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Article

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Phytotoxic Potential of Secondary Metabolites and Semisynthetic Compounds from Endophytic Fungus *Xylaria feejeensis* Strain SM3e-1b Isolated from *Sapium macrocarpum*

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ABSTRACT: Bioactivity-directed fractionation of the combined culture medium and mycelium extract of the endophytic fungus *Xylaria feejeensis* strain SM3e-1b, isolated from *Sapium macrocarpum*, led to the isolation of three known natural products: (4*S*,5*S*,6*S*)-4-hydroxy-3-methoxy-5-methyl-5,6-epoxycyclohex-2-enone, or coriloxine, **1**, 2-hydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione, **2**, and 2,6-dihydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione or fumiquinone B, **3**. This is the first report of compound **3** being isolated from this species. Additionally, four new derivatives of coriloxine were prepared: (4*R*,5*S*,6*R*)-6-chloro-4,5-dihydroxy-3-methoxy-5-methylcyclohex-2-enone, **4**, 2-hydroxy-3-methyl-5-(methylamino)cyclohexa-2,5-diene-1,4-dione, **5**, (4*R*,5*R*,6*R*)-4,5-dihydroxy-3-methoxy-5-methyl-6-(phenylamino)cyclohex-2-enone, **6**, and 2-((4-butylphenyl)amino)-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione, **7**. X-ray analysis allowed us to unambiguously determine the structures and absolute configuration of semisynthetic derivatives **4**, **5** and **6**. The phytotoxic activity of the three isolated natural products and the coriloxine derivatives is reported. Germination of the seed, root growth, and oxygen uptake of the seedling of *Trifolium pratense*, *Medicago sativa*, *Panicum miliaceum,* and *Amaranthus hypochondriacus* were significantly inhibited by all of the tested compounds. In general, they were more effective inhibiting root elongation than suppressing the germination and seedling oxygen uptake processes as shown by their IC_{50}.

KEYWORDS: *Xylaria feejeensis*; endophytic fungi; quinones; coriloxine; phytotoxicity; *Sapium macrocarpum*.

INTRODUCTION

Endophytic fungi are microorganisms that live in the tissues of living plants, causing no apparent symptoms of disease for the host.[1,2] Some endophytes protect the host plant from other organisms, such as pathogens, herbivores and insects; and can also improve the drought tolerance and nutrients uptake of the plant.[3] Besides, as part of the colonization process, endophytes have the ability to produce a wide diversity of phytotoxic secondary metabolites and exoenzymes. Also, the endophyte is capable of producing phytotoxic metabolites *in vitro* with activity against different invading organisms, including algae and plants.[4] For this reason, endophytic fungi have been identified as potential sources of active secondary metabolites that could be useful in agriculture, as fungicides, herbicides, insecticides, among others.[5,6] This is particularly important since the excessive use of agrochemical products has promoted a faster evolution of resistant forms of pests. Studying these metabolites may lead to the discovery and design of new herbicides with effective action towards targets for pest control.[7–11]

It is remarkable that almost every known plant species contains at least one species of endophyte fungus. Strobel and Daisy[2] have suggested strategies to identify those plants hosting endophytic fungi that produce bioactive secondary metabolites. The main strategies contemplate plants living in uncommon conditions with novel survival skills, plants that have a history being used by indigenous peoples, ancient and endemic plants, and, particularly interesting for this investigation, plants from areas of great biodiversity. Plants selected under these considerations are more likely to lodge endophytic fungi producing novel bioactive products than other plants.

The Reserva de la Biósfera Sierra de Huautla "REBIOSH" is a region rich in water resources, with a significant forest cover, consisting of a medium low oak forest and a low

deciduous forest.[12] According to the aforementioned criteria from Strobel and Daisy,[2] the reserve is an ideal environment for the collection of plants. For this reason, the tree *Sapium macrocarpum* Mull. Arg. (Euphorbiaceae) was randomly selected at the "REBIOSH" reserve as potential source of interesting endophytes.

The largest genus of the family Xylariaceae Tul.&C. Tul. (Xylariales, Sordariomycetes), *Xylaria* Hill ex Schrank, comprises more than 300 species,[13] including endophytes that can be found in a variety of tropical plants. A wide range of bioactive secondary metabolites, mainly with antimicrobial, antimalarial, and cytotoxic properties, are produced by members of the genus *Xylaria.*[14,15] In particular, from *Xylaria feejeensis* the following compounds have been isolated: integric acid,[16] xylaropyrone,[17] nonenolide xyolide,[18] pestalotin 4′-*O*-methyl-β-mannopyranoside 3*S*,4*R*-(+)-4-hydroxymellein, 3*S*,4*S*-(+)-4-hydroxymellein, 3*S*-(+)-8-methoxymellein, 2-hydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione, 4*S*,5*S*,6*S*-4-hydroxy-3-methoxy-5-methyl-5,6-epoxycyclohex-2-enone, and 4*R*,5*R*-dihydroxy-3-methoxy-5-methylcyclohexen-2-en-1-one.[19]

The variety of biological activities, and the structural diversity of the secondary metabolites from *Xylaria*, offer the opportunity to find new chemical skeletons that may be used as templates for the development of compounds potentially bioactive in the fields of medicine, agriculture, and industry.[2,5,15] However, only a few studies have been focused on the evaluation of the secondary metabolites of *Xylaria fejeensis* as promising natural-like herbicides. There is only one report describing the phytotoxic effect of coriloxine on *Lactuca sativa* seeds.[20]

In this context, we report the evaluation of the phytotoxic potential of three major secondary metabolites, obtained from the combined culture medium and mycelium extracts, of the fungus *Xylaria feejeensis* strain SM3e-1b isolated from *S. macrocarpum*, collected at "REBIOSH". The three compounds strongly inhibited the germintaion of the seed, root growth, and the oxygen uptake of the seedlings on four weeds: *T. pratense*, *M. sativa*, *P. miliaceum,* and *A. hypochondriacus.* Looking to find new compounds with improved activity, four new synthetic derivatives of coriloxine were prepared and their phytotoxic potential was also evaluated. Our results, show that all the molecules, natural and semisynthetic, were potent inhibitors of the three physiological process tested on the four weeds.

MATERIALS AND METHODS

General. A Fisher-Johns apparatus was used for the determination of the melting points, the reported values are uncorrected. KBr disks were used to obtain the IR spectra on a 599-B spectrophotometer (Perkin-Elmer, Waltham, MA). COSY, NOESY, HMBC and HSQC NMR spectra were recorded on a Bruker AVANCE IIIHD (Fällanden, Switzerland) using tetramethylsilane (TMS) as an internal reference, in methan(ol-d), at 500 (^{1}H) and 125 (^{13}C) MHz, on a Bruker DMX500 (Billerica, MA), in chloroform-d, either at 400 (^{1}H) or 125 (^{13}C) MHz, and a Bruker 300 (Fällanden, Switzerland), in chloroform-d, at 300 (^{1}H) or 75 (^{13}C) MHz. High-resolution mass spectra, HRMS (DART-TOF+), were acquired with an AccuTOF-JMS-T100LC (Jeol, Peabody, MA) spectrometer. Single crystals of compounds **4**, **5** and **6** were obtained and their X-ray analysis was performed on a Bruker D8 Venture κ-geometry diffractometer (Madison, WI) equipped with micro-focus X-ray source and

Helios multilayer mirror as monochromator, Cu kα radiation (λ = 1.54178Å). For open column chromatography, silica gel 60 (70–230 mesh) from Merck-Millipore was used (Billerica, MA). For analytical and preparative thin-layer chromatography separations, pre-coated silica gel 60 F_{254} plates (Merck) were used. Lithium perchlorate ($LiClO_4$, ACS reagent, ≥98.0 %), anhydrous methanol (MeOH, 99.8%), anhydrous dichloromethane (CH_2Cl_2 ≥ 99.8%), and anhydrous acetonitrile (MeCN, 99.8 %) were obtained from Sigma-Aldrich (St. Louis, MO) and used as received. Aniline (ACS reagent ≥ 99.5%) and 97% 4-butylaniline were obtained from Sigma-Aldrich and distilled immediately before use. 35% Methylamine solution in H_2O was obtained from Merck. Sodium methoxide (NaOMe, reagent grade, powder 95%) was used as received from Sigma-Aldrich. Silica gel (SiO_2 60-120 mesh) was used as catalyst. Electrochemical grade tetrabutylammonium hexafluorophosphate (Bu_4NPF_6 ≥ 99.0%) from Fluka (St. Louis, MO), was dried at 60 ^oC under vacuum prior to use. An Autolab PGSTAT302N potentiostat Metrohm Autolab (Kanaalweg, Utrecht, Netherlands) was used for the electrosynthesis of **2**. An $Ag/AgNO_3$ system was used as reference electrode (silver wire in 0.01 M silver nitrate / 0.10 M Bu_4NPF_6/ acetonitrile).

Fungus Isolation. The endophytic fungus strain SM3e-1b was isolated from symptomless healthy leaves of *S. macrocarpum*, collected at the "REBIOSH" at Quilamula (18° 30′ 4.1″ N 98° 51′ 52″ W and 18° 32′ 12.2″ N 99° 02′ 05″ W; 1080-1230 meters above sea level), State of Morelos, Mexico, in September 2010.

Complete intact leaves were rinsed with running water, and then with distilled water. Then, the surface was sterilized by soaking the leaves during 60 s in 75% ethanol and subsequently in 3.4% NaClO solution (65% Clorox) also for 60 s, rinsing with sterile

distilled water both between solutions and at the end.[21] The sterilized leaves were dried with sterile adsorbent paper and cut in 2 x 2 mm pieces at the central vein level. Afterwards, four pieces were placed in a Petri dish containing potato-dextrose-agar (PDA), which was previously treated with 500 mg/L chloramphenicol (Sigma-Aldrich) to prevent bacterial growth. Plates were incubated at 28 °C with 12:12 h light:dark cycles using a fluorescent light (T12 30W) (Philips, Chihuahua, Mexico). Petri dishes were observed daily and the individual hypha tip of the emerging colonies were re-inoculated in new PDA plates until pure cultures were obtained.[22–24]

The pure culture of the endophytic strain SM3e-1b is preserved on PDA slants at the Instituto de Química, and at the Instituto de Biología (Laboratorio de Micología C006), UNAM both in water agar (0.2%) at 4 °C, and in 30% glycerol potato-dextrose-broth (PDB) at – 80 °C. Dried cultures on PDA and oatmeal agar (OA) have been deposited, with accession number MEXU 27802, in the fungal collection of the Herbario Nacional de México (MEXU), UNAM.

Morphological and Molecular Identification of the Strain SM3e-1b**.** Based on the colony and mycelium characteristics,[25–27] the strain SM3e-1b was identified as *Xylaria* sp. In addition, the internal transcribed spacer region (ITS1-5.8S-ITS2) was sequenced and analyzed for identification of the species.[22–24] Nucleotide BLAST analysis of the SM3e-1b sequence (Genbank accession number KR025539) revealed *Xylaria feejeensis* as the closest match, with sequences identities ranging from 100 to 97%. The most similar sequence was that of *X. feejeensis* E6912b (accession HM992808) followed by several other sequences of *X. feejeensis* strains.

Fermentations and Extraction of Compounds from *X. feejeensis*. Three Fernbach flasks (3 L capacity) with 1.2 L of PDB were used for the culture of endophytic

fungus *X. feejeensis* strain SM3e-1b. Each Fernbach flask was inoculated with five 2.0 cm^2 agar plugs from a PDA stock culture of *X. feejeensis*, and were incubated for 30 days at 28 °C under static conditions with 12:12 h light:dark cycles. After this period, the mycelium and the culture medium were separated by filtration through cheesecloth. Culture medium was subsequently extracted with CH_2Cl_2 (× 6), and then with EtOAc (× 6). Both organic phases were combined, dried with anhydrous sodium sulfate and concentrated under vacuum, to yield 910 mg of a brown solid. The mycelium was macerated with 1.5 L of CH_2Cl_2 (× 6) and 1.5 L of EtOAc (× 6). The dichloromethane and ethyl acetate phases were mixed, dried with anhydrous sodium sulfate and concentrated under vacuum, to yield 3.58 g of a brown extract.[24,28]

Isolation of Compounds from *X. feejeensis*. The organic extracts from the culture medium and mycelium (~4g) were combined. A silica gel chromatographic column (420 g of SiO_2, 70–230 mesh) with a gradient of *n*-hexane-CH_2Cl_2 (100:0 to 0:100) and CH_2Cl_2-MeOH (99.9:0.1 to 50:50) resulted in 200 fractions (50 mL each), all of which were monitored by TLC; seven primary fractions (FI–FVII) were obtained from the combination of the fractions with similar patterns.

Phytogrowth-inhibitory activity using a bioassay on a Petri dish showed that all fractions were active (FI to FVII). From phytotoxic fraction IV (2.923 g), eluted with CH_2Cl_2-MeOH (99:1), the known epoxycyclohexenone derivative, (4*S*,5*S*,6*S*)-4-hydroxy-3-methoxy-5-methyl-5,6-epoxycyclohex-2-en-1-one or coriloxine **1**, was isolated. The pure compound (930 mg) was obtained after exhaustive washing with a mixture of *n*-hexane and dichloromethane (1:1), followed by recrystallization from CH_2Cl_2-MeOH (99:1). Active fractions IV (1.8 g) and V (0.29 g), eluted with CH_2Cl_2-MeOH (96:4), were purified by successive preparative TLC (CH_2Cl_2-MeOH; 99:1 × 3) yielding the known quinone

derivatives: 2-hydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione, **2**, (250 mg), and 2,6-hydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione or fumiquinone B, **3**, (100 mg). Coriloxine, **1**, (170 mg) was also obtained from these fractions.

Synthetic Derivatives of Coriloxine, 1.

Synthesis of (4*R*,5*S*,6*R*)-6-Chloro-4,5-dihydroxy-3-methoxy-5-methylcyclohex-2-enone, 4. 100 mg (0.59 mmol) of coriloxine were dissolved in methanol (2 mL) and 26 mg (0.12 mmol) of indium(III) chloride were added to the solution. The mixture was refluxed for 24 h, then 0.01 g (0.045 mmol) more of indium(III)chloride and 10 mg of silica gel (60-120 mesh) were added; then, the solution was stirred under reflux for an additional 24 h. The reaction was allowed to cool down to room temperature, filtered to remove the SiO_2, and the solvent was evaporated.[29] The residue was purified by chromatographic column on silica gel (70–230 mesh) using an isocratic system of CH_2Cl_2-MeOH (98:2), to yield 21.5 mg of a colorless crystalline solid, **4.**

2-Hydroxy-3-methyl-5-(methylamino)cyclohexa-2,5-diene-1,4-dione, **5.** In a small vial, 57.5 mg (0.34 mmol) of coriloxine were dissolved in 500 µL of water, then 37 mg (0.35 mmol) of lithium perchlorate and 40 µL (0.41 mmol) of 35% aqueous methylamine solution were added. The mixture was stirred for 40 min at room temperature.[30] A dark purple solution was obtained, the solvent was evaporated and the residue was purified by silica gel (70–230 mesh) column chromatography eluting with an isocratic system of EtOAc (100%), to yield 18.0 mg of a red crystalline solid, **5**.

Synthesis of (4*R*,5*R*,6*R*)-4,5-dihydroxy-3-methoxy-5-methyl-6-(phenylamino)cyclohex-2-enone, 6. 44.9 mg (0.26 mmol) of coriloxine, 7.0 mg of silica gel (60-120 mesh), and 2 mL of anhydrous dichloromethane were placed in a two-neck round bottom flask with a nitrogen inlet, and a reflux condenser attached to the flask. The

reaction mixture was stirred at room temperature under N_2 atmosphere during 15 min, then, 37 μL (0.40 mmol) of freshly distilled aniline were added.[31] After refluxing the reaction mixture for 12 h, thin layer chromatography revealed that while the aniline was completely consumed, there was coriloxine remaining. 20 μL (0.22 mmol) more of freshly distilled aniline were added and the mixture was refluxed for an additional period of 6 h, then stirred during 10 h at room temperature. The dark yellow solution was filtered and the solvent was evaporated. Extensive preparative TLC (*n*-hexane-EtOAc (30:70) of the crude, yielded 20.0 mg of a pink crystalline solid, **6**.

2-((4-Butylphenyl)amino)-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione, 7. 88.0 mg (0.52 mmol) of coriloxine, 14.0 mg of silica gel (60-120 mesh), and 4 mL of anhydrous dichloromethane were placed in a two-neck round bottom flask equipped with a reflux condenser and a nitrogen inlet. The reaction mixture was stirred at room temperature under N_2 atmosphere during 15 min; then, 120 μL (0.76 mmol) of freshly distilled *p*-butylaniline were added to the mixture. This solution was refluxed during 15 h and stirred at room temperature during additional 12 h.[31] The dark brown solution was filtered, and the solvent was evaporated. The crude product was purified by silica gel 60 (70–230 mesh) column chromatography eluting with an isocratic system of EtOAc (100%) to afford 26.6 mg of a purple amorphous solid, **7**.

2-Hydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione, 2. 84.9 mg (0.50 mmol) of coriloxine were dissolved in 1 mL anhydrous methanol, previously passed through a short column of activated alumina; then, 32 mg (0.59 mmol) of sodium methoxide were added and the reaction mixture was stirred during 2 h under nitrogen atmosphere at room temperature.[32] A dark brown solution was obtained, the solvent was evaporated and the crude residue was purified by silica gel 60 (70–230 mesh) column

chromatography eluting with an isocratic system of EtOAc (100%) to afford 10.2 mg of a yellow crystalline solid, **2**.

Electro-organic Synthesis of 2-Hydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione, 2. 50 mL of anhydrous acetonitrile, containing 0.1 M of tetrabutylammonium hexafluorphosphate as supporting electrolyte, were placed in a 75 mL two-compartments electrolysis cell (Bioanalytical Systems West Lafayette, IN). Oxygen was removed from the solution in the cell by bubbling nitrogen gas, previously saturated with acetonitrile. Then, 150 mg of coriloxine were added and the solution was stirred under N_2 flow for additional 15 min. The working electrode consisted of a large area reticulated vitreous carbon. The counter electrode (platinum wire) was placed in a separated compartment using a frit glass; $Ag/AgNO_3$ was used as reference electrode. A constant potential of -2.1 V *vs* $Ag/AgNO_3$ was applied under constant agitation at room temperature, N_2 flow continued throughout the experiment. The charge was monitored during the electrolysis; when the total charge flow reached 2 Faradays/mole (two electron per molecule), the reaction was stopped. The value of the potential applied during the electrolysis was previously determined by cyclic voltammetry experiments of coriloxine, and was set at approximately 100 mV after the peak potential of the reduction wave of coriloxine. A purple solution was obtained after the electrolysis, acetonitrile was evaporated and the residue was purified by column chromatography on silica gel eluting with a gradient of *n*-hexane-CH_2Cl_2 (100:0 to 0:100) and CH_2Cl_2-MeOH (99.9:0.1 to 98:2). The fraction eluted with CH_2Cl_2 (100%) was purified by preparative TLC (CH_2Cl_2-MeOH; 98:2) to yield 52 mg of a yellow crystalline solid, **2**.

Phytogrowth-Inhibitory Bioassays. The combined culture medium and mycelia organic extracts from *X. feejeensis*, natural compounds and semisynthetic compounds were

tested for their inhibitory effect on the germination of the seed, elongation of the root, and the oxygen uptake (respiration) of the seedlings of three dicotyledonous species, *Trifolium pratense* (Fabaceae) (red clover, peavine clover, and cow grass), *Medicago sativa* (Fabaceae) (California clover and buffalo grass), and *Amaranthus hypochondriacus* (Amaranthaceae) (amaranth); and one monocotyledonous plant, *Panicum miliaceum* (Poacea) (red millet). Seeds were obtained from Casa Cobo, S.A. de C.V. (Central de Abastos, Mexico City, Mexico).

A Petri dish bioassay was performed to evaluate the phytotoxic effects of different treatments on seedling growth.[23,24,28] The organic extract was evaluated at 50, 100, 200, and 400 µg/mL, and the natural compounds and semi-synthetic derivatives were evaluated at 20, 50, 150, and 200 µg/mL by dilution in agar (1%). For each treatment, the extract was dissolved in MeOH, not exceeding 0.5%, and added to ~40 °C sterile agar in 3-cm Petri dishes before its solidification. Rival® [glyphosate: *N*-(phosphonomethyl)glycine] (Monsanto, Sao Paulo, Brasil) and ExterPro® [hexazinone: (3-cyclohexyl-6-(dimethylamino)-1-methyl-1,3,5-triazine-2,4(1H,3H)-dione)] (Comercial Solrac, S.A de C.V., Mexico City, Mexico) at 20, 50, 100, 150, and 200 µg/mL were used as positive control; agar (1% with 0.5% MeOH) and pure agar (1%) as negative controls. Thirty seeds of every plant species were sown onto the agar in four replications for every treatment, in a completely randomized design. The agar plates, sealed with parafilm foil, were placed in a germination chamber at 28 °C under complete darkness. After treatment, germination of the seed, root growth, and oxygen uptake of the seedling were measured for *A. hypochondriacus* (24 h), and for *T. pratense*, *M. sativa* and *P. miliaceum* (48 h).

For the oxygen uptake experiments, a 10 mL glass chamber with 4 mL of air saturated deionized water at 28 °C was used; seedlings were transferred into the chamber

and different concentrations of either the extract or the pure compounds were added. Oxygen consumption was polarographically measured during 3 min, using a Clark-type electrode connected to a 5300A biological oxygen monitor (YSI Inc., Yellow Springs, OH). Oxygen requirement was determined as a percentage, considering the control as 100%. For each experiment, a certain number of seeds was selected so that appreciable change in O_2 uptake could be detected.[23,24,28]

Statistical Analysis. The effect of the different treatments (extract or pure compound) on the inhibition of the three physiological processes was analyzed by ANOVA, and Tukey statistical tests.[33] For each activity, Probit analysis[34] was used to determine the IC_{50} values (half maximal inhibitory concentration), based on the average percentage of inhibition for each concentration of the organic extract, and the natural and semisynthetic compounds. A *p* value of 0.05 or less (*) was used to denote statistical significance. All the calculations were performed with GraphPad Prism statistical computer software, ver. 6.0 (La Jolla, CA). Data are shown as mean ± standard deviation (SD).

Coriloxine, 1. Colorless crystals (EtOAc); mp 155–156 °C; IR (KBr) ν_{max} 3450, 1665, 1612, 1234 and 1080 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.66 (s, 3H, CH_3-5), 2.59 (d, *J*= 6.0 Hz, 1H, OH-4), 3.33 (d, *J*= 2.0 Hz, 1H, H-6), 3.77 (s, 3H, OCH_3-3), 4.49 (d, *J*= 6.0 Hz, 1H, H-4); 5.26 (d, *J*= 1.6 Hz, 1H, H-2) ppm; ^{13}C-NMR ($CDCl_3$, 100 MHz), δ18.9 (CH_3-5), 56.5 (OCH_3-3), 59.8 (C-5), 60.6 (C-6), 69.1 (C-4), 97.7 (C-2), 171.3 (C-3), 193.3 (C-1) ppm.

2-Hydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione, **2**. Yellow crystals (CH_2Cl_2-CH_3OH); mp 176-178 °C; IR (KBr) ν_{max} 3284, 3074, 1646, 1599 and 1297 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 1.95 (s, 3H, CH_3-5), 3.86 (s, 3H, OCH_3-3), 5.84

(s, 1H, H-2), 7.23 (s, 1H, OH-6); ^{13}C-NMR ($CDCl_3$, 75 MHz) 7.8 (CH_3-5), 56.8 (OCH_3-3), 102.2 (C-2), 114.8 (C-5), 151.6 (C-6), 161.4 (C-3), 181.9 (C-4), 182.6 (C-1) ppm.

Fumiquinone B, **3**. Purple amorphous solid; mp 253-255 °C; IR (KBr) ν_{max} 3387, 2961, 1678, 1605, 1370, 1284 and 1100; ^{1}H NMR (CH_3OD, 500 MHz) δ 1.79 (s, 3H, CH_3-5), 3.81 (s, 3H, OCH_3-3); ^{13}C-NMR ($CDCl_3$, 75 MHz); δ 8.18 (CH_3-5), 57.1 (OCH_3-3), 110.4 (C-5), 164.2 (C-2), 170.8 (C-6), 175.7 (C-1), 178.2 (C-3), 196.3 (C-4) ppm.

(4*R*,5*S*,6*R*)-6-Chloro-4,5-dihydroxy-3-methoxy-5-methylcyclohex-2-enone, 4. Colorless crystals (CH_2Cl_2); mp 185-188 °C; IR (KBr) ν_{max} 3592, 2942, 1677, 1621, 1241, 1188 and 848; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.42 (s, 3H, CH_3-5), 3.08 (s, 1H, OH-4), 3.35 (s, 1H, OH-5), 3.81 (s, 3H, OCH_3-3), 4.40 (s, 1H, H-6), 4.57 (s, 1H, H-4), 5.44 (s, 1H, H-2); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 21.1 (CH_3-5), 56.8 (s, 1H, OCH_3), 65.3 (C-4), 60.5 (C-5), 72.3 (C-6), 99.8 (C-2), 172.7 (C-3), 189.9 (C-1) ppm. HRMS (ESI) $[M + H]^+$ *m*/*z* 207.04223 (calcd for $C_8H_{12}ClO_4$, 207.04241).

2-Hydroxy-3-methyl-5-(methylamino)cyclohexa-2,5-diene-1,4-dione, **5.** Red crystalline solid (CH_3OH); mp 195-198 °C; IR (KBr) ν_{max} 3558, 3364, 2981, 2940, 1671, 1621, 1504, 1315, 1240, 1125, 1055, 1006 and 845; ^{1}H NMR ($CDCl_3$, 300 MHz); δ 1.92 (s, 3H, CH_3-5), 2.92 (d, *J*=5.7 Hz, 3H, CH_3-3), 5.35 (s, 1H, H-2), 5.51 (s, 1H, NH-3), 6.43 (s, 1H, OH-6), ^{13}C RMN ($CDCl_3$, 75 MHz); 9.0 (CH_3-5), 31.2 (N-CH_3-3), 111.4 (C-5), 112.4 (C-2), 151.1 (C-3), 155.2 (C-6), 182.0 (C-1), 183.4 (C-4) ppm. HRMS (ESI) $[M + H]^+$ *m*/*z* 168.06659 (calcd for $C_8H_{10}NO_3$, 168.06607).

(4*R*,5*R*,6*R*)-4,5-Dihydroxy-3-methoxy-5-methyl-6-(phenylamino)cyclohex-2-enone (6). Pink crystalline solid (CH_2Cl_2-CH_3OH); mp 289-294 °C; IR (KBr) ν_{max} 3558, 3364, 2940, 1671, 1621, 1240, 1123, 1055, 1006 and 845; ^{1}H NMR ($CDCl_3$, 300 MHz) δ

1.19 (s, 3H, CH_3-5), 3.38 (s, 1H, -NH-6), 3.79 (s, 3H, OCH_3-3), 4.29 (s 1H, H-4), 4.62 (s 1H, H-6), 5.46 (s, 1H, H-2), 6.80 (t, *J*= 6.6 Hz, 1H, H-4′), 6.90 (t, *J*= 8.0 Hz, 2H, H-2′, 6′), 7.20 (t, *J*= 8.0 Hz, 2H, H-3′, 5′). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 20.3 (CH_3-5), 56.7 (OCH_3-3), 64.1 (C-6), 74.7 (C-5), 75.2 (C-4), 101.4 (C-2), 119.3 (C-4′), 114.8 (C-2′, 6′), 129.3 (C-3′, 5′), 149.1 (C-1′), 172.6 (C-3), 194.7 (C-1) ppm. HRMS (ESI) $[M + H]^+$ *m*/*z* 264.12342 (calcd for $C_{14}H_{18}NO_4$, 264.12358).

2-((4-Butylphenyl)amino)-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione, 7. Purple amorphous solid; mp 290-295 °C; IR (KBr) ν_{max} 3331, 2960, 2932, 1650, 1591, 1235, 1076 and 842; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.93 (t, *J*= 7.4 Hz, 3H, CH_3-4′′), 1.36 (m, 2H, CH_2-3′′), 1.57 (s, 3H, CH_3-5), 1.59 (m, 2H, CH_2-2′′), 2.6 (t, *J*= 7.7 Hz, 2H, CH_2-1′′), 3.85 (s, 3H, OCH_3-3), 5.82 (s, 1H, H-2), 6.90 (d, *J*= 8.4 2H, H-2′, 6′), 7.13 (d, *J*= 8.4 2H, H-3′, 5′), 7.36 (s, 1H, NH-6). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 12.6 (CH_3-5), 13.9 (C-4′′), 22.2 (C-3′′), 33.5 (C-2′′), 35.0 (C-1′′), 56.4 (OCH_3-3), 102.9 (C-2), 111.2 (C-5), 123.1 (C-2′, 6′), 128.6 (C-3′, 5′), 136.7 (C-4′), 139.7 (C-1′), 141.1 (C-6), 161.3 (C-3), 181.1 (C-4), 184.2 (C-1) ppm. HRMS (ESI) $[M + H]^+$ *m*/*z* 300.15997 (calcd for $C_{18}H_{22}NO_3$, 300.15997).

X-ray Crystallography of Compounds, 4-6. Colorless crystals of 0.26 × 0.20 × 0.20 mm, and 0.376 × 0.148 × 0.094 mm dimensions were obtained from slow evaporation of CH_2Cl_2 solutions for **4** and **6**, and a red crystal of dimension 0.226 × 0.118 × 0.070 mm for **5**, with empirical formulas $C_8H_{11}ClO_4$, $C_8H_9NO_3$, and $C_{14}H_{17}NO_4$, and Mr =206.62, Mr = 167.16, and Mr = 293.28, for **4**, **5**, and **6**, respectively. Compound **4** crystallized in a orthorhombic crystal system, $P2_12_12_1$ (No. 19), with cell parameters, a = 7.7512(4) Å, b = 10.3272(6) Å and c = 11.7465(7) Å, V = 940.29(9) Å^3 Z = 4, D_{cal}=1.46 Mg/m^3, mμ 3.481

328 mm^{-1}, F(000) = 432. Compound **5** crystallized in a triclinic crystal system, P-1 (No. 2) with
329 cell parameters a= 6.8367(5) Å, b= 7.4505(5) Å, c =8 2103 Å, α= 79.128(4)°, β=
330 81.026(4)°, γ = 71.417(4)°, V =387.23(5) $Å^3$, Z =2, D_{cal}=1.434 Mg/m^3, mμ 0.935 mm^{-1},
331 F(000) = 176. Compound **6** crystallized in a monoclinic crystal system $P2_1$ (No. 4), with
332 cell parameters a = 12.4136(6) Å, b = 9.6126(5) Å, c = 22.0258(11) Å, β= 93.1066(19)°,V
333 = 2624.4(2)$Å^3$, Z = 8, D_{cal}=1.333 Mg/m^3, mμ = 0.810 mm^{-1}, F(000) = 1120. All compounds
334 were irradiated with Cu Kα radiation (λ =1.54178Å) on the Bruker D8 Venture κ-geometry
335 diffractometer with micro-focus X-ray source, and Helios multilayer mirror as
336 monochromator, using an APEX 2 program[35] at 296(2) K. Data reduction was achieved
337 using the SAINT program.[35] 7291, 2756 and 29262 reflections were collected, from which
338 1707 (R_{int} = 0.0582), 1351 (R_{int} = 0.0784), and 8252 (R_{int} = 0.0855) reflections were
339 independent. Structures were solved using direct methods and then refined with the
340 SHELXS[36] and SHELXL[36] programs with full-matrix least-squares on F^2 respectively.
341 ORTEP-3 software was used for the figures.[37] Four crystallographic independent molecules
342 were found in the asymmetric unit in compound **6**.

343 The final values S=1.091, R1=0.0324, wR2=0.0928 were based on 1668 reflections
344 observed, 126 parameters for **4**. S=1.071, R1=0.0595, wR2=0.1707 based on 1049
345 reflections observed, 117 parameters for **5.** S=1.032, R1 = 0.0446, wR2 = 0.1222, based on
346 7713 reflections observed, 729 parameters for **6**. The largest different peak and hole for **4**,
347 **5**, and **6** were 0.243, -0.198 $eÅ^{-3}$, 0.250, -0.245 $eÅ^{-3}$ and 0.221, and -0.223 $e.Å^{-3}$
348 respectively.

349

350 **RESULTS AND DISCUSSION**

The tree *S. macrocarpum* was randomly selected at the "REBIOSH" reserve;[12] following the ecological criteria, with symptomless healthy leaves and without herbivore damage.[2,38] This selection was mainly based on the recommendations of Strobel and Daisy cited in the introduction. *S. macrocarpum* is a common tree from humid and dry tropical forests of central North Pacific, and from the region spanning from southern Mexico to Costa Rica. It is commonly called "Amate prieto", "Hincha huevos", "Palo lechón", "Amantillo", "Chileamate", "Chonte", "Hierba de la flecha", "Higuerillo bravo", "Lechón", "Mataise" or "Venenillo".[39] The recommended uses are for making paper, in constructions, as poles, boxes, and for medicinal use.[39,40]

Fermentation of *X. feejeensis* Strain SM3e-1 and Phytogrowth Inhibitory Activity of the Organic Extracts. The endophytic fungus *X. feejeensis* strain SM3e-1 isolated from *S. macrocarpum* was grown under static conditions in liquid substrate fermentation on PDB. The culture medium and the mycelia were extracted with CH_2Cl_2 and EtOAc. TLC analysis of the organic extracts exhibited a similar profile and the presence of the same major secondary metabolites. The initial phytotoxic activity of the combined extracts of the mycelia and culture medium was evaluated on the germination of the seed, root growth, and oxygen uptake of the seedling of four weeds: *T. pratense*, *M. sativa*, *P. miliaceum,* and *A. hypochondriacus*. The extract showed potential phytotoxicity with significant inhibitions in a concentration-dependent way; and, in general was more potent as germination and seedling respiration inhibitor than as root elongation inhibitor (Figures 3A-C). Table 1 compiles the IC_{50} values of the phytotoxic effect of the organic extract on the three physiological processes on the four different seeds. The results show that *A. hypochondriacus* seeds were the most affected, with IC_{50} values <400 µg/mL (highest concentration tested) for the three processes. Root growth of *P. miliaceum* and seedling

respiration of *M. sativa* also showed the IC_{50} values <400 µg/mL (highest concentration tested).

Isolation of Phytotoxic Compounds produced by *X. feejeensis* strain SM3e-1. Bioassay-guided fractionation from the culture medium and mycelium extracts from *X. feejeensis* strain SM3e-1 using chromatographic procedures led to the isolation of three known natural products: one epoxycyclohexenone derivative, (4*S*,5*S*,6*S*)-4-hydroxy-3-methoxy-5-methyl-5,6-epoxycyclohex-2-enone, or coriloxine, **1**, and two quinone derivatives; 2-hydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione, **2**, and 2,6-dihydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione or fumiquinone B, **3** (Figure 1). The spectroscopic characteristics of compounds **1**, **2**, and **3**, including IR, ^{1}H NMR, and ^{13}C NMR, are identical to those previously reported for these known compounds.[41–43] Compound **3** is new to this species.

Semisynthetic Derivatives of Coriloxine, 1.

Four coriloxine derivatives were prepared according to the synthetic approaches depicted in Figure 2, also described on the Materials and Methods section. Compounds **4**-**7** were identified from their IR and ^{1}H NMR spectroscopic data. Single crystal diffraction confirmed the structure of compounds **4**, **5** and **6**. Figure 4 shows an ORTEP drawing of these structures. In addition, anomalous dispersion effects in diffraction measurements on the crystal of **4** and **6** allowed to assess the absolute configuration. It is important to notice that these coriloxine derivatives have not been reported before.

Treatment of coriloxine with an aqueous solution of methylamine in presence of a catalytic amount of $LiClO_4$ produced compound **5**.[30] For compounds **6** and **7** a catalytic amount of SiO_2 was used to promote the opening of the epoxide ring[44]; in both cases the

nucleophilic attack of the aromatic amine (aniline or *p*-butylaniline) took place at the less sterically hindered carbon atom.

When designing semisynthetic derivatives with herbicidal activity, it is important that they satisfy the requirements established by Lipinski's and Tice's rules,[45,46] thus, before preparing the derivatives depicted in Figure 2, it was confirmed that they posses adequate physicochemical properties for their application as herbicides. Besides, since lipophilicity is a key parameter that rules the pharmacokinetic behavior of agrochemicals, by enhancing their *in vivo* transport and passive diffusion across membranes, we designed derivatives with different degrees of lipohilicity, using methylamine, aniline and butylaniline to open the epoxide, derivatives **5** – **7**.

In our attempts to open the epoxide ring of coriloxine, an electrochemical reduction of compound **1** was carried out in anhydrous acetonitrile under N_2 atmosphere. Surprisingly, these conditions led to an oxidized coriloxine derivative, compound **2**. The formation of **2** can be rationalized as follows: the electrochemical reduction of the epoxy moiety proceeds through a two-electron process that promotes the ring opening, yielding the corresponding hydroxy derivative, **1′** (Figure 5);[47] then, by a keto-enol equilibrium, the hydroquinone of **2** is formed, **2′**, which is easily oxidized by the O_2 in the air during workup. Compound **2** was also obtained when coriloxine was reacted with a strong organic base, such as sodium methoxide. Although, NaOMe is both a strong base and a good nucleophile, no nucleophilic addition of the methoxy group was observed under our experimental conditions. In another attempt to open the epoxide by the introduction of a methoxyl group, coriloxine was reacted with methanol using a catalytic amount of indium(III)chloride and SiO_2;[29] again, methoxy addition was not achieved; instead, we obtained derivative **4**. Knowing that about 65% of all the known agrochemicals have

422 halogens in their structure,[48] we considered important to evaluate the activity of the
423 chlorine derivative **4**.

424 **Phytogrowth-Inhibitory Activity of the Secondary Metabolites and**
425 **Semisynthetic Derivatives of Coriloxine, 1.** The inhibitory effect of the natural
426 compounds **1**−**3**, and the semisynthetic derivatives of coriloxine, compounds **4**−**7**, was
427 evaluated in the same manner than the extracts for the three physiological processes on the
428 four weeds. Figure 3 shows the phytotoxic effect, expressed as inhibition percentage, for
429 the different compounds tested at 200 μg/mL. As it is observed in the Figure, all the
430 compounds exhibited a significant phytotoxic effect on the growth of the four target species
431 and their root elongation inhibition activity is more effective than their ability to inhibit
432 germination and seedling respiration (Figure 3A-C).

433 For the root elongation process of the four weeds, the seven evaluated compounds
434 had significant inhibition percentages between 19 and 100% at 200 μ g/mL. The greatest
435 effect was on root elongation of *A. hypochondriacus* (Figure 3B) with inhibitions ranging
436 from 45 to 100%; compounds **1** and **6** caused 100% inhibition, and **3** and **4** caused 80 and
437 96% inhibition respectively; compound **5** had no significant effect. Positive controls,
438 Rival® (31.8%) and ExterPro® (56.2%), are less effective than compounds **1**, **3**, **4**, and **6**.
439 Additionally, IC_{50} values of these compounds were found to be between 0.1 and 0.4 mM,
440 whereas the positive controls Rival® and ExterPro® values were >1.2 and >0.8 mM
441 respectively (Table 1).

442 On the other hand, the evaluated compounds reduced the germination of the four
443 weeds; however, the percentages of inhibition were low. Again, *A. hypochondriacus* was
444 the most affected weed with percentages of inhibition from 19 to 100% (Figure 3A).

Compounds **1** and **6** caused 100% of inhibition; IC_{50} values were found to be 0.7 mM and 0.2 mM respectively, while positive control Rival® presents an IC_{50} value >1.2 mM (Table 1).

Finally, with exception of the seeds of *M. sativa*, the compounds also inhibited the seedling respiration significantly at 200 µg/mL, though percentages of inhibition were low. Natural compounds **1**−**3** inhibited seedling respiration of *A. hypochondriacus* by ≥60% at 200 µg/mL, showing greater efficiency than the positive controls Rival® (6.0%) and ExterPro® (12.0%) at the same concentration (Figure 3C). IC_{50} values of compounds **1-3** were found to be 1.1, 1.1, and 0.8 mM respectively, whereas positive controls Rival® and ExterPro® were >1.2 mM and >0.8 mM (Table 1). Products **4** and **6** did not have a significant effect on the oxygen consumption of *A.hypochondriacus, P. miliaceum* and *T. pretense*.

Table 1 summarizes the phytotoxic effect, expressed as IC_{50} values, of the three isolated compounds, and the four coriloxine derivatives. The results show that all the tested compounds have a high phytotoxic activity, inhibiting the three physiological processes on the four seeds in a concentration-dependent way. The IC_{50} values confirmed that, in general, the evaluated compounds strongly inhibit the root growth, and have a weaker effect on the germination and seedling respiration processes.

In conclusion, this work demonstrates that the combined culture medium and mycelium extract, the three secondary metabolites from *X. feejeensis* strain SM3e-1 (**1**−**3**), and the four semisynthetic derivatives of coriloxine (**4**−**7**), possess phytotoxic properties against the evaluated physiological processes of three dicotyledon species, *T. pratense*, *M. sativa*, and *A. hypochondriacus;* and one monocotyledon species, *P. miliaceum*. Based on the results from this investigation, additional studies will be performed in order to have a

469 better understanding of the action mechanisms associated with the phytotoxic effects of the
470 phytotoxins, semisynthetic derivatives and organic extracts produced by endophytic fungus
471 *Xylaria feejeensis* strain SM3e-1b isolated from *S. macrocarpum*.
472
473 **ASSOCIATED CONTENT**
474 **Supporting Information**
475 A comparative Nucleotide BLAST analysis of the SM3e-1b sequence is available free of
476 charge via the Internet at http://pubs.acs.org. Crystallographic data for the semisynthetic
477 derivatives of coriloxine, compounds **4**, **5** and **6** have been deposited at the Cambridge
478 Crystallographic Data Centre (CCDC) as supplementary publication # CCDC 1450883,
479 CCDC 1450884 and CCDC 1450885 respectively. Copies of the data can be obtained, free
480 of charge, from http://www.ccdc.cam.ac.uk/data_request/cif.
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Notes

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FIGURE CAPTIONS

Figure 1. Structures of phytotoxic compounds isolated from the endophytic fungus *Xylaria feejeensis* strain SM3e-1b.

Figure 2. Schematic diagram of the synthetic pathway of compounds **4**, **5**, **6**, and **7**.

Figure 3. Phytotoxic effect of organic extract, secondary metabolites and semisynthetic compounds from endophytic fungus *Xylaria feejeensis* strain SM3e-1b (200 μg/mL) on *A. hypochondriacus, T. pratense*, *M. sativa*, and *P. miliaceum*: **A**. seed germination, **B**. root elongation and **C**. seedling respiration. Vertical bars represent SD, n =4; (∗) $P < 0.05$.

Figure 4. X-ray crystal structures of (4*R*,5*S*,6*R*)-6-chloro-4,5-dihydroxy-3-methoxy-5-methylcyclohex-2-enone, **4**, (4*R*,5*R*,6*R*)-4,5-dihydroxy-3-methoxy-5-methyl-6-(metylamino)cyclohex-2-enone, **5**, and (4*R*,5*R*,6*R*)-4,5-dihydroxy-3-methoxy-5-methyl-6-(phenylamino)cyclohex-2-enone, **6**.

Figure 5. Mechanism for the electrochemical formation of compound **2**.

Table 1. Phytogrowth-inhibitory Activity of Secondary Metabolites and Semisynthetic Compounds from *Xylaria feejeensis* strain SM3e-1b on the Germination, Root Elongation, and Seedling Respiration of *Amaranthus hypochondriacus*, *Trifolium pratense*, *Medicago sativa* and *Panicum miliaceum*.

Compound	Seed	IC_{50} (mM)		
		Germination	Root growth	Seedling Respiration
Organic extract*	*A. hypochondriacus*	160.1	221.5	279.3
	P. miliaceum	>400	305.4	>400
	T. pratense	>400	>400	>400
	M. sativa	>400	>400	231.2
1	*A. hypochondriacus*	0.7	0.1	1.1
	P. miliaceum	0.8	0.4	>1.2
	T. pratense	0.7	0.6	>1.2
	M. sativa	>1.2	1.1	>1.2
2	*A. hypochondriacus*	0.4	0.9	1.1
	P. miliaceum	>1.2	1.1	1.1
	T. pratense	>1.2	0.9	>1.2
	M. sativa	>1.2	1.0	>1.2
3	*A. hypochondriacus*	1.0	0.4	0.8
	P. miliaceum	>1.1	1.0	>1.1
	T. pratense	>1.1	>1.1	>1.1
	M. sativa	>1.1	0.9	>1.1
4	*A. hypochondriacus*	0.9	0.2	>1.0
	P. miliaceum	>1.0	>1.0	0.8
	T. pratense	>1.0	0.9	>1.0
	M. sativa	>1.0	>1.0	>1.0
5	*A. hypochondriacus*	>1.2	>1.2	>1.2
	P. miliaceum	>1.2	>1.2	>1.2
	T. pratense	>1.2	1.0	1.1
	M. sativa	>1.2	>1.2	>1.2
6	*A. hypochondriacus*	0.2	0.3	>0.8
	P. miliaceum	>0.8	0.5	>0.8
	T. pratense	>0.8	0.4	0.7
	M. sativa	>0.8	0.7	>0.8
7	*A. hypochondriacus*	>0.7	>0.7	>0.7

	P. miliaceum	>0.7	0.6	>0.7
	T. pratense	>0.7	0.6	>0.7
	M. sativa	>0.7	>0.7	>0.7
Rival®	*A. hypochondriacus*	>1.2	>1.2	>1.2
	P. miliaceum	>1.2	0.4	>1.2
	T. pratense	>1.2	>1.2	1.1
	M. sativa	>1.2	>1.2	>1.2
ExterPro®	*A. hypochondriacus*	0.4	>0.8	>0.8
	P. miliaceum	>0.8	0.2	>0.8
	T. pratense	>0.8	>0.8	>0.8
	M. sativa	>0.8	0.7	>0.8

Rival®= glyphosate: *N*-(phosphonomethyl)glycine (positive control); ExterPro®= hexazinone; 3-Cyclohexyl-6-(dimethylamino)-1-methyl-1,3,5-triazine-2,4(1H,3H)-dione. *Expressed in µg/mL

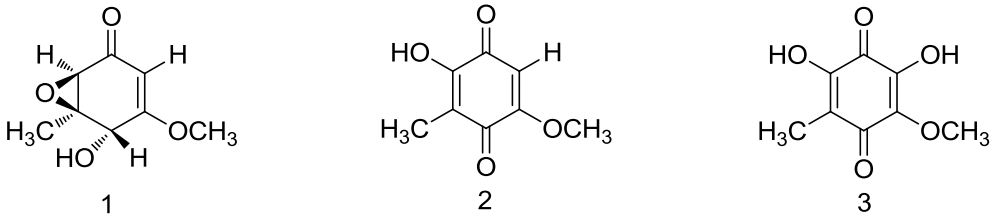


Figure 1.

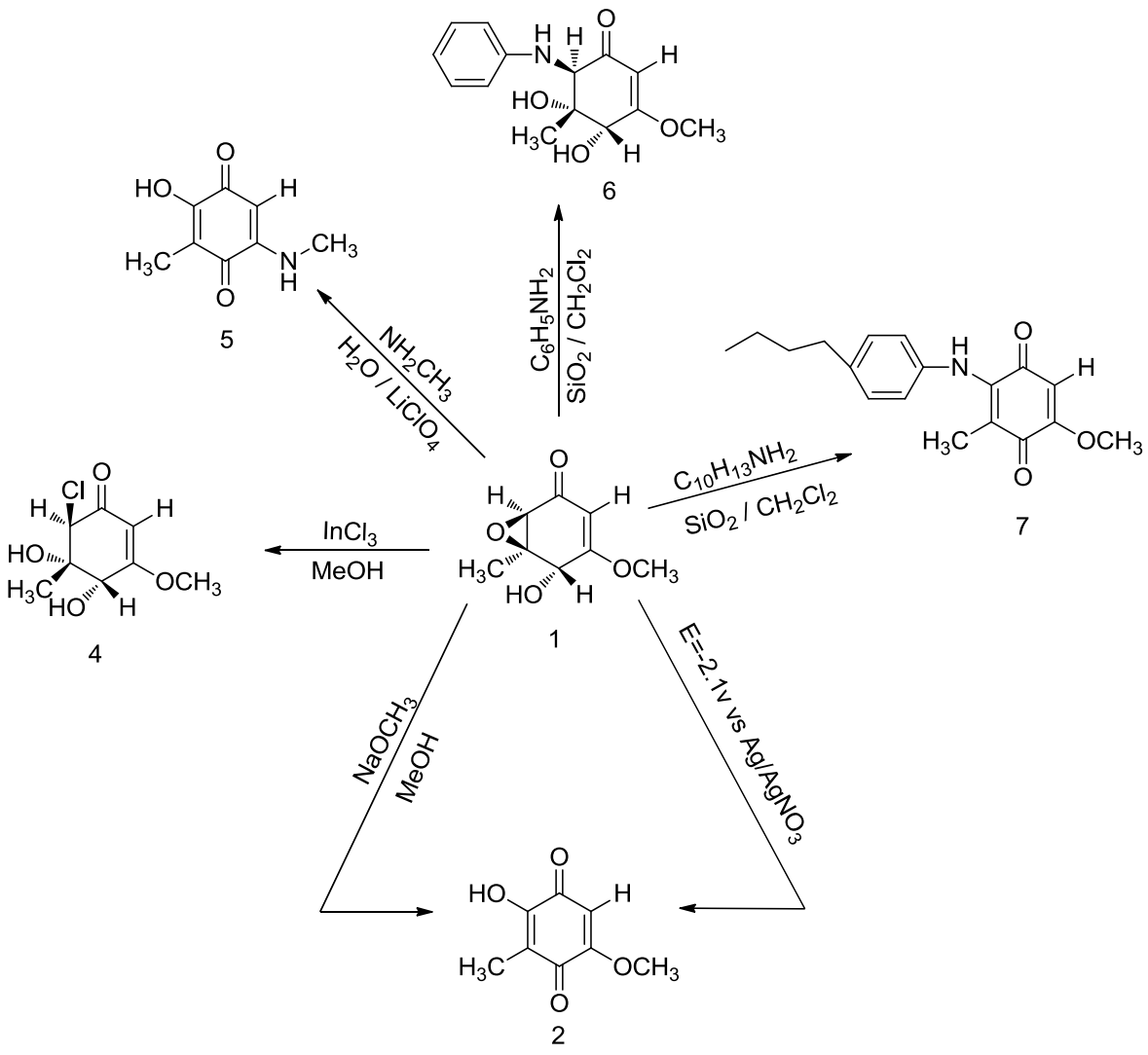

6
5
7
4
1
2
NH_2CH_3
H_2O / $LiClO_4$
$C_6H_5NH_2$
SiO_2 / CH_2Cl_2
$C_{10}H_{13}NH_2$
SiO_2 / CH_2Cl_2
$InCl_3$
MeOH
$NaOCH_3$
MeOH
E=-2.1v vs Ag/$AgNO_3$

Figure 2.

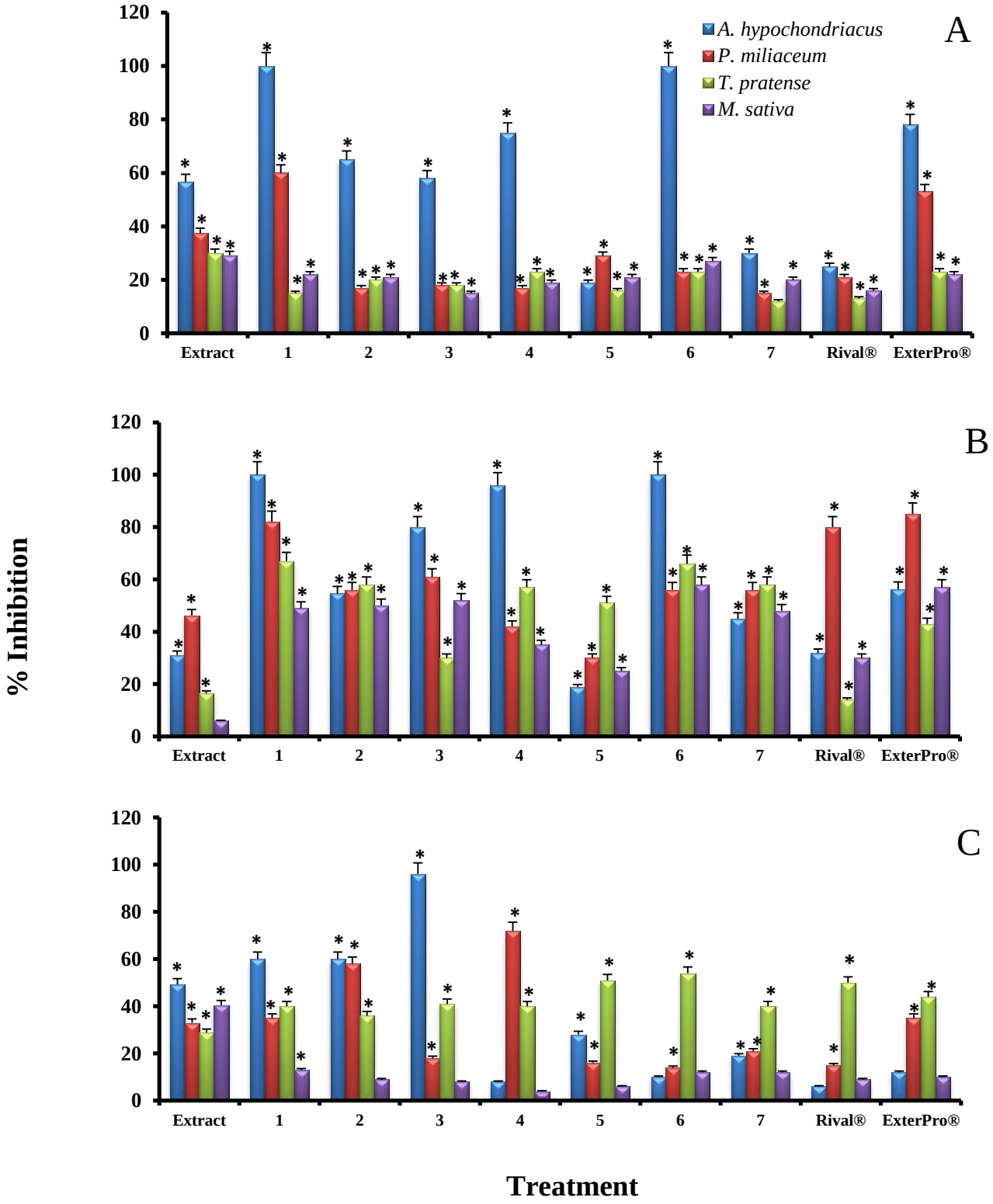
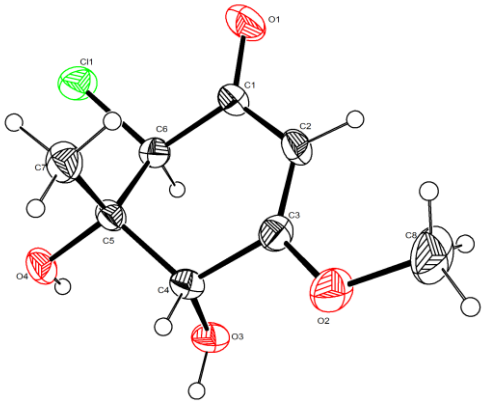

A
B
C
A. hypochondriacus
P. miliaceum
T. pratense
M. sativa
120
100
80
60
40
20
0
Extract
1
2
3
4
5
6
7
Rival®
ExterPro®
% Inhibition
Treatment

Figure 3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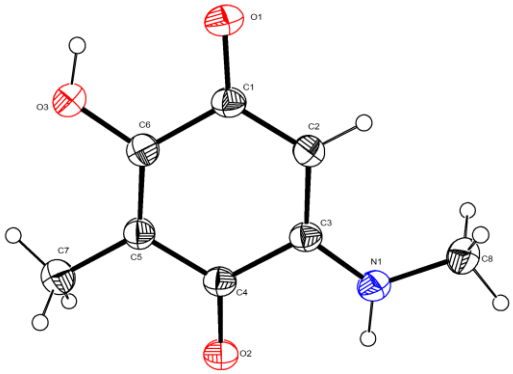
Cl1
C1
C2
C6
C7
C3
C5
C8
O4
C4
O2
O3

4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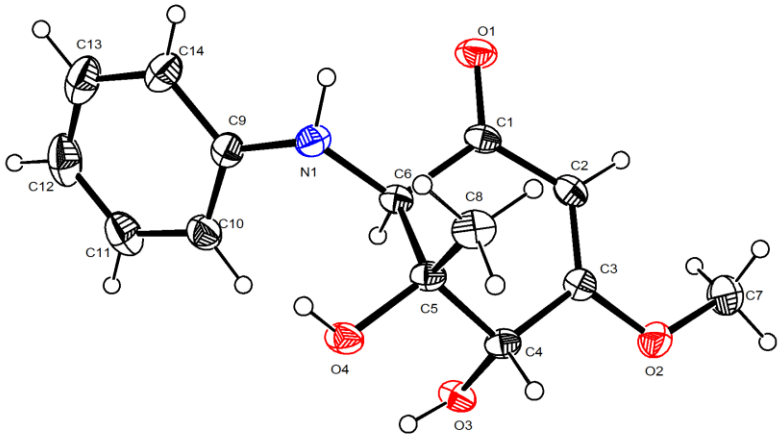
O3
C1
C6
C2
C7
C5
C3
N1
C8
C4
O2

5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C14
O1
C9
C1
N1
C6
C2
C12
C8
C10
C11
C3
C7
C5
C4
O4
O2
O3

6

Figure 4.

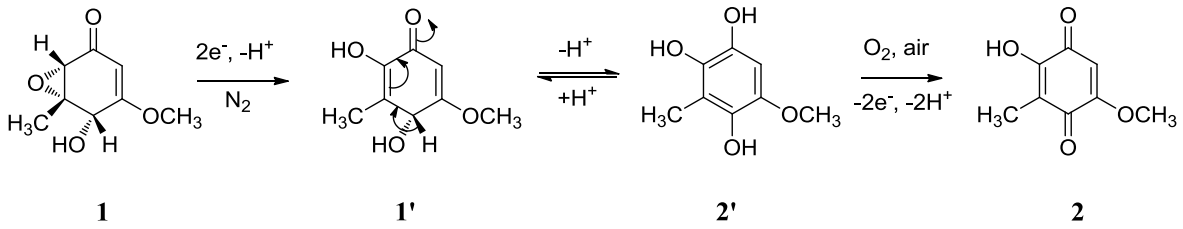
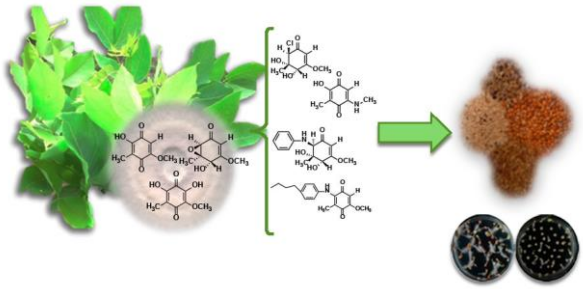


Figure 5.

TOC graphic



or