantify flavonoids and total phenols. **Results**. A total of six different strains of insect-associated fungi were identified, including the genera *Cordyceps*, *Beauveria* and *Clonosthachys*. The strain CIMA_225, identified as *Cordyceps takaomontana*, represent the first report of these fungi associated with a Lepidoptera pupa in the ecological reserve La Martinica, Banderilla, Veracruz, Mexico. The *C. takaomontana* culture broth extract presented higher antioxidant capacity than the control antioxidant Trolox, against ABTS and DPPH radicals, this antioxidant capacity was related to the content of flavonoids and total phenols on the extract. Biomass extracts from samples CIMA_256 to CIMA 258, identified as *Beauveria bassiana* and culture broth extracts from CIMA_259 (*Clonosthachys rosea*) and CIMA_260 (*C. rogersoniana*) presented higher percentages of inhibition of acetylcholinesterase when compared to galantamine as positive control. **Implications**. These strains can be considered candidates for a deeper chemical-biological study and contribute to the knowledge of novel sources of bioactive fungal metabolites. **Conclusion**. The insect-associated fungi extracts reported in this work showed antioxidant and acetylcholinesterase inhibitory potential, therefore their metabolites can be used in biomedical and insecticidal applications.

Key words: Bioprospecting; Bioactive fungi; *Cordyceps* sp.; *Beauveria* sp.; *Clonostachys* sp.

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RESUMEN

Antecedentes. La zona central del estado de Veracruz, México, incluye áreas de bosque mesófilo de montaña (bosque de niebla), en este hábitat hay interacciones microbianas como la de hongos asociados a insectos, entre ellos, *Isaria*, *Paraisaria*, *Ophiocordyceps*, *Paecilomyces* y *Cordyceps*. **Objetivo.** Aislar hongos entomopatógenos del bosque de niebla de Veracruz y determinar su potencial antioxidante e inhibidor de acetilcolinesterasa. **Metodología.** Se recolectaron pupas e insectos cubiertos de micelio y aquellos que presentaron cuerpos fructíferos. A partir de ello, se purificaron seis hongos entomopatógenos y se identificaron por morfología y secuenciación de la región ITS del ADNr y análisis de máxima verosimilitud e inferencia bayesiana. Estas cepas se propagaron mediante cultivos en lote utilizando fermentación líquida, posteriormente se extrajo el caldo de cultivo y biomasa, para evaluar la capacidad antioxidante, inhibición de acetilcolinesterasa y cuantificar flavonoides y fenoles totales. **Resultados.** Se identificaron seis cepas de hongos asociados a insectos, incluyendo los géneros *Cordyceps*, *Beauveria* y *Clonosthachys*. *Cordyceps takaomontana* CIMA_255, es el primer reporte asociado a una pupa de lepidóptera en la Reserva Ecológica La Martinica, Banderilla, Veracruz, México. El extracto de caldo de cultivo de *C. takaomontana* presentó capacidad antioxidante superior al antioxidante control Trolox, en contra de radicales ABTS y DPPH, dicho potencial antioxidante se relacionó con el contenido de fenoles totales. Los extractos de biomasa de *Beauveria bassiana* CIMA_256 a CIMA_258 y los extractos de caldo de cultivo de *Clonosthachys rosea* CIMA_259 y *C. rogersoniana* CIMA_260, mostraron los más altos porcentajes de inhibición de AChE comparado con el control de galantamina. **Implicaciones.** Estas cepas son candidatos para su estudio químico-biológico y con ello contribuir al conocimiento de nuevas fuentes de obtención de metabolitos bioactivos. **Conclusión.** Los extractos de hongos asociados a insectos reportados en este trabajo, mostraron potencial antioxidante e inhibidor de acetilcolinesterasa, por consiguiente, sus metabolitos pueden utilizarse en aplicaciones biomédicas e insecticidas.

Palabras clave: Bioprospección; Hongos bioactivos; *Cordyceps* sp.; *Beauveria* sp.; *Clonostachys* sp.

INTRODUCTION

The study of entomopathogenic fungi has been centered on certain genera like *Cordyceps*, *Metacordyceps*, *Elaphocordyceps* and *Ophiocordyceps*, which includes the anamorphic species (*Beauveria* Vuill., *Isaria* Pers., *Lecanicillium* W. Gams et Zare; *Metarhizium* Sorokin; *Tolypocladium* W. Gams, *Hirsutella* Pat., *Hymenostilbe* Petch, *Paraisaria* Samson et B.L. Brady, *Synglíocladium* Petch., *Paecilomyces* Bainier., *Polycephalomyces* Kobayasi y *Verticillium* Nees) (Hodge, 2003; Sung *et al*., 2007; Pérez-Villamares *et al*., 2017). Nevertheless, the fungi that are associated with insects in other ways like opportunistic pathogens or saprophytes and share the same habitat are understudied. Moreover, entomopathogenic and other insect-associated fungi have a main role in the decomposition and availability of chitin in the environment (Deaver *et al*., 2019). Among the microorganisms that could be associated with insects are mainly bacteria, viruses and fungi. The association could be pathogenic or symbiotic (mutualism, commensalism or parasitism) and normally are transmitted between insects, by a plant host or by the soil (Coolen, der-Molen Magda and Welte, 2022).

The central zone of Veracruz state in Mexico includes zones with the presence of cloud forests in localities such as Xalapa (19º32’24’’N 96º55’39’’O), Banderilla (19° 35′ 27″N, 96°56′37″O) and Coatepec (19°27′13″ N, 96°57′ 32″O), humid and warm climates are present in these areas with an altitude between the 900 and 2300 m, which favor the good growth of vegetation and funguses and the interactions between those microorganisms and some other life forms. In this area *Isaria fumosorosea* (Pérez-Silva, 1978), *Paraisaria gracilis*, *Ophiocordyceps entomorhiza* (Chacón and Guzmán, 1995), *Ophiocordyceps melolonthae* (Guzmán *et al*., 2001), *Paecilomyces tenuipes* (Lopez and Garcia, 2002), and *Cordyceps dipterigena* (Lopez and García, 2009) has been reported. Consequently, the region presents a high importance as a source of fungal resources with bioactive potential, particularly since there are reports of the search for bioactive compounds from the genus *Isaria*. In addition, *Isaria tenuipes* Peck (synonym: *Cordyceps tenuipes*) is a multi-infectious fungus that can parasitize insects from Order Lepidoptera, such infective capacity is due to certain active compounds as isariotonines, bauvericine and bauveriolides among others. Currently, *I. tenuipes* can be cultivated in controlled conditions and it is considered an edible and medicinal fungus due to its diverse beneficial pharmacological activities such as antiaging, antioxidant, antibacterial, antidepressive, antitumoral, blood glucose and lipid-lowering effects (Prommaban *et al*., 2022). Additionally,

Isaria farinose possesses antioxidant properties as well as the capacity to inhibit the growth of cancer cells (Chhetri *et al*., 2020). Furthermore, bioactivities like hypoglycemic, anti-inflammatory, antitumoral, antibacterial, antifungal, antioxidant and immunoprotective have been attributed to *Cordyceps militaris* (Das *et al*., 2021).

The species that conform the genera *Beauveria* are entomopathogenic fungi with a worldwide distribution that can parasitize more than 700 species of insects. Stuart *et al*. (2023) evaluated an interaction between a fungal consortium of *B. bassiana* and *Duponchelia foyealis* (Zeller) caterpillars observing that the main mechanisms employed by strains of *Beauveria* spp. consist in the production of antioxidant molecules and metabolites that increase the fungal virulence suggesting that the antioxidants contribute to evading the insect's immunological defenses. Also, Zhang *et al*. (2022) observed a reduction of acetylcholinesterase (AChE) activity of *Megalurithrips usitatus* after the application of *B. bassiana*. As well, species of *Clonostachys* have been considered as biological control agents for phytopathogenic fungi, nematodes and insects. Concerning the biological control on insects, compounds like ilicicolines C and E, and colletochlorin B has been related to the inhibitory activity of AChE (Han *et al*., 2020; Sun *et al*., 2020). Therefore, the objective of this work was to isolate entomopathogenic fungi from the cloud forest from Veracruz and determine their antioxidant and acetylcholinesterase inhibitor potential.

MATERIALS AND METHODS

Area of study

Three areas with a high coverage of cloud forest were selected: Macuiltepetl Hill (19°32’54” N, 96°55’09” W) and los Tecajetes Park (19°31′55” N, 96°55′51” E) in Xalapa, La Martinica Ecological reserve (19°35'20" N, 96°56'46" W) in Banderilla, Botanical Garden “Javier Clavijero” (19°29′0″ N, 97°9′0″ W) and Briones forest (19°50’70” N, 96°95’04” W) in Coatepec, all located in Veracruz State, Mexico. For the recollection of the samples, the sites previously mentioned were visited in different seasons between 2019 and 2021 in search of infected insects, implementing a type of opportunistic sampling. Finally, Petri dishes whit divisions and moisture paper were employed to transport the samples to the laboratory.

Mycelial isolation of insect-associated fungi

From the exposed mycelium or fruiting bodies on the collected pupae and insects, a sample was taken and inoculated into Petri dishes containing Potato Dextrose Agar (PDA) with 0.2 g/L of chloramphenicol and subsequently were incubated in the dark at 25 ± 2 °C until mycelial development. The resultant mycelial colonies were purified by the hyphal tip in Petri dishes with PDA medium under the same conditions previously mentioned.

Morphological identification of fungal strains

The collected exemplars were characterized by macroscopic observations considering the size of the stroma, color and position of the perithecium in the stromatic tissue or synnema present in the sample (Pérez-Villamares *et al*., 2017), the mycelial cultures were registered in our collection. The microscopic revision contemplates the measurement of diverse structures including perithecium, conidiophores, phialides and conidia. The identification of the species was carried out with support of specialized literature (Watanabe, 2002; Yokoyama, Yamagishi and Hara, 2005; Shrestha *et al*., 2014).

Molecular identification of mycelial culture of insect-associated fungi

DNA extraction was carried out employing XNAP-REDExtract-N-Amp™ Plant PCR Kit (Sigma-Aldrich). For each fungal strain after seven days of growth in PDA a disc of 0.5 mm was placed with 20 µL of extraction solution in Eppendorf tubes, after were incubated in a thermocycler (Bio-Rad mod T100) for 10 min at 65 °C and 10 min at 95 °C. When time had passed, 20 µL of dilution solution was added and let rest for 30 min at room temperature. Finally, 40 µL was transferred to clean tubes disposing of the organic phase and storage at 4 °C. From the genomic DNA of each fungal isolate, the ITS 1 and 2 regions of the nuclear ribosomal RNA (RNAnr) were amplified by direct PCR employing ITS1F and ITS4 primers as described by Mata *et al*. (2016). PCR verification was performed by comparing the amplicon size (650 bp) using a 1 kb DNA molecular marker. The amplicons were purified with Wizard (Promega®, USA) kit following the manufacturer's instructions. After this, were sequenced in Laboratorio de Secuenciación Genómica de la Biodiversidad del Instituto de Biología de la UNAM, obtaining sequences in both directions in an ABI3100 sequencer by sequencing reaction Big Dye Terminator 3.1 (Applied

Biosystems). The resulting sequences were compared with the NCBI GenBank database by Nucleotide Blast software (table 1) and were aligned on online software MAFFt v.7 (Katoh, Rozewicki and Yamada, 2019). Subsequently, were analyzed employing a maximum likelihood method on RAxMl v.7.2.6 (Stamatakis and Alachiotis, 2010), with the substitution model GTR+G and a bootstrap of 1000. A Bayesian inference analysis on MrBayer v.3.2.1 (Ronquist *et al*., 2012) was also made according to the evolutive model of Akaike obtained on jModelTest v.2.1.4 (Darriba *et al*., 2012), setting as starting values of maximum likelihood (BS) > 50% and Bayesian Posterior Probability (BPP) > 0,75.

Inoculum preparation and submerged fermentation

Each of the six molecularly identified fungal strains was propagated in 100 mL of Sabouraud Dextrose Yeast (SDY) Broth (g/L: dextrose 20, yeast extract 5 and peptone 5) at pH 5.6 in an Erlenmeyer flask of 500 mL, employing five discs of agar with mycelium after 20 days of growth on SDY agar, and was incubated for 15 days at 25 ± 2 °C. Once the pre-inoculum was obtained a 500 mL of submerged culture was carried out in five Erlenmeyer flasks containing 100 mL of Potato Dextrose Agar and 5 mL of pre-inoculum, then were incubated in a dark room for 14 days at 120 rpm, and 14 days in static way at 25 ± 2 °C. Once the incubation period of the submerged culture of the fungi was over, the biomass was separated from the culture broth by vacuum filtration. Subsequently, the two phases were frizzed and lyophilized, the dehydrated products were extracted by maceration and ultrasound bath with a mixture of chloroform:methanol (1:1) (Couttolenc *et al*., 2022). The organic extracts were evaporated to dryness in a rotatory evaporator at reduced pressure and storage at 4 °C until performance of the antioxidant capacity and acetylcholinesterase inhibition.

Antioxidant assays

The radical scavenging potential of the insect-associated fungal extracts was evaluated by ABTS and DPPH assays according to Couttolenc *et al*. (2022). Meanwhile, the metal ion-reducing antioxidant power of the insect-associated fungal extracts was evaluated by CUPRAC and FRAP methods.

[azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)] *ABTS assay.* To the formation of ABTS·+ radical, an equal amount of ABTS 7.4 mM and potassium persulphate ($K_2S_2O_8$) were mixed and let react for 16 h. Then the work solution was prepared with 1 mL of the mixture in 60 mL of methanol. For each lecture, 2.85 mL of work solution was transferred to the reaction cell a mixed with 150 µL of fungal extract or Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) standard and let react for 5 min, after that time the absorbance was measured at 734 nm. The calibration curve was made with Trolox as standard at concentrations of 6.25, 12.5, 25, 50 and 100 (µM). The fungal extracts were assayed at 1mg/mL, and the effective concentration 50 (EC_{50}) was calculated through the linear equation and is expressed as Trolox equivalents.

[1,1-diphenyl-2-picrylhydrazyl] DPPH assay. In this assay, a DPPH solution at concentration 0.1 mM in methanol/water 80% (v/v) was employed. For each measurement, 2.85 mL of DDPH solution was mixed with 150 µL of fungal extract or Trolox standard and the mixture was incubated at room temperature for 30 minutes and protected from light. After that time, the decrement of absorbance was measured at 517 nm. The calibration curve was obtained through Trolox standard at concentrations of 25, 50 75, 100, 150 and 200 µM. The fungal extracts were tested at a concentration 1 mg/mL and the EC_{50} was calculated based on the standard curve and are expressed as Trolox equivalents.

(Cupric Reducing Antioxidant Capacity) CUPRAC assay. Equal volumes (1 mL) of fungal extracts, $CuCl_2$ (10 mM), acetate buffer (1 mM, pH 7.0) and neocuproine (7.5 mM) were mixed and gently agitated, then the mixture was incubated in dark at room temperature (Apak *et al*., 2004). After 30 minutes, the absorbance was measured at 450 nm. The Trolox standard curve was made with standard concentrations of 50, 100, 200, 400 and 600 µM, and the concentrations of equivalents were calculated through linear regression.

[Ferric Reducing Antioxidant Power] FRAP assay. A work solution composed of acetate buffer (300 mM, pH 3.6), TPTZ solution 10 mM (in HCl 40 mM) and $FeCl_3 \cdot 6H_2O$ 20 mM in a ratio 10:1:1 respectively was prepared just before the start of the test and was incubated at 37 °C for 10 minutes. For the determination of the antioxidant capacity 900 µL of work solution was mixed with 120 µL of fungal extract of antioxidant standard and let react for eight minutes then the absorbances was determined at 593 nm (Knežević *et al*., 2018). The assay was carried out employing work solution as blank and ascorbic acid 5 µM as positive control.

For calculations of ion concentration, a standard curve of $FeSO_4 \cdot 7H_2O$ was made.

Flavonoid and Total Phenolic content

The Flavonoid content (FVC) was determined according to Chang *et al*. (2020) with some modifications and adapted to 96 wells microplates. A mixture composed of 30 µL of fungal extract (1 mg/mL) or quercetin standard (Sigma-Aldrich) with 90 µL of ethanol 95% (v/v), 6 µL of aluminum chloride 10% (v/v), 6 µL of sodium acetate 1 M and 168 µL of deionized water. The mixture was left to react for 40 minutes and after this time the absorbance was measured at 450 nm employing deionized water as blank. A standard curve of quercetin was constructed with concentrations of 2.5, 5, 10, 20, 40, 80 and 100 mg/L. The results were expressed as quercetin equivalents per milligram of extract (QE/mg).

For the determination of the Total Phenolic content (TPC), 200 µL of fungal extract of gallic acid standard was mixed with 2.6 mL of deionized water and 200 µL of Folin-Ciocalteu, after six minutes 2 mL of sodium carbonate 10.75% (w/v) was added to the mixture, after 30 minutes of reaction the decrement of absorbance at 760 nm was observed. The standard curve was made with gallic acid (on methanol 50%) at concentrations of 10, 20, 50, 70, 100 and 200 mg/L. The results were expressed as gallic acid equivalents per mg of sample (GAE/mg) (Couttolenc *et al*., 2022).

In vitro Inhibition of Acetylcholinesterase

The inhibition of acetylcholinesterase (IAChE) was carried out according to the Ellman method as reported by Ingkaninan *et al*. (2003) with some modifications. A starting solution consisting of 10 µL of fungal extract or standard, 20 µL of AChE and 150 µL of dithiobenzoate 6 mM on PBS 0,01 M, pH= 7.4 (DTNB) was made. Then the reaction started when 20 µL of acetylcholine iodide 15 mM was added. The absorbance of the mixture was read after 10 minutes at 414 nm. A solution of galantamine 3.5 mM was used as positive control. The results were expressed as percentage of IAChE as was calculated as shown in equation 1.

$$IAChE\ (\%) = \left(\frac{1 - ATest}{ABlank}\right) X\ 100$$

RESULTS AND DISCUSSION

Isolation and identification of insect-associated fungi

During the period between October 2019 to September 2021 a total of 45 samples, mainly pupae (with stromatic bodies and mycelium), also adult forms of beetles, earwigs, bumblebee, leafhopper another unidentified insect. From these samples 125 isolates were obtained, which were grouped in 24 morphotypes according to mycelial characteristics. Thus, through their microscopical and morphological characteristics 12 species of the phylum Ascomycota, among the identified genera are *Cordyceps*, *Beauveria*, *Clonosthachys*, *Pestaloptiosis*, *Neopestalotiopsis*, *Trichoderma*, *Monascus*, *Hypocrea* and *Fusarium* (Watanabe, 2002; Yokoyama, Yamagishi and Hara, 2005). Of them, 55% belong to the order Hypocreales and the remaining 45% belong to the Order Xylariales, Amphispharіales and Eurotiales. According to their previously reported information each fungal isolate was classificatied into the groups of entomopathogenic, opportunistic pathogens and saprophytes. For the entomopathogenic genera (*Cordyceps*, *Beauveria*, *Clonosthachys*) a molecular identification was carried out to confirm identity, the results of the comparison with NCBI Blast are shown in table 1 with their corresponding accession number.

Strains CIMA_255 and CIMA_259 were collected in Ecologic Reserve La Martinica (19°35'20" N, 96°56'46" W), CIMA_256 to CIMA_258 in Macuiltepetl hill (19°32’54” N, 96°55’09” W) and CIMA_260 in Briones forest (19°50’70” N, 96°95’04” W) all located in Veracruz State, Mexico. The phylogenetic reconstruction was made with molecular data of entomopathogenic and pathogenic fungi from the Cordycipitaceae, Clavicipitaceae and Bionectriaceae families representing the order Hypocreales. The maximum likelihood and the Bayesian Inference analysis results in the grouping of strain CIMA_255 was grouped in the same clade as *Cordyceps takaomontana* with values of 97/1. In addition, CIMA_256, CIMA_257 and CIMA_258 in a clade include sequences belonging to the complex *Beauveria caledonica*, with BS/BPP values of 99/1. Finally, strain CIMA_259 was related to the clade of *Clonostachys rosea* with values of 92/0.99 and CIMA_260 in the clade of *C. rogersoniana* with values of 100/0.98 (Figure 1).

Table 1. Insect-associated fungal isolates and their nearest BLAST result (ITS).

Strain key	Species	Host	Reference accession number	BLAST % Query coverage	BLAST % Identity	Accession Number
CIMA_255	*Cordyceps takaomontana*	Pupa/lepidoptera	MF361953.1	99.0	99.2	PP907162.1
CIMA_256	*Beauveria caledonica*	Hymenoptera	MT180427.1	99.0	99.0	PP938453.1
CIMA_257		Unidentified		100.0	99.2	PP938455.1
CIMA_258		Lepidoptera		100.0	99.2	PP952121.1
CIMA_259	*Clonostachys rosea*	Hymenoptera	MH862010.1	99.0	99.7	PP938457.1
CIMA_260	*Clonostachys rogersoniana*	Pupa/lepidoptera	OQ910711.1	93.0	100.0	PP938529.1

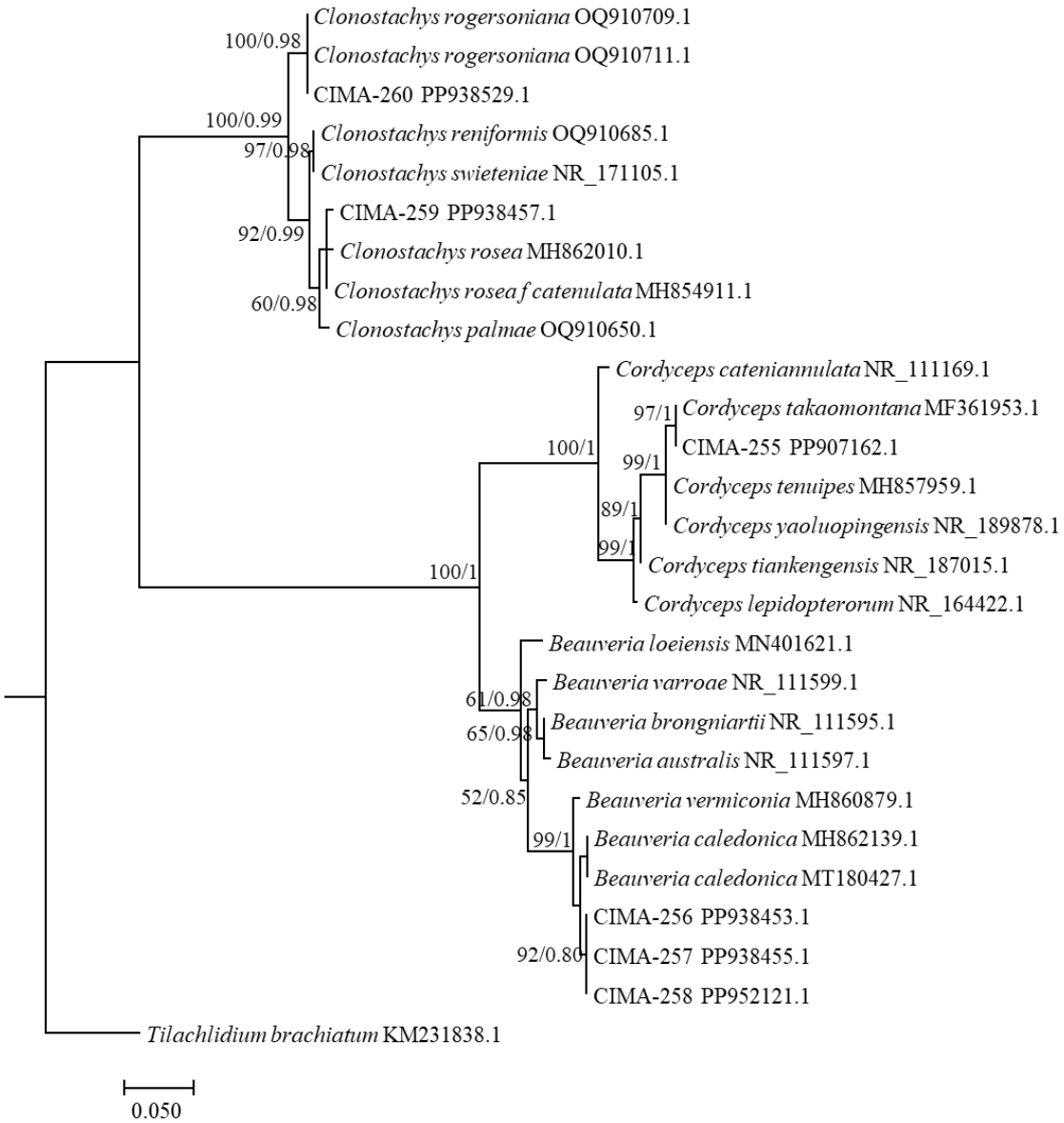

Figure 1. Maximum likelihood (ML) and Bayesian inference phylogeny of isolates representing the order Hypocreales, rooted to the outgroup species *Tilachlidium brachiatum*. Maximum likelihood bootstrap (BS) values >50% and Bayesian posterior probabilities (BPP) >0.75. Bar length indicates the number of substitutions.

Cordyceps takaomontana (Yakush et Kumaz) is a parasitic fungus of the pupae stage of some lepidoptera and was described for the first time by Kobayasi (1941). This species has been reported in Asian countries like Indonesia, Japan (Nikoh and Fukatsu, 2000), Australia and Ghana, as well, in South American countries: Brazil (D'Alessandro *et al*., 2014), Colombia (Castro-Pérez *et al*., 2013), Argentina (Darwinion Institute of Botanic, 2019), Bolivia, Ecuador and Paraguay, in Europe: Germany, Belgium, France, the Netherlands and United Kingdom (Samson, 1974) and North America (Bissett, 1979). In Mexico, the presence of the fungi has been reported by Pérez-Villamares *et al*. (2017) in Mexico State, therefore, the strain CIMA_255 identified as *C. takaomontana* coincides with previous reports for the anamorphic form, developing synnema with yellow foot and the upper section white with ramifications and dusty aspect (Pérez-Villamares *et al*., 2017; Del Valle Catania and Robledo, 2019). Therefore, this is the first report of *C. takaomontana* in the state of Veracruz, thus expanding knowledge about its distribution in Mexico.

The species of genera *Beauveria* are cosmopolitan entomopathogenic fungus which parasitize more than 700 species of insects. Since the establishment of the genera *Beauveria* in 1912 by Vuillemin, its taxonomic status was particularly based on classical morphological characteristics (Imoulan *et al*., 2017). Species like *Beauveria bassiana*, *B. brongniartii*, *B. velata*, and *B. amorpha* were originally described as pathogens of diverse species of insects (Samson and Evans, 1982). On the other hand, species like *B. vermiconia* and *B. caledonica* behave as saprophytes isolated from volcanic soils in Chile and moor soil in Scotland. Nevertheless, Glare, Jackson and Cisternas A. (1993) informed that *B. vermiconia* was a natural pathogen of *Hylamorpha elegans* (Coleoptera: Scarabaediae) larvae in Osorno, Chile. Also, has been demonstrated that *B. caledonica* is a natural pathogen to beetles from New Zealand and Scotland (Glare *et al*., 2008). In the same study, the authors regrouped two isolates labeled as *B. bassiana* (ARSEF 1567) and *B. amorpha* (ARSEF 2251) into a clade of *B. caledonica*, suggesting that the species has the potential to act as a pathogen for some insects, which is consistent with our isolates CIMA_256, CIMA_257 and CIMA_258, which we isolated from Hymenoptera and Lepidoptera cadavers.

The fungi *Clonostachys rosea* is known as a parasite of other fungi and nematodes as well as a saprophyte from soil, besides, Toledo *et al*. (2006) consider it as an entomopathogenic fungus of two leafhopper pests, *Oncometopia tucumana* and *Sonesimia grossa* (Hemiptera:Cicadellidae).

Similarly, Vega (2008) reports diverse fungal entomopathogens genera including *Acremonium*, *Beauveria*, *Cladosporium*, *Clonostachys* and *Isaria* as endophytic in coffee plants collected in Colombia, Hawaii and Puerto Rico. Of these, *Beauveria bassiana* and *Clonostachys rosea* showed entomopathogenic capacity against the coffee drill, *Hypothenemus hampei* (Ferrari). Furthermore, Yang *et al*. (2021) reported *Clonostachys rosea* as an inductor of mortality in adults of *Diaphorina citri*. Additionally, Rodrigues *et al.* (2022) report for the first time *C. eriocamporesii* and *C. byssicola* as natural pathogens of *Aedes aegyptii*. The above suggests the entomopathogenic capacity of the strains CIMA_259 and CIMA_260 identified as *Clonostachys rosea* and *C. rogersoniana* isolated from Lepidoptera dead husk, however, more *in vivo* studies are required to demonstrate it.

Antioxidant activity

The results obtained from the antioxidant capacity expressed as Trolox equivalents (TEAC) of the 12 extracts, both biomass and broth, corresponding to samples CIMA_255 to CIMA_260 both in radical scavenging (ABTS and DPPH) and metal ion reduction (FRAP and CUPRAC) methodologies are shown in table 2. For the ABTS radical scavenging capability assays the calculated EC_{50} for Trolox was 383.6 µM, being the extract from the broth of *Cordyceps takaomontana* (386.8 µM) the only sample to present a higher value than Trolox and the closest values were both extracts of *Clonostachys rosea* and the broth extract from *C. rogersoniana*. In addition, on the DPPH assay, the broth extracts of *Cordyceps takaomontana*, *Clonostachys rogersoniana* and *Beauveria caledonica* (CIMA_256) exhibit values of 284.7, 113.6 y 80.2 µM TEAC, respectively and the biomass extract of *B. caledonica* (CIMA_257) shown a value of 104.3 µM all of them superior to the calculated IC_{50} for Trolox standard.

For the evaluation of the antioxidant capacity regarding the reduction of copper ions, on the CUPRAC assay, an interval of Trolox concentrations from 50 to 600 µM was employed to compare the activity of the extracts, obtaining that the biomass extracts of *Clonostachys rosea* presents the higher value among the extracts (857.4 µM TE) followed by *Beauveria caledonica* (CIMA_257), *Cordyceps takaomontana* and broth

extracts of *Beauveria caledonica* (CIMA_256 and CIMA_258) with values of 592.8, 539.9, 542.2 and 522.5 µM of TE respectively. On the other hand, for the FRAP assay a solution of ascorbic acid 5 µM was used as a positive control, the results show that biomass extract of *Beauveria caledonica* (CIMA_257) was the only one with a higher activity than the positive control with 251.6 µM of Fe^{+2}, other samples with values close to the positive control were biomass of *Clonostachys rosea* and *Cordyceps takaomontana* (184.2 and 134.1 µM of Fe^{+2} respectively).

The antioxidant capacity observed on the insect-associated fungi extracts could be related to the flavonoids and total phenolic content (FVC and TPC) present in them. In Figure 2 can be observed the values of the FVC in a range of concentration from 183.1 to 325.3 EQ/mg of extract, and the TPC with a range from 110.7 to 1045.1 GAE/mg of extract. In this aspect, the culture media of *Cordyceps takaomontana* (CIMA_255), *Beauveria caledonica* (CIMA_256), *Clonostachys rogersoniana* (CIMA_260) and the biomass extracts from *Beauveria caledonica* (CIMA_258) and *Clonostachys rogersoniana* (CIMA_260) stand out with values of TPC of 1045.1, 546.2, 405.0, 376.2 and 367.3 GAE/mg respectively which justify the antioxidant capacity observed.

The antioxidant capacity and the therapeutic potential of fungi of the genera *Cordyceps* is well known (Olatunji *et al*., 2018; Das *et al*., 2021; Krishna, Ulhas and Malaviya, 2024). A first report informs that culture of *C. sinensis* extracts possess potent antioxidant and anti-lipidic peroxidation activity as well as the capacity to inhibit the cholesterol esters accumulation on macrophages by the suppression of LDL oxidation (Yamaguchi *et al*., 2001). Starting with that, Huang *et al*. (2013), produced exopolysaccharides through liquid fermentation of *C. sinensis* (Cs-Hkl), which exhibit significant antioxidant potential dependent on the protein content. In addition, Dong, Yang and Lian (2014) reported that *C. militaris* mycelium exhibits a higher chelating capability, reducing power and total antioxidant capacity compared to its fruiting body. In other work, Wang *et al*. (2015) compared the antioxidant activity of a wild *C. sinensis* against cultured mycelium, observing that aqueous extracts from the mycelium have a higher capacity to scavenge DPPH and hydroxyl radicals as well as higher reducing power. Moreover, Chiriví *et al*. (2017) reported the presence of polyphenols in *C. nidus* through a metabolomic profile, which opens an opportunity to explore with more detail the antioxidant capacity and the conditions that enhance that characteristic. Furthermore, mycelial extract from *Isaria tenuipes* (anamorph of *Cordyceps tenuipes*) cultured on liquid media shows a high antioxidant activity against DPPH radicals (IC_{50} ~ 0.0581 mg/mL) (Kenkhunthot and Labua, 2020). In addition, Prommaban *et al*. (2022) reported antioxidant activity against ABTS and DPPH radicals (IC_{50} = 0.22 and 0.05 mg/mL respectively) as well as ferric ion reducing capacity (EC: 95.3 $FeSO_4$/g of extract) from the fruiting body of *I. tenuipes*. Stuart *et al*. (2023) evaluated the interaction between a fungal consortium of *Beauveria bassiana* and *Duphonchelia foyealis* (Zeller) caterpillars observing that the main mechanisms employed by *Beauveria* spp. strains to enhance their virulence consist in the production of antioxidant molecules and other metabolites, suggesting that the antioxidants contribute to evading the defenses of the insect immunological system. On the other hand, *Beauveria bassiana* endophyte strain enhances *in vitro* antioxidant capacity (FRAP and TEAC) of *B. bassiana* inoculated lettuce extracts (Macuphe, Oguntibeju and Nchu, 2021). The interaction between irrigation interval and the inoculation of *B. bassiana* significantly affects the flavonol content of onion bulbs and their antioxidant activity on FRAP assay (Gana, Etsassala and Nchu, 2022).

Inhibition of acetylcholinesterase

The assay was standardized according to the Ellman method with some modifications. The results obtained from biomass and broth extracts from insect-associated fungi are displayed in Figure 3. First, a percentage of inhibition of 39.7% corresponding to the galantamine 3.5 µM as positive control could be observed. Regarding the broth extracts, *Clonostachys rosea* CIMA_259 and *C. rogersoniana* CIMA_260 were the closest to the galantamine with values of 27.7 and 25.3% respectively. On the other hand, among biomass, the extracts corresponding to *Beauveria bassiana* CIMA_256 to CIMA_258 and *Clonostachys rosea* CIMA_259 were the ones that exhibited the higher activity with values of 26.0, 24.6, 23.7 and 18.9% respectively.

Table 2. Antioxidant capacity of insect-associated fungal extracts to inhibit ABTS and DPPH radicals and reduction of Cu^{+2} metals expressed in Trolox equivalents (TEAC µM/mg extract) and FRAP expressed in ascorbic acid equivalents.

Strain key	Species	Extract type	Radical inhibition		Metal reduction	
			ABTS	DPPH	CUPRAC	FRAP
CIMA_255	*Cordyceps takaomontana*	Biomass	168.1 ± 2.0 [b]	10.6 ± 0.1[e]	539.9 ± 13.5[c]	134.1 ± 12.2[c]
		Broth	**386.8 ± 6.0[a]**	**284.7 ± 0.7[a]**	370.8 ± 14.1[b]	39.4 ± 45.1[a,b]
CIMA_256	*Beauveria caledonica*	Biomass	62.5 ± 12.1[d]	11.0 ± 0.1[e]	112.7 ± 0.4[e]	62.5 ± 4.7[e]
		Broth	74.6 ± 2.3[d]	**80.2 ± 3.9[c]**	542.2 ± 19.1[a]	36.36 ± 2.5[a,b]
CIMA_257	*Beauveria caledonica*	Biomass	53.3 ± 6.6 [d]	**104.3 ± 2.4[a]**	592.8 ± 14.1[b]	**251.6 ± 16.2[a]**
		Broth	64.5 ± 8.6[d]	41.9 ± 1.6[e]	62.8 ± 5.4[e]	32.1 ± 2.2[b]
CIMA_258	*Beauveria caledonica*	Biomass	57.7 ± 7.3[d]	32.5 ± 0.4[d]	258.3 ± 2.9[d]	104.1 ± 7.8[d]
		Broth	62.8 ± 7.1[d]	62.7 ± 1.9[d]	522.5 ± 8.8[a]	100.3 ± 12.7[a]
CIMA_259	*Clonostachys rosea*	Biomass	232.7 ± 1.2 [a]	54.3 ± 0.1[c]	**857.4 ± 15.5[a]**	184.2 ± 5.1[b]
		Broth	266.4 ± 0.4[b]	7.5 ± 1.4[f]	195.7 ± 6.0[d]	12.1 ± 4.2[b]
CIMA_260	*Clonostachys rogersoniana*	Biomass	137. 8 ± 0.8 [c]	57.7 ± 1.2[b]	107.8 ± 1.8[e]	86.3 ± 6.0[d,e]
		Broth	234.9 ± 3.0[c]	**113.6 ± 1.1[b]**	261.7 ± 15.0[c]	47.7 ± 36.3[a,b]
Antioxidant control	IC_{50} Trolox (µM)		383.6	63.4	$C_{máx}$=600	
	Ascorbic acid (5 µM)					189.8

Note: Results are expressed as mean ± standard deviation (SD) with n=3. $C_{máx}$=Maximum concentration of the linear interval. Different letters indicate significant differences. Where a is the highest value and e is the lowest value for biomass extracts; and a is the highest value and f is the lowest value for broth extracts; according to Tukey test (α= 0.05). The font type is highlighted in bold, for those fungal extract values higher than the control antioxidant.

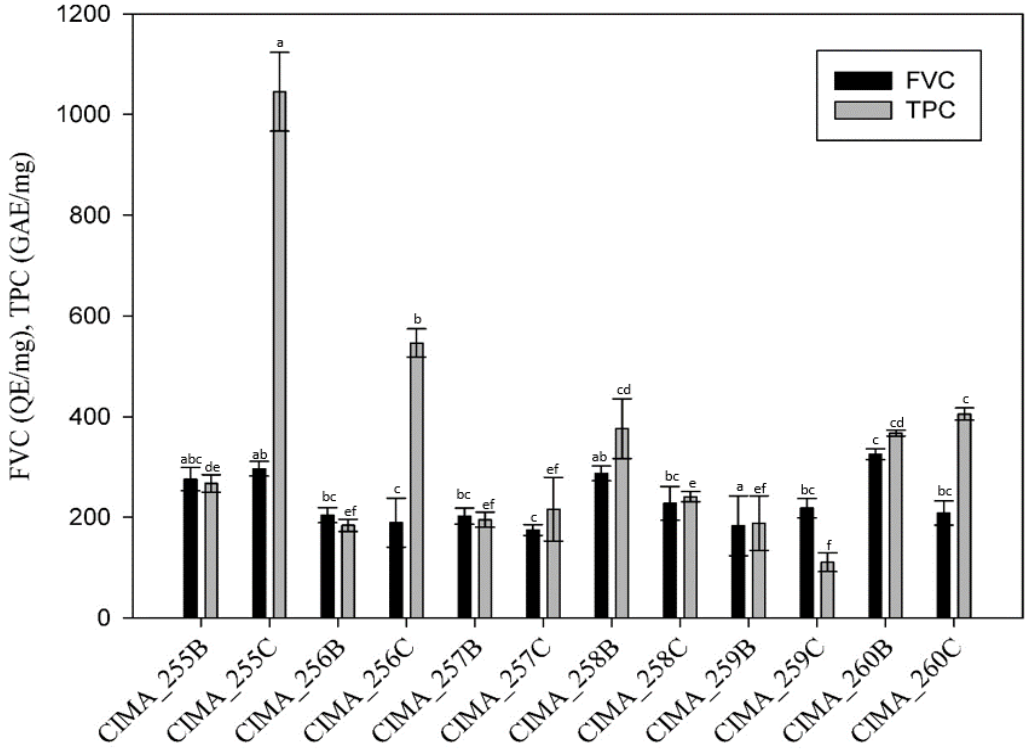

Figure 2. Flavonoid content (FVC) and Total Phenolic content (TPC) expressed as QE/mg extract and GAE/mg extract, respectively, of strains CIMA_255 to CIMA_260 (B= Biomass extract, C= Broth extract). Where a is the highest value and c is the lowest value for FVC; and a is the highest value and f is the lowest value for TPC; according to Tukey test (α= 0.05).

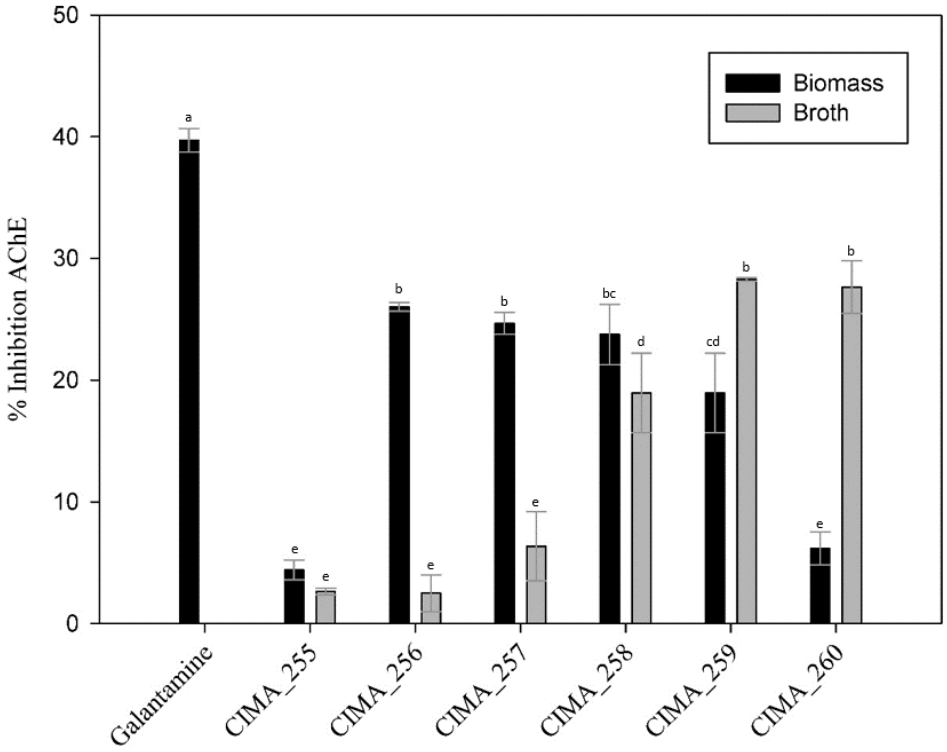

Figure 3. Percentage of inhibition of AChE from biomass and culture media from insect associated fungi. Galantamine 3.5 mM was employed as positive control. Where a is the highest value and e is the lowest value for biomass and broth extracts; according to Tukey test (α= 0.05).

The acetylcholinesterase (AChE) had an essential role in the neurotransmission of the cholinergic synapse catalyzing the hydrolysis of acetylcholine. The inhibitor of this enzyme binds to the active site provoking an accumulation of acetylcholine in the synapse that impede the cell repolarization in various organisms. In this sense, *Beauveria bassiana* is an entomopathogenic fungi, considered as biocontrol agent against a variety of pest insects, as example the control that exert isolates of *B. bassiana* over adults and pupae of *Ceratitis capitata* in laboratory as well as in greenhouse conditions (Rabea *et al*., 2015). Experiments carried out by Wu *et al*. (2019) demonstrated that the combination of *B. brongniartii* and matrine presented a synergic effect against *Spodoptera litura*. Also, Zhang *et al*. (2022) observed a reduction of the AChE of *Megalurothrips usitatus* after the application of *B. bassiana* coinciding with the findings of Zibaee, Bandani and Tork (2009) who observed a similar inhibition of the AChE activity when *B. bassiana* and their metabolites against the solar pest *Eurygaster integriceps*. *Clonostachys* sp. is an excellent agent of biological control, particularly *C. rosea* plays a crucial role on the control of some phytopathogen fungi, nematodes and insects, of the latter *Myzus persicae*, *Rhopalosiphum padi*, *Thrips tabaci* and *Varroa destructor* has been reported (Sun *et al*., 2020). Moreover, ilicicololines C, E and coletoclorine B from fungi *Nectria* (synonym *Clonostachys*) shows inhibitory activity of AChE with IC_{50} values of 30-36 µg/mL (Han *et al*., 2020). All the above, place the strains *Cordyceps takaomontana* CIMA_255, *B. bassiana* CIMA_256 to CIMA_258, *Clonosthachys rosea* CIMA_259 and *C. rogersoniana* CIMA_260 as candidates for further chemical-biological studies to contribute to the knowledge of new natural sources for metabolites with biological activities like antioxidants and enzymatic inhibition such as AChE.

CONCLUSIONS

The interaction of fungi associated with insects stimulates the production of bioactive metabolites that can be exploited. Thus, *Cordyceps takaomontana* CIMA_255, the strains of *Beauveria bassiana* CIMA_256 to CIMA_258, *Clonosthachys rosea* CIMA_259 and *C. rogersoniana* CIMA_260, proved to be a good source of antioxidant metabolites and acetylcholinesterase inhibitors. In addition, this study reports for the first time *Cordyceps*

takaomontana CIMA_255, associated with a lepidopteran pupa collected in the La Martinica Ecological Reserve in Veracruz, Mexico. The above highlights the need to investigate the chemical composition of the fungal extracts reported here, to discuss the structure-activity relationship and possible mechanisms of action.

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Compliance with ethical standards. The authors declare that all the research was carried out following the relevant ethical standards and regulations in force.

Data availability. Data are available upon request to the corresponding author (cespinoza@uv.mx).

Author contribution statement (Credit). Celeste Ricaño-Rodríguez-Methodology, Visualization, and elaboration of phylogenetic reconstructions, **Irene Lagunes-**Validation, Writing an original draft, Revision of the manuscript, **Manuel E. Medina**-Validation, Writing an original draft, Revision of the manuscript, **A. Couttolenc-**Manuscript writing, elaboration of phylogenetic reconstructions, Formal Analysis, **Rosario Medel-Ortiz**-Formal Analysis, Validation, **C. Espinoza-**Project leader, Conceptualization, Formal Analysis, Validation, Writing an original draft.

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