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Bioactive Metabolites from the Fungus *Nectria galligena*, the Main Apple Canker Agent in Chile

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The phytopathogenic fungus *Nectria galligena* Bres. is the most common canker disease agent of hardwood trees. The terpenoids colletochlorin B, colletorin B, ilicicolin C, E, and F, as well as the phytotoxin α,β-dehydrocurvularin have been isolated from liquid cultures of *N. galligena* obtained from the xylem of infected apple trees in central Chile. Ilicicolin C and F and α,β-dehydrocurvularin were active against *Pseudomonas syringae* with IC_{50} values of 28.5, 28.5, and 14.2 μg/mL, respectively, in the same range as streptomycin and penicillin G (11 and 15 μg/mL, respectively). All of the compounds showed moderate inhibitory activity toward the enzymes acetylcholinesterase (AChE) and β-glucuronidase. The most active enzyme inhibitors were colletochlorin B and ilicicolin C and E, with IC_{50} values of 30−36 μg/mL in the AChE assay and 32−43 μg/mL in the β-glucuronidase test. All of the chlorinated compounds showed some toxicity toward human lung fibroblasts, with IC_{50} values in the range of 64−120 μg/mL. α,β-Dehydrocurvularin proved to be the most toxic compound, showing IC_{50} values less than 12 μg/mL. The effect of the isolated compounds on seed germination and radicle and epicotyl growth was assessed in lettuce and millet seeds. At 100 and 200 μg/disk, α,β-dehydrocurvularin significantly reduced radicle length and epicotyl growth in *Lactuca sativa*. This is the first report on the occurrence of colletochlorin B, colletorin B, ilicicolin C, E, and F, as well as α,β-dehydrocurvularin associated to *N. galligena*.

KEYWORDS: ***Nectria galligena*; Ascomycete; apple canker; colletochlorin B; colletorin B; ilicicolin C, E, and F; α,β-dehydrocurvularin; biological activity**

INTRODUCTION

Nectria galligena Bres. (Ascomycota, Nectriaceae) is a phytopathogenic fungus that causes canker on tree and shrub species. The disease is characterized by the production of swollen dead areas of bark over which the periderm loosens and breaks away on twigs, branches, and trunks. These cankers are formed mainly at leaf scars wherever injuries occur (e.g., tree-tie or pruning wounds and bark fissures caused by frost and drought). They appear first as slightly sunken areas on the bark, becoming target-shaped or elongated through several growing seasons. The pathogen may be associated with hardwood trees, reducing log quality and value, and is also commonly found on apple (*Malus* x *domestica* Borkh) trees (*1*). In Chile, *Nectria* canker is found on apple nurseries and commercial orchards with serious economic effects. Severe outbreaks occur after cool and rainy weather conditions during leaf fall (April−July) at the growing regions of the country (*2*). Disease incidences as high as 68% have been reported on Richard Red apples in Southern Chile (*3*).

N. galligena is an ascomyces fungus with its asexual stage (anamorph) corresponding to *Cylindrocarpon heteronemum* (*4*). Species belonging to genus *Nectria* including *N. haematococca* are known to produce metabolites such as the naphthoquinones fusarubin and its derivatives, javanicin, bostricoidin, fusarubinoic acid, nectriafurone, and other pigments (*5*). These compounds displayed antimicrobial, insecticidal, and phytotoxic activity (*6*).

The aim of the present work was to grow into liquid culture local strains of *N. galligena*, to isolate their metabolites, and to assess the activity of the metabolites on seed germination, radicle and epicotyl growth, as well as toward selected enzymes and microorganisms. The cytotoxicity of the isolated compounds was also evaluated on human lung fibroblasts.

MATERIALS AND METHODS

Isolation and Culture of *N. galligena*. The fungus *N. galligena* Bres. was isolated from apple tree bark collected in the localities of El Olivar and Requinoa, VI Región, central Chile, in April of 2003. The fungus was cultured in two different liquid media, potato-glucose (PG, 20 g of dehydrated mashed potatoes and 20 g of glucose in 1 L of

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water at pH 5.5) and malt extract/glucose/peptone (MGP, 20 g of malt extract, 20 g of glucose, and 1 g of peptone in 1 L of water at pH 5.5). The fungus was cultured at 24 °C on a shaker platform at 150 rpm. After glucose consumption, the medium containing the metabolite soup and fungus (7 L of PG and 9.5 L of MGP medium for each isolate) was extracted separately with ethyl acetate (EtOAc, 4 × 500 mL). The organic phase was dried over anhydrous sodium sulfate, and the extracts were taken to dryness under vacuum at temperatures lower than 40 °C.

Antibacterial and Antifungal Activity. The antifungal activity against *Alternaria alternata* (Centro Micológico, Universidad de Rosario, Rosario, Argentina) and *Botrytis cinerea* (Instituto de Investigaciones Agropecuarias del Gobierno de Chile, INIA) was determined in triplicate experiments by the microdilution method (*7*−*9*), and results are presented as the minimum inhibitory concentration (MIC). The spores were cultured on Saboureaud medium at 25 °C for 7 days. The compounds were assessed in the dilution interval of 250−15 μg/mL, and the standard antifungal compounds were assessed in the range of 250−3.9 μg/mL. Spores were obtained from well-grown and sporulating fungal cultures maintained in potato−glucose−agar medium by suspention in sterile distilled water, filtration on glass wool, and centrifugation. The spores were counted in a Neubauer chamber and diluted with sterile distilled water to a final concentration of 10^4−10^5 spores/mL. The assay was carried out in 96-well microtiter plates. Some 100 μL of the spore suspention was incubated with 100 μL of the compound sample suspended in Saboureaud medium. The final volume of the mixture was 200 μL. A spore germination control and a Saboureaud medium control were included in all of the experiments as well as the standard fungicides iprodione [3-(3,5-dichlorophenyl)-*N*-isopropil-2,4-dioxoimidazolidine-1-carboxamide] (Rukon, Aventis CropScience, France) and myclobutanil [α-butyl-α-(4-chlorophenyl)-1*H*-1,2,4-triazol-1-propanonitrile] (Systhane, Dow AgroSciences). The MIC is defined as the lowest concentration of the compound showing no visible spore germination after the incubation time (7 days).

Antibacterial assays against the phytopathogenic Gram-negative bacteria *Erwinia carotovora* subsp. *carotovora*, isolated from *Capsicum annuum* (Talca) and *Pseudomonas syringae* pv. *tomato* (INIA) were carried out by the doubling dilution method using the procedure reported by Eloff (*10*) in 96-well microtiter plates.

Bacterial suspensions were obtained from overnight cultures in Luria Broth Base nutrient broth (Gibco BRL, Scotland) cultured at 25 °C and diluted to approximately 10^5 colony-forming units (CFU)/well in fresh medium. The compounds were dissolved to give 1 mg/mL in methanol (MeOH) as a stock solution. Stock solutions of compounds were diluted to give serial 2-fold dilutions that were added to each medium resulting in concentrations ranging from 250 to 1.92 μg/mL. The final concentration of MeOH in the assays did not exceed 2%. The plates were kept at 25 °C overnight (12 h). After incubation, 20 μL of 0.5 mg/mL aqueous 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, MO) was added in each well and re-incubated for 30 min to detect living bacteria. The absorbance was read in a universal microplate reader (Bio-Tek Instruments, Inc., VT) at 515 nm. The results were transformed to the percentage of controls, and the median inhibitory concentration (IC_{50}) values were graphically obtained from the dose−response curves. Penicillin G (Sigma−Aldrich, St. Louis, MO) and streptomycin (Laboratorio Chile, Santiago, Chile) were used as standard antibacterials.

Phytotoxic Effect. This assay was carried out according to Cutillo et al. (*11*). All of the compounds were dissolved in MeOH and applied to a Whatman 4 paper disk (1.5 cm diameter). The paper disk received either the compounds at 200, 100, and 50 μg/disk, the solvent (MeOH), or the reference pre-emergence herbicide Herbadox 330 EC (BASF, Germany): *N*-(1-ethylpropyl)-3,4-dimethyl-2,6-dinitrobenzamine, pendimethalin, 330 g/L active constituent. Seeds of the dicot *Lactuca sativa* var. Great Lakes and the monocot *Panicum millaceum* were used for the test. The seed viability was tested before the experiments and was 85 and 92%, respectively. Some 15 seeds were deposited on the dry paper disks, and 200 μL of water was added to each vial. Experiments were carried out in triplicate. Vials were placed in a humid chamber to avoid water evaporation. Seeds were stored for 72 h in the dark at a temperature of 25 °C and then exposed to light for 24 h. The parameters measured were germination percentage and radicle and epicotyl length. Controls were performed using distilled water and the reference herbicide Herbadox 330 EC.

***β*-Glucuronidase Inhibition Assay.** β-Glucuronidase activity was measured spectrophotometrically using *p*-nitrophenyl-β-D-glucuronide as a substrate as reported by Kawasaki et al. (*12*). The standard inhibitor glucosaccharo-1:4 lactone was used as a reference compound. Compounds were tested at 50 μg/mL. When the enzyme inhibition was >50% at this concentration, further dilutions were undertaken and the corresponding IC_{50} values were determined by means of regression analysis. The enzyme, obtained from bovine liver (Sigma G-0501, type B-10), substrate, and standard inhibitor were purchased from Sigma Chemical Co., St. Louis, MO.

Acetylcholinesterase (AChE) Inhibition Assay. The assay for measuring AChE inhibitory activity was carried out according to Ellman et al. (*13*) and adapted to 96-well microtiter plates by López et al. (*14*). The enzyme was obtained from electric eel (C-3389, type VI-S, Sigma Chemical Co., St. Louis, MO). Some 50 μL of the sample dissolved in phosphate buffer (8 mM K_2HPO_4, 2.3 mM NaH_2PO_4, 150 mM NaCl, and 0.05% Tween 20 at pH 7.6) and 50 μL of the AChE solution (0.25 unit/mL) in the same phosphate buffer were added to the wells, and the plates were incubated for 30 min at room temperature. After the incubation time, 100 μL of the substrate solution [40 mM Na_2HPO_4, 0.2 mM 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), and 0.24 mM acetylthiocholine iodide (ACTI) in distilled water at pH 7.5] was added. The absorbance was read in a Bio-Tek Instrument microplate reader at 405 nm after 3 min. The enzyme activity was calculated as a percentage compared to a control using only the buffer and enzyme solution. The compounds were assayed at 100 μg/mL, and the alkaloid galanthamine was used as the reference compound. When the enzyme inhibition was >50% at 100 μg/mL, dilutions were performed to determine the corresponding IC_{50} values. The IC_{50} values were calculated by means of regression analysis from three individual determinations.

Cytotoxicity. The cytotoxic effect of the compounds, expressed as cell viability, was assessed on the permanent fibroblast cell line derived from human lung (MRC-5) (ATCC Nr. CCL-171). MRC-5 fibroblasts were grown as monolayers in minimum essential Eagle medium (MEM) with Earle's salts (*15*), 2 mM L-glutamine, and 1.5 g/L sodium bicarbonate, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator with 5% CO_2 in air at 37 °C. Cells were seeded at a density of 2.5×10^3 cells/well in 96-well plates. Confluent cultures of MRC-5 cells were treated with medium containing the compounds at concentrations ranging from 1000 to 3.8 μM. The substance was first dissolved in DMSO and then in MEM supplemented with 2% FBS. The final concentration of DMSO in the test medium and controls was 1%. Cells were exposed for 24 h to the test medium with or without the compound (control). Each drug concentration was tested in quadruplicate and repeated 3 times in separate experiments. At the end of the incubation, the neutral red uptake (NRU) assay was carried out as described by Rodríguez and Haun (*16*). To calculate the IC_{50} values, the results were transformed to the percentage of controls and the IC_{50} values were graphically obtained from the dose−response curves. The IC_{50} value is defined as the concentration of the compound that reduces cell viability in 50%.

Structural Identification of the Compounds. Nuclear magnetic resonance (NMR) spectra were obtained with a multinuclear Bruker Avance 400 spectrometer operating at 400 MHz for protons and 100 MHz for ^{13}C. $CDCl_3$ was used as the solvent. The UV spectra were obtained using a Helios α V-3.06 UV/vis spectrophotometer. Mass spectra were measured with a VG EBE Autospec Micromass at 70 eV. Infrared (IR) spectra on KBr disks were run on a Nicolet Nexus 470 or a Bruker FT-IR spectrophotometer. Melting points were measured in capillar tubes in an Electrothermal 9.100 equipment. Optical rotations were measured in a Jasco DIP 370 digital polarimeter.

Statistical Analysis. Regarding the phytotoxicity experiments, statistical differences between treatments and their respective control were determined by one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test. The level of significance was set at $p < 0.05$.

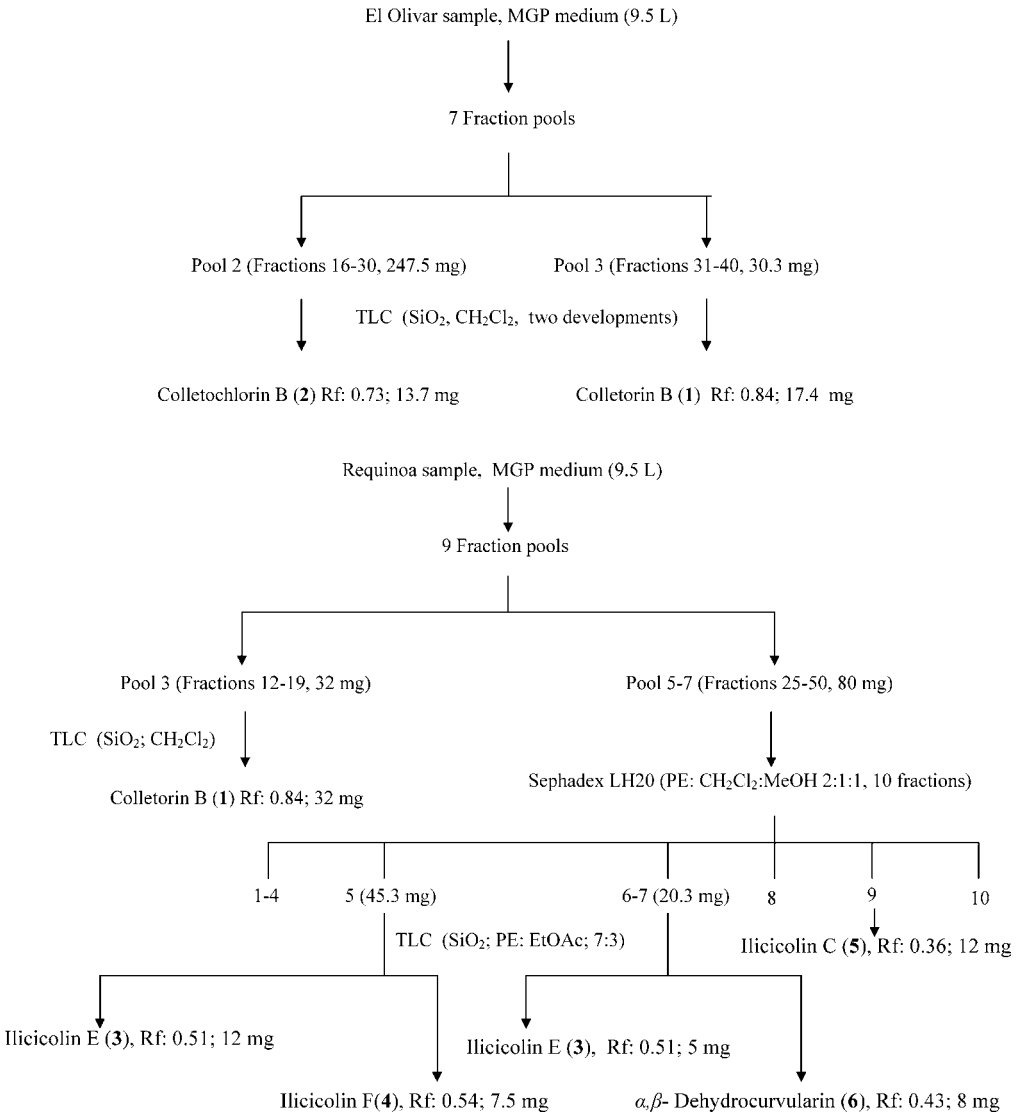

Figure 1. Isolation of compounds **1**–**6** from MGP medium.

RESULTS AND DISCUSSION

Extracts. From the PG medium (7 L for each isolate) some 1.2 g of EtOAc solubles were obtained from the El Olivar and 1.3 g of EtOAc solubles from the Requinoa sample. From the isolates cultured in MGP medium (9.5 L for each isolate) some 2.8 and 1.8 g of EtOAc solubles were obtained for the Requinoa and El Olivar isolates, respectively.

Fungal Culture in MGP Medium. Both EtOAc extracts from El Olivar and Requinoa were compared by analytical thin-layer chromatography (TLC) on silica gel plates (Sigma−Aldrich Chemie, Steinheim, Germany) showing different patterns. Therefore, extracts were separately worked-up. The EtOAc extract from El Olivar (1.8 g) was chromatographed on a silica gel (Merck 7734, particle size 0.063−0.200 mm) column (60 cm length, internal diameter of 3 cm) eluting with a petroleum ether, petroleum ether/dichloromethane (CH_2Cl_2), CH_2Cl_2, and methanol (MeOH) gradient. The volume for each solvent or solvent mixture is given in parentheses followed by the number and sequence of fractions. Petroleum ether (1 L, fractions 1−8), 9:1 (v/v) petroleum ether/CH_2Cl_2 (0.5 L, fractions 9−15), 8:2 (v/v) petroleum ether/CH_2Cl_2 (0.5 L, fractions 16−25), 7:3 (v/v) petroleum ether/CH_2Cl_2 (0.5 L, fractions 26−32), 6:4 (v/v) petroleum ether/CH_2Cl_2 (0.5 L, fractions 33−44), 1:1 (v/v) petroleum ether/CH_2Cl_2 (1 L, fractions 45−53), 4:6 (v/v) petroleum ether/CH_2Cl_2 (0.5 L, fractions 54−62), 3:7 (v/v) petroleum ether/CH_2Cl_2 (0.5 L, fractions 63−70), 2:8 (v/v) petroleum ether/CH_2Cl_2 (0.5 L, fractions 71−80), 1:9 (v/v) petroleum ether/CH_2Cl_2 (0.5 L, fractions 81−92), CH_2Cl_2 (1 L, fractions 93−101), 1:1 (v/v) CH_2Cl_2/MeOH (1 L, fractions 102−112), and MeOH (1 L, fractions 113−120). Fractions were pooled according to the TLC patterns as follows: pool 1, fractions 1−15; pool 2, fractions 16−30; pool 3, fractions 31−40; pool 4, fractions 41−49; pool 5, fractions 50−79; pool 6, fractions 80−99; and pool 7, fractions 100−120. The isolation of the secondary metabolites is summarized in **Figure 1**.

The EtOAc extract from the Requinoa fungus (2.8 g) was chromatographed on a silica gel (Merck 7734, particle size of 0.063−0.200 mm) column (70 cm length, internal diameter of 3 cm) and eluted with a petroleum ether, petroleum ether/CH_2Cl_2, CH_2Cl_2, and MeOH gradient. The volume for each solvent or solvent mixture is given in parentheses followed by the number and sequence of fractions. Petroleum ether (1 L, fractions 1−2), 9:1 (v/v) petroleum ether/CH_2Cl_2 (0.75 L, fractions 3−6), 8:2 (v/v) petroleum ether/CH_2Cl_2 (0.75 L, fractions 7−11), 7:3 (v/v) petroleum ether/CH_2Cl_2 (0.75 L, fractions 12−16), 6:4 (v/v) petroleum ether/CH_2Cl_2 (0.75 L, fractions 17−20), 1:1 (v/v) petroleum ether/CH_2Cl_2 (1 L, fractions 21−25), 4:6 (v/v) petroleum ether/CH_2Cl_2 (0.75 L,

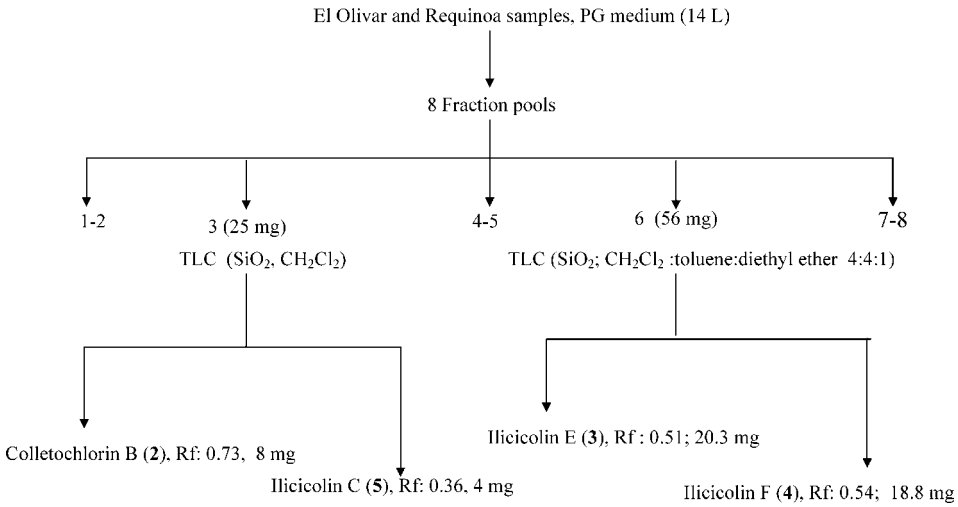

Figure 2. Isolation of compounds **2**–**5** from PG medium.

fractions 26−32), 3:7 (v/v) petroleum ether/CH_2Cl_2 (0.75 L, fractions 33−37), 2:8 (v/v) petroleum ether/CH_2Cl_2 (0.75 L, fractions 38−42), 1:9 (v/v) petroleum ether/CH_2Cl_2 (0.75 L, fractions 43−47), CH_2Cl_2 (1 L, fractions 48−52), 1:1 (v/v) CH_2Cl_2/MeOH (1 L, fractions 53−57), and MeOH (1 L, fractions 58−60). Fractions were pooled according to the TLC patterns as follows: pool 1, fractions 1−6; pool 2, fractions 7−11; pool 3, fractions 12−19; pool 4, fractions 20−24; pool 5, fractions 25−31; pool 6, fractions 32−38; pool 7, fractions 39−50; pool 8, fractions 51−56; and pool 9, fractions 57−60. The isolation of the secondary metabolites is summarized in **Figure 1**.

Fungal Culture in PG Medium. The EtOAc extracts obtained in PG medium (1.2 g for El Olivar and 1.3 g for the Requinoa sample) were compared by TLC using different solvent systems and showed the same TLC pattern. Therefore, both extracts were combined and worked-up together. The total extract (2.5 g) was chromatographed on a silica gel (Merck 7734, particle size of 0.063−0.200 mm) column (60 cm, internal diameter of 3 cm) and eluted with a petroleum ether, petroleum ether/CH_2Cl_2, CH_2Cl_2, and MeOH gradient. The volume for each solvent or solvent mixture is given in parentheses followed by the number and sequence of fractions. Petroleum ether (1 L, fractions 1−3), 9:1 (v/v) petroleum ether/CH_2Cl_2 (0.75 L, fractions 4−8), 8:2 (v/v) petroleum ether/CH_2Cl_2 (0.75 L, fractions 9−12), 7:3 (v/v) petroleum ether/CH_2Cl_2 (0.75 L, fractions 13−17), 6:4 (v/v) petroleum ether/CH_2Cl_2 (0.75 L, fractions 18−20), 1:1 (v/v) petroleum ether/CH_2Cl_2 (1 L, fractions 21−25), 4:6 (v/v) petroleum ether/CH_2Cl_2 (0.75 L, fractions 26−32), 3:7 (v/v) petroleum ether/CH_2Cl_2 (0.75 L, fractions 33−37), 2:8 (v/v) petroleum ether/CH_2Cl_2 (0.75 L, fractions 38−43), 1:9 (v/v) petroleum ether/CH_2Cl_2 (0.75 L, fractions 44−49), CH_2Cl_2 (1 L, fractions 50−54), 1:1 (v/v) CH_2Cl_2/MeOH (1 L, fractions 55−59), and MeOH (1 L, fractions 60−62). Fractions were pooled according to the TLC patterns as follows: pool 1, fractions 1−8; pool 2, fractions 9−18; pool 3, fractions 19−22; pool 4, fractions 23−28; pool 5, fractions 29−37; pool 6, fractions 38−45; pool 7, fractions 46−56; and pool 8, fractions 57−62. The isolation of the secondary metabolites is summarized in **Figure 2**.

Six metabolites previously described from other microorganisms were identified in *N. galligena* by comparison of their ^1H and ^{13}C NMR data with the literature. The spectroscopic data are in agreement with the proposed structures. The isolated compounds and the references are listed below. Colletorin B (**1**) and colletochlorin B (**2**) (*17*−*19*). Ilicicolin E (**3**) (*19*−*22*), ilicicolin F (**4**) (*20*, *22*, *23*), ilicicolin C (**5**) (*19*−*22*), and α,*β*-dehydrocurvularin (**6**) (*24*, *25*). The structures of compounds **1**−**6** are presented in **Figure 3**. The ^1H and ^{13}C NMR assignments for compounds **1**−**6** are presented in **Tables 1**−**3**. Copies of the spectra are available from the authors.

Cylindrocarpon lucidum (Ascomycota, Hypocreales) has been reported as a producer of ascochlorine-derived compounds known as cylindrols (*21*). Derivatives of ilicicolin E (**3)** and ilicicolin F **(5)** were isolated from *N. coccinea* (*5*, *20*), *Fusarium* (*23*), and *Ascochyta viciae* (*18*, *19*). Ascochlorin was previously isolated and characterized from *Acremonium luzulae* (*26*) and reported as an antibiotic from *Ascochyta viciae* (*27*). Ascochlorin has an inhibitory effect on mitochondrial respiration as well as inhibitory action on the growth of the tobacco mosaic virus (*28*) and showed a significant inhibitory effect on the Newcastle disease virus growth in cultured cells (*28*) and antifungal activity (*29*). Compounds belonging to the ascochlorin family showed a wide range of inhibitory effects on farnesyl protein transferase (FPTase) activity (*21*, *30*). Ascochlorin and 8,9-dehydroascochlorin showed a significant inhibitory effect on the activity of testosterone-5α-reductase and presented antitumor activity (*22*). Ilicicolin C, E, and F isolated from *N. galligena* are close related to ascochlorin and might be a good source of industrial scaffolds for this interesting metabolite. Colletorin B was previously isolated from *Cephalosporium diospyri* (*17*), while colletochlorin B was reported from *Colletotrichum nicotianae* (*18*). Colletochlorin B and ascochlorin were also obtained from *Nigrosabulum globosum* (*29*). Colletochlorin B and derivatives have been shown to be powerful inducers of differentiation of human promyelocytic leukemia cells and a morphogen factor that induces differentiation of *Dictyostelium discoideum* (*31*).

Ilicicolins A−H were reported from *Cylindrocladium ilicicola* (*20*), while ilicicolin F was isolated from *Verticillium hemipterigenum* (*5*) and *Fusarium* sp. (*23*). Ilicicolin E was first described from *Verticillium* sp., and ilicicolin C is a metabolite produced by *N. coccinea* and *Fusarium* sp. (*5*). Ilicicolins D, E, and F were previously isolated from an unidentified *Nectria* species and evaluated against plant pathogenic fungi, showing good fungicidal activity (*32*). Ilicicolins were also reported to be fungicidal to the oomycetes *Phytophthora infestans* and *Plasmopara viticola*, and according to Yamaguchi (*33*), they can be considered interesting leads for further synthetic and structure−activity studies. Ilicicolins isolated from an unidentified *Fusarium* species were reported to display anti-*Tetrahymena pyriformis* activity (*23*). α,*β*-Dehydrocurvularin was reported from *Alternaria zinniae* (*24*) and *Aspergillus* sp. (*5*, *25*) and exhibits phytotoxic, antifungal, and cytotoxic effects (*25*).

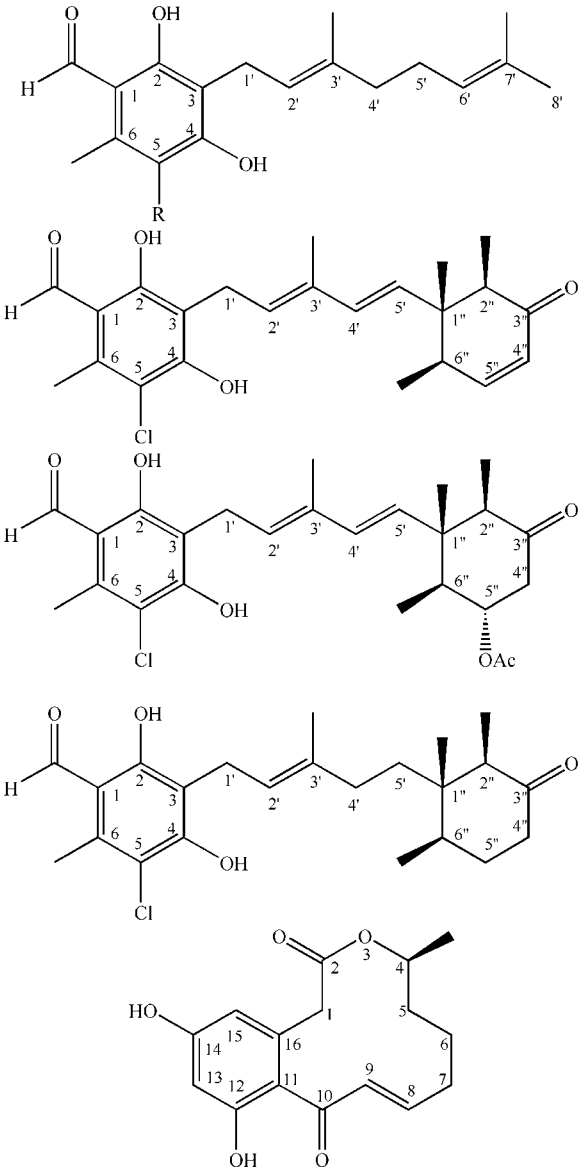

Figure 3. Secondary metabolites isolated from *N. galligena*.

In the present work, five prenylated phenols and α,β-dehydrocurvularin were isolated from liquid cultures of the phytopathogenic fungus *N. galligena*. The metabolites **2**−**5** were isolated from the extract obtained from PG broth, while compounds **1**−**6** were produced in MGP medium. One of the aims of the study was to compare the production of secondary metabolites from two different accessions of the fungus. The Requinoa sample was isolated from a Golden Delicious apple tree, while the El Olivar fungus was obtained from a Granny Smith apple tree. While the El Olivar sample produced the "open" chain metabolites **1**−**2** in MGP medium, from the Requinoa isolate, colletorin and ilicicolin derivatives as well as the phytotoxin α,β-dehydrocurvularin were obtained. The different compounds were obtained because of different liquid media and individual variation of the fungal isolates.

In our search for bioactive compounds from microbial origin, the isolated compounds were assessed for several biological activities. The antibacterial and antifungal effect was determined to disclose the potential of the fungal compounds to control phytopathogenic fungi and bacteria. The seed germination assay allowed us to obtain information on the phytotoxicity of the compounds as well as the influence of the fungal metabolites on seedling root and epicotyl growth. Using both monocot and dicot seeds, the test gives information on the potential herbicidal effect of the assayed products. The cytotoxicity on human fibroblast measures the toxicity of the products on a mammal cell line, thus allowing a comparison of the active concentrations on different test systems with the mammal cell toxicity.

AChE inhibitors are the only approved drugs for treating patients with mild to moderately severe Alzheimer's disease, a disorder associated with progressive degeneration of memory and cognitive function (*14*). The β-glucuronidase is an exoglycosidase enzyme that catalyzes the cleavage of *O*-glucuronosyl bonds. It has been reported that, in certain diseases such as cancer, inflammatory joint disease, some hepatic diseases, and AIDS, the β-glucuronidase activity increases. Endogenous biliary β-glucuronidase deconjugates the glucuronides of bilirubin and causes the development of cholelithiasis in human bile. Many β-glucuronidase inhibitors such as 8-hydroxytricetin-7-glucuronide, isovitexin, trihydroxy-pipecolic acid, and scoparic acid A and C have already been isolated from different plants, and some are used clinically (*34*). We expected to find some effect on seed germination and cytotoxicity because of the damage produced to the host plant.

All of the assayed compounds showed low antifungal activity toward *Botrytis cinerea*. Compound **6** presented a MIC of 62.5

Table 1. ^{1}H NMR Assignments for Compounds **1**–**5** (400 MHz in $CDCl_3$; δ Values in ppm, *J* in Hz)

H	1	2	3	4	5
1-CHO	10.11 s	10.19 s	10.18 s	10.19 s	10.19 s
2-OH	12.80 s	12.73 s	12.75 s	12.75 s	12.73 s
4-OH	6.34 brs	6.50 brs	6.45 brs	6.42 brs	6.45 brs
5	6.25 s				
6-CH_3	2.53 s	2.65 s	2.64 s	2.65 s	2.65 s
1′	3.44 d (6.9)	3.44 d (7.3)	3.58 d (7.3)	3.58 d (6.9)	3.43 d (6.9)
2′	5.30 t (6.9)	5.27 t (7.3)	5.59 t (7.3)	5.59 t (6.9)	5.29 t (6.9)
3′-CH_3	1.85 s	1.83 brs	1.97 brs	1.97 s	1.85 s
4′	2.04–2.19 m	2.04 m	6.04 d (16)	5.97 d (15.7)	2.00 m
					1.87 m
5′	2.04–2.19 m	2.10 m	5.44 d (16)	5.37 d (15.7)	1.40 m
6′	5.09 t (6.6)	5.10 dd			
		(6.9, 5.9)			
7′-CH_3	1.63 s	1.62 s			
8′	1.71 s	1.69 s			
2″			2.49 q (6.8)	2.47 m	2.47 q (6.9)
4″			6.03 dd	2.91 dd (13.7,	1.87 m
			(10.3, 2.9)	5.9), 2.40 m	1.64 m
5″			6.60 dd	4.93 ddd	2.36 m
			(10.3, 2.0)	(11.7, 10.8, 5.9)	
6″			2.68 dq	2.03 dq	2.03 m
			(7.6, 2.0)	(11.7, 5.9)	
1″-CH_3			0.84 s	0.78 s	0.60 s
2″-CH_3			1.02 d (6.8)	0.91 d (6.9)	0.94 d (6.9)
6″-CH_3			0.96 d (7.6)	0.91 d (6.9)	0.92 d (6.9)
OAc				2.10 s	

Table 2. ^{13}C NMR Assignments for Compounds **1**–**5** (100 MHz in $CDCl_3$; δ Values in ppm)

H	1	2	3	4	5
1	113.3 s	113.9 s	113.8 s	113.0 s	111.9 s
1-CHO	193.6 d	191.7 d	193.2 d	193.9 d	193.0 d
2	163.2 s	163.2 s	162.2 s	164.0 s	162.3 s
3	116.7 s	114.3 s	113.8 s	113.0 s	113.9 s
4	156.0 s	157.9 s	156.2 s	157.0 s	163.7 s
5	113.3 d	112.6 s	113.7 s	113.0 s	110.7 s
6	137.0 s	138.7 s	134.4 s	137.0 s	138.7 s
6-CH_3	15.8 q	21.0 q	14.5 q	12.6 q	16.5 q
1′	22.0 t	21.7 t	22.3 t	22.6 t	21.3 t
2′	121.1 d	121.3 d	134.2 d	128.2 d	121.3 d
3′	135.4 s	138.8 s	134.4 s	137.9 s	141.9 s
3′-CH_3	15.4 q	14.7 q	14.5 q	12.6 q	18.0 q
4′	41.5 t	40.9 t	134.4 d	134.2 d	35.6 t
5′	27.2 t	25.2 t	134.0 d	134.2 d	32.7 t
6′	122.0 d	124.8 d			
7′	133.2 s	131.2 s			
7′-CH_3	15.1 q	18.2 q			
8′	26.8 q	25.4 q			
1″			48.0 s	45.4 s	43.9 s
2″			51.9 d	53.9 d	50.6 d
3″			209.7 s	207.9 s	214.3 s
4″			128.0 d	47.2 t	41.6 t
5″			152.2 d	73.7 d	30.9 t
6″			42.0 d	45.6 d	36.2 d
1″-CH_3			12.6 q	11.5 q	15.4 q
2″-CH_3			8.9 q	8.9 q	15.1 q
6″-CH_3			15.2 q	14.5 q	7.6 q
OAc				170.1 s, 21.1 q	

Table 3. ^{1}H and ^{13}C NMR Assignments for Compound **6** (400 and 100 MHz, respectively, in $CDCl_3$; δ Values in ppm, *J* in Hz)

	^{1}H	^{13}C
1	4.08 d (17.6), 3.57 d (17.6)	43.9 t
2		171.8 s
4	4.92 m	73.0 d
5	1.95 m, 1.68 m	34.0 t
6	2.02 m, 1.68 m	24.0 t
7	2.54 m, 2.40 m	32.7 t
8	6.66 ddd (15.4, 8.8, 3.9)	148.3 d
9	6.69 dd (15.4, 1.3)	131.5 d
10		196.3 s
11		166.3 s
12	6.35 d (1.96)	103.3 d
13		161.0 s
14	6.30 d (1.96)	113.6 d
15		137.7 s
16		114.4 s
Me	1.30 d (6.5)	19.9 q

μg/mL against *Alternaria alternata*, while compounds **2**−**4** showed a MIC of 125 μg/mL and compounds **1** and **5** were less active (MIC > 250 μg/mL). The positive controls were the fungicides Systhane and Rukon with MICs of 31.3 and 3.9 μg/mL against *A. alternata* and 15.6 and 31.3 μg/mL toward *B. cinerea*, respectively. The six isolated compounds were slightly active against *Erwinia carotovora*, with IC_{50} values > 250 μg/mL. The most active compounds toward *Pseudomonas syringae* were **4**, **5**, and **6**, with IC_{50} values of 28.5, 28.5, and 14.2 μg/mL, respectively. Under our assay conditions, the IC_{50} values of streptomycin and penicillin G against *P. syringae* were 11.1 and 15.6 μg/mL, respectively, while for *E. carotovora* the values were 15.6 and 122.7 μg/mL.

The effect of the compounds **1**−**6** on seed germination and radicle and epicotyl growth was assessed in lettuce and millet seeds. Significant reduction in germination of the *L. sativa* seed was detected with metabolites **4** and **6**, at 200 μg/disk, while a significant reduction in germination of the *P. millaceum* seed was detected with metabolite **6** at 200 μg/disk (**Figure 4A**). The radicle in *L. sativa* was significantly reduced in length by compounds **3** and **6** at 100 and 200 μg/disk (**Figure 4B**). At the assayed concentrations, none of the compounds elicited a significant effect on the radicle length of *P. millaceum*. Compounds **1**, **2**, and **6** presented a significant reduction in epicotyl growth of *L. sativa* seedlings at 100 and 200 μg/disk. A significant reduction in epicotyl growth of *P. millaceum* seedlings was detected with metabolites **3** and **5** at 200 μg/disk and compounds **4** and **6** at 100 and 200 μg/disk (**Figure 4C**). All natural products were much less active than the reference herbicide Herbadox 330 EC, which reduced germination by 50% at 12 and 5.3 μg/disk for lettuce and millet, respectively.

Compounds **2**, **3**, and **5** showed moderate inhibitory activity toward AChE, with IC_{50} values of 30−32 μg/mL (**Table 4**). The compounds **1**−**3** and **5** showed a moderate inhibitory effect on the enzyme β-glucuronidase, with IC_{50} values of about 33−

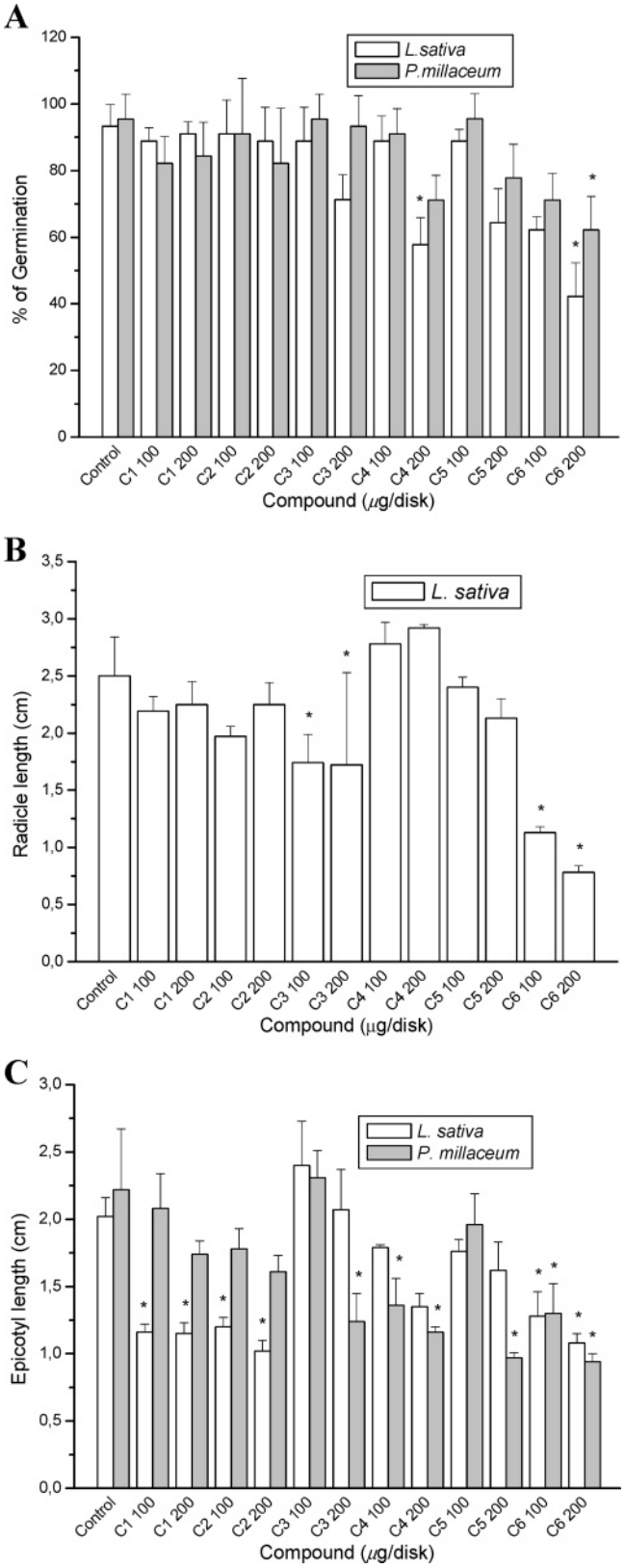

Figure 4. Effect of the secondary metabolites of *N. galligena* on seed germination (A), radicle (B), and epicotyl length (C) of *L. sativa* (dicot, A, B, and C) and *P. millaceum* (monocot, A and C) after a 96-h treatment. Experiments were carried out in triplicate. The data represent the mean ± SD, with $n = 15$. ANOVA followed by LSD test. An asterisk indicates $p < 0.05$.

43 μg/mL (**Table 4**). Considering the compounds **1**−**5**, with a common phenolic moiety, the lowest toxicity was found for compound **1** lacking the chlorine atom, while compounds **2**−**4** with a chlorine at C-5 were more cytotoxic. α,β-Dehydrocurvularin proved to be the most cytotoxic compound from *N. galligena* cultures, with an $IC_{50} < 12$ μg/mL (**Table 4**).

Table 4. Activity of Compounds **1**–**6** toward the Enzymes β-Glucuronidase and AChE and Cytotoxicity on Human Lung Fibroblasts

	β-glucuronidase IC_{50}		AChE IC_{50}		cytotoxicity IC_{50}	
compounds	(μg/mL)	(μM)	(μg/mL)	(μM)	(μg/mL)	(μM)
1	33	109	86	286	>1000	504
2	32	99	32	100	58	180
3	43	108	30	73	62	153
4		>300		>300	64	138
5	42	103	36	90	120	285
6		>300		>300	<12	<40
reference inhibitors						
glucosaccharo-1:4 lactone		4.7				
galanthamine				1		

This is the first report on the production of compounds **1**−**6** by *N. galligena*. In the paper by Bal-Tembe et al. (*32*), ilicicolins were isolated from an unidentified *Nectria* species, while Ellestadt et al. (*23*) obtained six terpenoids of mixed biosynthetic origin, including ilicicolins, from an unidentified *Fusarium* species. The bioactivities reported for the isolated compounds as well as the results of our study suggest the potential of the *Nectria* metabolites as leads for further studies, including structural modifications and biochemical studies on their mode of action.

A comparison of the active metabolites of *N. galligena* from different hosts and locations should be undertaken to assess the possible variability between the fungal isolates and relate the patterns, if possible, with host effects. Furthermore, the host response is of interest to get a clearer picture of this fungus−plant interaction.

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