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Bioactive metabolites from the Endophytic Fungus *Aspergillus sp.* SPH2

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Abstract: In the current study, an ethyl acetate extract from the endophytic fungus *Aspergillus sp.* SPH2 isolated from the stem parts of the endemic plant *Bethencourtia palmensis* was screened for its biocontrol properties against plant pathogens (*Fusarium moniliforme*, *Alternaria alternata* and *Botrytis cinerea*), insect pests (*Spodoptera littoralis*, *Myzus persicae*, *Rhopalosiphum padi*), plant parasites (*Meloidogyne javanica*) and ticks (*Hyalomma lusitanicum*). SPH2 gave extracts with strong fungicidal and ixodocidal effects at different fermentation times. The bioguided isolation of these extracts gave compounds **1-3**. Mellein (**1**) showed strong ixodocidal effects and was also fungicidal. This is the first report on the ixodocidal effects of **1**. Neoaspergillic acid (**2**) showed potent antifungal effects. Compound **2** appeared during the exponential phase of the fungal growth while neohydroxyaspergillic acid (**3**) appeared during the stationary phase, suggesting that **2** is the biosynthetic precursor of **3**. Additional molecular ions compatible with pyrazynes that were detected during the exponential phase included flavacol, and aspergilliamide while ochramide A was mostly detected during the stationary phase of the fermentation. Moreover, polyketids were also detected during the stationary phase of the fermentation curve (dihydroaspyrone, aspyrone, asperlactone) and the alkaloid circumdatin H. Ochratoxin A was not detected. Therefore, SPH2 could be a potential biotechnological tool for the production of ixodocidal mellein.

Keywords: Endophyte; *Aspergillus ochraceus*; antifungal; neoaspergillic acid; ixodocidal; mellein

1. Introduction

Endophytes are a particularly interesting group of microorganisms that can be isolated from asymptomatic plant tissue. Among fungal endophytes, most species belong to the ascomycota and deuteromycota phyla and might be the producers of several groups of new, unique secondary metabolites [1].

The specific relationship between the host plant and its endophytes includes complex biochemical interactions [2, 3]. Endophytes have adapted themselves to their special microenvironments by genetic variation, including uptake of some plant DNA into their own genomes [4]. After long-term coexistence with their host, endophytes can synthesize biologically active substances similar to the secondary metabolites produced by host plants [5, 6, 2]. The potential of fungal endophytes for producing novel biologically active compounds with promising medicinal or agricultural applications has been demonstrated [7, 8]. These molecules can play an important role in communication between organisms, in plant protection and plant adaptation to habitat and environmental changes [8].

Little is known regarding endophyte biogeography and community assembly. An important role for biome and host phylogeny has been shown for bioactivity (against Chagas and malaria) of

endophytic fungi from tropical angiosperms and ferns, showing higher degree of bioactivity in taxa sourced from cloud forests compared to lowland tropical forests [9]. In this context, targeting plants with unique traits, such as production of unusual secondary metabolites or found in restricted habitats, has been suggested [10].

The Macaronesian region consists of the Azores and Madeira (Portugal) and the Canary Islands (Spain). The three archipelagos share a volcanic origin, a contrasting landscape and a gentle climate. These features have created an ideal environment for a particularly rich biodiversity. The Canaries host unique forests habitats home to the richest biodiversity of the region, and one of the richest in the world. Around 25 % of the flora species are endemic [11], and therefore hosts of a potentially rich endophytic microbiome.

Endophytes isolated from endemic species of the Laurel forest of the Canarian flora reported as being insecticidal, produced extracts with crop protection effects such as insect antifeedant and antifungal [7]. Additional endemic plants with reported insect antifeedant effects included species of the genus *Bethencourtia* [12, 13]. This genus, endemic to the Canary Islands, consists of three species: *Bethencourtia hermosae* (Pit), *Bethencourtia palmensis* (Nees) Choisy and *Bethencourtia rupicola* (B. Nord.) B. Nord [14]. Previous studies on *B. palmensis* showed the presence of silphinene sesquiterpenes with strong insect antifeedant effects acting on insect GABA receptors [13]. Therefore, the plant *B. palmensis* was chosen for the isolation of fungal endophytes with the ability to produce secondary metabolites with biopesticidal properties (as antifungal, nematocidal, insect antifeedant and ixodocidal agents).

In this work, we report on the isolation of the endophytic fungal strain SPH2 from *Bethencourtia palmensis* and the isolation and characterization of the bioactive compounds (against the fungal pathogens *Alternaria alternata*, *Fusarium oxysporum* and *Botrytis cinerea* and the tick *Hyalomma lusitanicum*). Additionally, a metabolomic study of the SPH2 time-course fermentation has been carried out.

2. Materials and Methods

Plant material

Bethencourtia palmensis was collected in Barranco del Rio, Abona (Tenerife) (28°34'10"N 16°18'48"W). The samples were placed into sterile polybags and transported, under refrigeration, in a box container until isolation processing within 48 hours of collection.

Isolation of endophytic fungus SPH2

Endophytic fungi were isolated according to Kumar et al., (2013) [15]. The stem surface was sterilized with 70% ethanol for 2 min followed by 1% sodium hypochlorite for 3 min. Sterilized stems were dried on sterile blotting sheet and then chopped in sterile Petri plate and transferred to Potato Dextrose Agar plates (PDA) with 50 mg/L. of chloramphenicol to inhibit bacterial growth. These plates were incubated at 24°C for 3–15 days in a growth chamber. The fungal colonies were transferred to fresh PDA plates to get pure cultures. SPH2 was initially identified by microscopic examination and later identified by molecular methods. The culture was maintained on PDA by routine subculturing.

Molecular characterization of SPH2

The genomic DNA of the pure fungal isolate SPH2 was extracted using DNeasy Plant mini kit (Qiagen GmbH, Hilden Cat. No 69104) by following the manufacture's instruction. The extracted DNA was used for PCR amplification by primer ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') according to Kumar et al., (2011) [16]. Genomic DNA (100-200 ng) was amplified on a PTC-200 Thermal Cycler (MJ Research) in a 25 µL final volume with the AmpONE Taq DNA polymerase PCR kit (GeneAll) for 35 cycles (95 °C, 1 min; 50°C, 20 sec; 72°C, 1.5 min) after an initial denaturation (95°C, 2 min) and followed by a final extension (72°C, 7

min). Amplicons were checked by agarose gel (1%) electrophoresis, purified using the EXO-SAP-IT kit (Affimetrix-USB), and sequenced on an AB 3500 Genetic Analyzer (Thermo Fisher) at the University of La Laguna (Spain) genomic service. A BLASTN search of the sequence against the NCBI nucleotide identified strain SPH2 as *Aspergillus* sp., similar to these in the group Circumdati (*A. ochraceus* y *A. westerdijkiae*).

Cultivation of SPH2 for extract preparation

SPH2 was cultivated on PDA solid medium for 8 days at 25°C. Sterile water (10 mL) was added to each Petri dish to obtain a suspension of superficial mycelium, and then transferred to an Erlenmeyer flask (250 mL) with 50 mL of a modified Czapek-Dox-Yeast liquid media ([Cz-L: NaNO₃ (2 g/L), KH₂PO₄ (5 g/L), MgSO₄ (0.5 g/L), FeSO₄ (0.01 g/L), ZnSO₄ (0.003 g/L), yeast extract (1 g/L) and glucose (60 g/L)]. This suspension was cultivated for 3 days at 25°C under continuous agitation and was used as pre-inoculum. Two Erlenmeyer flasks (500 mL) with 200 ml of fresh Cz-L medium each were inoculated with 15 ml of pre-inoculum and incubated at 25°C under continuous agitation for 4 and 10 days.

Extract preparation:

The filtered culture media was submitted to exhaustive liquid/liquid extraction with ethyl acetate (EtOAc), dried over SO₄Na₂ and concentrated under reduced pressure to give crude SPH2 extracts (yield of 177 or 660 mg/L for 4 and 10 days, respectively) for bioguided fractionation.

Time-course fermentation

A total of 27 Erlenmeyer flasks were prepared with 100 ml of culture media (three replicates / sample) and inoculated with 500 mg mycelium from the pre-inoculum suspension. Three flasks were sampled on days 1, 3, 4, 6, 7, 8, 10, 12 and 13 of incubation. The culture media was separated from the mycelium by filtration with a Buchner filter and the mycelium from each sample was frozen with liquid nitrogen, lyophilized and weighted.

Bioassays

Antifungal bioassay

The fungal species *Fusarium moniliforme*, *F. solani*, *F. oxysporum*, *Alternaria alternata* and *Botrytis cinerea* were provided by the fungal collection of IPNA-CSIC (Spain). The mycelial growth inhibition test was carried out in 12-well plates (Falcon) by a modified agar-dilution method with the addition of 0.05 mg/mL of methyltetrazolium salts (MTT). Extracts and pure compounds dissolved in ethanol (EtOH) were tested at different concentrations (extracts at 1, 0.5, 0.25 and 0.1 mg/mL; compounds at 0.5, 0.1, 0.25 and 0.05 mg/mL) and were incorporated into the culture medium before plates were poured. A series of test solutions into PDA (potato dextrose agar) and MTT were prepared for each concentration tested and then 300 µL were added to each well. All treatments were replicated four times and EtOH was used as negative control. After incubation in darkness at 27°C for 48 hours, fungal colonies were digitalized and measured with the application ImageJ (<http://imagej.nih.gov/ij/>). Percent inhibition (%I) was calculated as: $\%I = (C - T/C) \times 100$, where C is the diameter of the control colonies and T is the diameter of the test colonies [17]. Data was analyzed with Statgraphics statistical analysis software (Centurion XVIII) and EC₅₀ values (effective dose to obtain 50% of inhibition) were determined by means of a regression curve mycelial growth inhibition versus log dose.

Ixodical activity

Hyalomma lusitanicum engorged female ticks were collected in central Spain (Finca La Garganta, Ciudad Real) from their host (deer) and maintained at 22-24 °C and 70% RH until oviposition and egg hatch. Resulting larvae (4-6 week old) were used for the bioassays [18]. Briefly, 50 µL test solution were added to 25 mg of powdered cellulose at different concentrations and the solvent was

evaporated. For each test, three replicates with 20 larvae each were used. Dead ticks were counted using a binocular magnifying glass 24 h after contact with the treated cellulose under the environmental conditions described. Larvicidal activity data are presented as percent mortality corrected according to Schneider-Orelli's formula. Effective lethal doses (LC_{50} and LC_{90}) were calculated by Probit Analysis (5 serial dilutions, STATGRAPHICS Centurion XVI, version 16.1.02).2.10

Insecticidal and nematocidal activities

See Supplementary materials for material and methods.

Isolation and identification of the bioactive compounds

Column chromatography (CC): Sephadex LH-20 (Sigma-Aldrich). TLC: silica gel (105554 and 105744; Merck); compounds were visualized on TLC under a UV lamp and oleum solution. Preparative HPLC: Varian Prostar 210 HPLC Pump equipped with normal phase (Ace 5 SIL 127-2510) and reverse phase (Interchrom KR/25M Kromasil 4g Si) columns. Gas chromatography mass spectrometry (GC-MS): Shimadzu GC-2010 gas chromatograph coupled to a Shimadzu GCMS-QP2010 Ultra mass detector (electron ionization, 70 eV) and equipped with a 30 m \times 0.25 mm i.d. capillary column (0.25 μ m film thickness) Teknokroma TRB-5 (95%) Dimethyl-(5%) dimethylpolysiloxane. NMR Spectra: Bruker AMX 500 MHz spectrometer with pulsed-field gradient, solvent as internal standard ($CDCl_3$, at δ_H 7.26 and δ_C 77.0); the programs used for DEPT, 1H , 1H -COSY, NOESY, HSQC and HMBC experiments furnished in the Bruker software. EI and HR-ESI-MS: Micromass Autospec and Micromass LCT Premier spectrometers in m/z .

Bioguided extract fractionation

An antifungal EtOAc extract (4 days, 146 mg) was chromatographed on a Sephadex LH-20 column (140 mL) eluted with n-hexane: Cl_2CH_2 :MeOH (2:1:1) mixture to afford two fractions. The active fraction 1 (20 mg) was further purified by semi-preparative HPLC (Ultrasphere ODS, 10.0 \times 250 mm, 5 μ) eluted with an isocratic mixture of MeOH:H₂O (80:20) at a flow rate of 3mL/min to yield compound **2** (6 mg).

Compound identification

Compound **1** was analyzed by GC-MS with the following working conditions: split ratio (20:1), injector temperature 300°C, temperature of the transfer line connected to the mass spectrometer 250°C, initial column temperature 70°C, then heated to 290°C at 6°/min. Electron ionization mass spectra and retention data were used to assess the identity of compounds by comparing them with those of standards or found in the Wiley 229 and NIST Mass Spectral Database. Further, the retention times of authentic compound (isolated in this work and purchased from Cayman Chemical Company (Ann Arbor, Michigan, USA) were also used to confirm the identity of the compound. Compound **1** was identified as mellein.

(-)-Mellein (**1**): $[\alpha]_D^{25} -77.5$ (c 0.08, $CHCl_3$); 1H NMR (500 MHz, $CDCl_3$), δ : 11.02 (1H, s, -OH), 7.41 (1H, t, J = 7.9 Hz, H-6), 6.89 (1H, d, J = 8.4 Hz, H-7), 6.69 (1H, d, J = 7.4 Hz, H-5), 4.74 (1H, dq, J = 12.7, 6.4 Hz, H-3), 2.93 (2H, dd, J = 7.3, 2.1 Hz, H-4), 1.54 (3H, d, J = 6.3 Hz, -CH₃). ^{13}C NMR (125 MHz, $CDCl_3$), δ : 169.9 (C-1), 162.2 (C-8), 139.4 (C-10), 136.1 (C-6), 117.9 (C-5), 116.2 (C-7), 108.3 (C-9), 76.1 (C-3), 34.6 (C-4), 20.7 (-CH₃).

Compounds **2** and **3** were identified as neoaspergillic acid and neohydroxyaspergillic acid. Their molecular formulas ($C_{12}H_{20}N_2O_2$ and $C_{12}H_{20}N_2O_3$) were determined by ESI-HREIMS (m/z 223.1447 $[M-H]^-$ and 263.367 $[M+Na]^+$). Their 1H NMR and ^{13}C NMR spectra are shown in Table 1).

Table 1. ¹H NMR and ¹³C NMR spectra of compounds **2** and **3** in CD₃OD.

| Position | 2 | | 3 | |
|----------|-----------------------------------|-----------------------|-----------------------------------|-----------------------|
| | δ_H (J in Hz) ^a | δ_C , mult | δ_H (J in Hz) ^a | δ_C , mult |
| 2 | | 158.8, C | | 158.2, C |
| 3 | | 148.5, C | | 149.2, C |
| 5 | 7.26, s | 126.9, CH | 7.49, s | 125.7, CH |
| 6 | | 142.5, C | | 143.4, C |
| 7 | 2.63, d (7.1) | 38.1, CH ₂ | 4.75, br s | 74.0, CH |
| 8 | 2.19, dq (13.8, 6.9) | 26.4, CH | 2.32, br s | 32.5, CH |
| 9 | 0.89, d (6.6) | 22.9, CH ₃ | 0.90, m | 19.2, CH ₃ |
| 10 | 0.89, d (6.6) | 22.9, CH ₃ | 0.90, m | 17.7, CH ₃ |
| 11 | 2.60, d (7.3) | 42.6, CH ₂ | 2.64, d (7.3) | 42.6, CH ₂ |
| 12 | 2.11, dt (13.6, 6.8) | 28.4, CH | 2.14, br s | 28.6, CH |
| 13 | 0.89, d (6.6) | 22.9, CH ₃ | 0.90, m | 22.9, CH ₃ |
| 14 | 0.89, d (6.6) | 22.9, CH ₃ | 0.90, m | 22.9, CH ₃ |

Compound quantification

GC-MS analysis of the extracts from the time-course fungal fermentation were carried out at the same working conditions as above. Electron ionization mass spectra and retention data were used to assess the identity of compounds by comparing them with those of standards or found in the Wiley 229 and NIST Mass Spectral Database. Further, the retention times of authentic compound (isolated in this work) were also used to confirm the identity compounds **1-3**. Extract solutions (4 µg/µL) were dissolved in dichloromethane (DCM) for sample injection (1 µL / injection). The concentrations of **1-3** were calculated based on a calibration curve built with serial dilutions (1.0, 0.5, 0.25, 0.125 and 0.0625 µg/µL) of an external standard (bornyl acetate, Sigma Aldrich) and their peak area without a correction factor.

The time-course extracts were also analyzed by HPLC-MS on a Shimadzu LC-20AD HPLC coupled to a LCMS-2020 QP mass spectrometer using an electrospray ionization (ESI) interface and CTO-10AS VP column oven. Sample injections (5 µL) were carried out by a SIL-20A XR auto sampler. Samples were separated in an ACE 3 C18 column (150 x 4.6 mm, 3 µm particle size) and ACE 3 C18 analytical guard cartridge at 25°C. The mobile phase consisted of (A) MiliQ water and containing 0.1% acetic acid and (B) methanol (HPLC-MS grade) and containing 0.1% acetic acid. The solvent gradient was 38-100% B in 45 min, 100% B for 10 min and 38% B for 7 min. Solvent and N (drying gas) flow rates were 0.5 mL/min and 15 L/min respectively. The electrospray capillary potential was set to +4.50 kV and ESI was conducted in the Full Scan positive mode (m/z = 100-700) with a potential of 1.40 kV and a capillary temperature of 250°C. Extract (0.25 µg/µL) stock solutions were dissolved in MeOH for sample injection.

3. Results

In this work the endophytic fungus SPH2 was isolated from a stem portion of the endemic plant *Bethencourtia palmensis* and identified as *Aspergillus* sp., isolate SPH2, similar to these in the group Circumdati (*A. ochraceus* y *A. westerdijkiae*).

3.1. Fermentation and compound identification

The time-course fermentation of SPH2 showed a maximum of mycelial weight at days 4 and 13, reaching the stationary phase by day 7. The extract yield reached a stable production by day 7 with a second increase on day 12 (Figure 1).

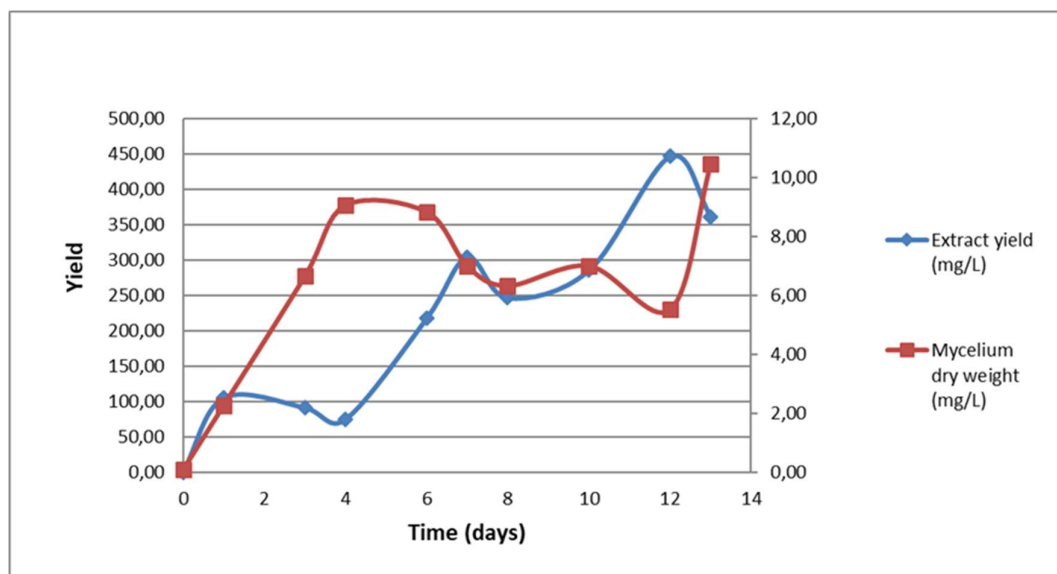


Figure 1. Time course of SPH2 mycelial and extract yield Table 1. This is a table. Tables should be placed in the main text near to the first time they are cited.

The ethyl acetate extracts from SPH2 were screened for their biocontrol properties against plant pathogens (*Fusarium moniliforme*, *Alternaria alternata* and *Botrytis cinerea*), insect pests (*Spodoptera littoralis*, *Myzus persicae*, *Rhopalosiphum padi*), a plant parasitic nematode (*Meloidogyne javanica*) and ticks (*Hyalomma lusitanicum*). Extracts from days 3-13 showed significant fungicidal and ixodocidal effects. The antifungal effects on *B. cinerea* and *A. alternata*, the most sensitive species, were stronger between days 3 and 7 of incubation, while the ixodocidal effects started on day 7 (the most active extract) and were maintained until day 13 (Table 2). These extracts were not antifeedant or nematocidal (Supplementary materials, Tables S1 and S2).

Table 2. Ixodocidal activity on *H. lusitanicum* larvae (mortality) and antifungal (mycelial growth inhibition) on *F. oxysporum*, *A. alternata*, *B. cinerea*, of the time-course SPH2 extracts.

| Days | <i>H. lusitanicum</i> | <i>F. oxysporum</i> | <i>A. alternata</i> | <i>B. cinerea</i> |
|------|---------------------------------------|---------------------------------------|---------------------|-------------------|
| | LD ₅₀ (µg/mg) ^a | EC ₅₀ (mg/mL) ^b | | |
| 3 | >20 | 0.27 (0.24-0.30) | 0.10 (0.07-0.13) | 0.04 (0.01-0.12) |
| 4 | >20 | 0.25 (0.20-0.31) | 0.14 (0.11-0.18) | 0.05 (0.02-0.10) |
| 6 | >20 | 0.31 (0.26-0.38) | 0.16 (0.12-0.21) | 0.03 (0.02-0.05) |
| 7 | 2.85 (2.61, 3.18) | 0.45 (0.31-0.65) | 0.06 (0.05-0.09) | 0.02 (0.01-0.05) |
| 8 | 7.82 (6.91, 8.75) | 0.31 (0.25-0.39) | 0.25 (0.18-0.34) | 0.12 (0.08-0.18) |
| 10 | 6.63 (5.92-7.49) | 0.21 (0.18-0.25) | 0.16 (0.12-0.22) | 0.16 (0.12-0.22) |
| 12 | 3.30 (3.09, 3.52) | 0.39 (0.31-0.48) | 0.24 (0.20-0.29) | 0.24 (0.20-0.29) |
| 13 | 7.18 (6.67, 7.78) | 0.87 (0.62-1.24) | 0.44 (0.37-0.51) | 0.22 (0.19-0.26) |

^aLethalcalculated by Probit analysis

^bEffective doses calculated by linear regression

The bioguided fractionation of SPH2 extracts resulted in the isolation of compounds **1-3** (Figure 2). Compound **1** was identified as mellein based on its GC-MS and spectroscopic data [19, 20]. Compounds **2** and **3**, previously isolated as natural products from *Aspergillus ochraceus* [21], were identified as neoaspergillic and neohydroxyaspergillic acids respectively, based on their spectroscopic data [22, 23, 24].

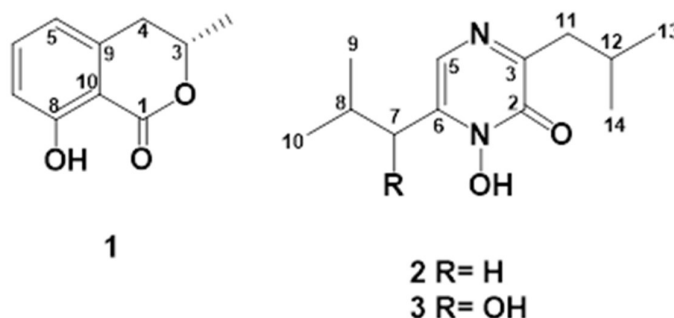


Figure 2. Chemical structures of compounds 1-3.

Compound **2** (neoaspergillic acid) was an effective antifungal against *A. alternata*, *B. cinerea* and *F. oxysporum* respectively, followed by mellein (**1**) (Table 3).

Table 3. Antifungal effects (mycelial growth inhibition) of compounds **1-3**.

| Compound | Effective doses (mg/mL) ^a | Mycelial growth inhibition | | |
|----------|---|----------------------------|---------------------|-------------------|
| | | <i>F. oxysporum</i> | <i>A. alternata</i> | <i>B. cinerea</i> |
| 1 | EC ₅₀ | 0.34 (0.27-0.43) | 0.44 (0.34-0.57) | 0.29 (0.23-0.37) |
| 2 | EC ₅₀ | 0.07 (0.04-0.10) | 0.01 (0.00-0.02) | 0.04 (0.03-0.04) |
| 3 | EC ₅₀ | >0.5 | 0.20 (0.17-0.25) | >0.5 |

^aEffective doses calculated by linear regression

Mellein (**1**), isolated from the ixodicidal fraction, showed a strong effect against *H. lusitanicum* larvae (LD₅₀ = 0.48 µg/mg, 0.44-0.51 95% Confidence Limits), being 10 times more effective than thymol (LD₅₀ = 2.94 µg/mg, 2.08-3.54 95% Confidence Limits).

Quantification of **1-3** and Metabolomics

The time-course production of **1-3** was quantified by GC-MS. Neoaspergillic acid (**2**) was the first compound detected (days 3 to 7), ranging between 4.11-11.7% weight, with the highest content found for day 7 (Figure 3). Neohydroxyaspergillic acid (**3**) appeared between days 8-13, ranging between 0.3-0.9% weight, with the highest content found for day 10. Mellein (**1**) was present between days 7-13 ranging between 3.9-17.7% weight, with the highest content found for day 7 (Figure 3).

Additional metabolites described in *A. sp.* have been detected by HPLC-MS (Table 4), including molecular ions compatible with dihydroaspyrone [25] (days 7-13, 4.4-10.5% of relative abundance); aspyrone [26] (days 7-13, 5.4-13.7%); asperlactone [26] (days 7-8 and 12-13, 0.4-3.8%); circumdatin H [27] (days 6-13, 0.9-4.6%), ochramide A [28] (days 3-13, 0.3-7.8%), flavacol [27, 29] (days 3-13, 0.9-4.7%) and aspergilliamide [29] (days 3-13, 0.3-7.2%).

| Rt (min) | Base Peak m/z | [M] ⁺ | Area (%) | | | | | | | | Compound match |
|-------------|---------------------|---------------------------|----------|------|------|------|-----------|-----------|-----------|-------|-------------------|
| | | | D3 | D4 | D6 | D7 | D8 | D10 | D12 | D13 | |
| 4,65 | 187 | 186 [M+H] ⁺ | | | | 4.40 | 6.45 | 6.92 | 6.11 | 10.47 | Dihydroaspyrone |
| 5,24 | 185 | 184 [M+H] ⁺ | | | | 5.42 | 5.41 | 11.7 4 | 13.7 4 | 7.81 | Aspyrone |
| 6,25 | 185 | 184 [M+H] ⁺ | | | | 0.39 | 1.73 | | 3.84 | 1.36 | Asperlactone |
| 17,87 | 348 | 347 [M+H] ⁺ | | | 4.58 | 1.67 | 4.68 | 2.70 | 0.94 | 4.39 | Circumdatin H |
| 21,94 | 179 | 178 [M+H] ⁺ | | | | 1.63 | 1.61 | 2.39 | 1.94 | 1.20 | Compound 1 |
| 22,58 | 241 | 240 [M+H] ⁺ | | | | | 14.8 8 | 13.7 0 | 3.08 | 8.87 | Compound 3 |
| 23,73 | 225 | 224 [M+H] ⁺ | 0.27 | 0.60 | 0.29 | 0.83 | 3.64 | 6.09 | 7.83 | 6.52 | Ochramide A |
| 30,00 | 209 | 208 [M+H] ⁺ | 4.25 | 4.17 | 2.39 | 3.85 | 0.92 | 0.58 | 0.66 | 4.73 | Flavacol |

| | | | | | | | | | | | |
|-------|-----|--------------------|-------|-------|-------|------|------|------|------|------|-----------------|
| 31,36 | 225 | 224 | 25.97 | 26.58 | 29.34 | 26.6 | 8.69 | 1.90 | 0.92 | 0.43 | Compound 2 |
| | | [M+H] ⁺ | | | | 7 | | | | | |
| 35,39 | 229 | 228 | 5.70 | 7.23 | 5.35 | 2.45 | 1.33 | 0.29 | 0.43 | 0.77 | Aspergilliamide |
| | | [M+H] ⁺ | | | | | | | | | |

4. Discussion

In this work the endophytic fungus SPH2 has been isolated from the plant *Bethencourtia palmensis* and identified as *Aspergillus* sp. based on the similarity of the SPH2 ITS with isolates from the group Circumdati of *Aspergillus* (*A. ochraceus*, *A. westerdijkiae*)

The genus *Aspergillus* is one of the most common among endophytic fungi associated with marine and terrestrial hosts. Endophytic *Aspergillus* have been described from the arctic tundra to the tropics [1]. Specifically, *A. ochraceus* has been isolated from the fern *Selaginella stauntoniana* [30]; and a wide range of plants including *Euphorbia geniculata* [31] *Polygonatum cyrtonema* [32], alfalfa (*Medicago sativa*) [33], bamboo [34], the cactus *Cereus jamacaru* [35], *Bauhinia forficata* [36]; orchids (*Bulbophyllum neilgherrense* and *Vanda testacea*) [37], and *Catha edulis* [38]. *A. ochraceus* has also been isolated as an endosymbiont from marine organisms such as the coral *Dichotella gemmacea* [39] and the marine brown alga *Sargassum kjellmanianum* [40, 41].

The time-course fermentation of SPH2 gave extracts with strong fungicidal and ixodicidal effects at different fermentation times. The bioguided isolation of these extracts gave compounds 1-3. Mellein (1) belongs to the subgroup of 3,4-dihydroisocoumarins. R-(-)-mellein is produced by fungi, plants insects and bacteria, being fungi the main natural source of this compound [42]. Overall, eleven out of 27 species in the *Aspergillus* section Circumdati produce mellein [43]. Among the different biological activities of 1, phytotoxicity and antifungal are the main ones reported [42]. In this work, mellein (1) showed moderate antifungal effects against *F. oxysporum*, *A. alternata* and *B. cinerea*. Similarly,previous reports showed moderate activity of 1 against *B. cinerea* [44, 19, 45], *F. oxysporum* and *F. solani* [46]. Mellein (1) also showed potent ixodicidal effects against larvae of the hard tick *Hyalomma lusitanicum*. In arthropods, 1 seems to play an ecological role for some insect species: as an attractant to the beetle *Tribolium confusum* [47], and as a defensive exudate [48; 49]. Furthermore, it has been suggested that the termite *Reticulitermes speratus* used 1 as an antifungal protectant [50]. On the other hand, 1 has been described as being larvicidal against *Aedes aegypti* [51]. However, this is the first report on the ixodicidal effects of mellein (1). Ticks are the second most important group of disease vectors after mosquitoes because they can transmit a great variety of pathogens to human and animals and are in expansion due to climate change [52]. For example, *Hyalomma* ticks, vectors of the Crimea Congo Haemorrhagic Fever virus, have spread from their original distribution (African and Mediterranean environments) to other European countries, becoming an increasing public health concern [53, 54, 55, 56]. Therefore, new effective and safer tick control agents are needed.

Compound 2, with antifungal effects, was identified as neoaspergillic acid and compound 3 as hydroxyneoaspergillic acid. Neoaspergillic and hydroxyneoaspergillic acids (2, 3) have been isolated from members of *Aspergillus* section Circumdati and related to the presence of homologs of the aspergillic acid gene cluster 11, responsible for their biosynthesis [57]. The aspergillic acid group of mycotoxins include a number of closely related pyrazine metabolites with antibiotic properties [58]. Neoaspergillic acid (2) has been reported to have antitumoral [59, 60] and antibacterial effects [61, 60]. Compound 2 has also been reported to have weak antifungal effects (against *Candida albicans* and *Aspergillus terreus*) [59]. This work has shown potent antifungal effects of compound 2 against phytopathogenic fungi (*Alternaria alterna*, *Botrytis cinerea* and *Fusarium oxysporum*) for the first time.

The time-course quantification of 1-3 showed that the pyrazine neoaspergillic acid (2) was the major compound that appeared during the exponential phase of the fungal growth (days 3-7), while

neohydroxyaspergillic acid (**3**) appeared during the stationary phase (days 8-13), suggesting that **2** is the biosynthetic precursor of **3**. We conclude that exponential phase extracts were antifungal because of their content in **2**. Additional molecular ions compatible with pyrazynes that were detected during the exponential phase included flavacol, and aspergilliamide while ochramide A was mostly detected during the stationary phase of the fermentation. Flavacol has reported cytotoxicity [29] and moderate respiratory chain inhibition effects [27].

Mellein (**1**) was detected during days 7-13. These extracts were antifungal and effective against the tick *Hyalomma lusitanicum* because of their content in mellein (**1**). Additional molecular ions compatible with polyketids were also detected during the stationary phase of the fermentation curve (dihydroaspyrone, aspyrone, asperlactone). Aspyrone and asperlactone have reported insect growth regulation effects on *Tribolium castaneum*, acting as an agonist of the juvenile hormone (JH III) [26] and, therefore, could contribute to the overall ixodocidal effects of these extracts. A molecular ion compatible with the alkaloid circumdatin H was also detected during the stationary phase. Circumdatin H, isolated from *A. sp.*, was a strong inhibitor of the electron transfer chain (NADH oxidase) with effects close to those of rotenone [27] and could also be related to the ixodocidal activity of these extracts.

Aspergillus species section Circumdati, produce ochratoxin A, a nephrotoxic mycotoxin [62, 43]. However, in this study, we have not detected any ochratoxin A compatible molecular ions by HPLC-MS in SPH2 extracts.

5. Conclusions

In this work the endophytic fungus SPH2 has been isolated from the plant *Bethencourtia palmensis* and identified as *Aspergillus sp.*. The time-course fermentation of SPH2 gave extracts with strong fungicidal and ixodocidal effects at different fermentation times. The bioguided isolation of these extracts gave compounds **1-3**. Mellein (**1**) was shown for the first time to have strong ixodocidal effects, in addition to being fungicidal. Neohydroxyaspergillic acid (**2**) showed potent antifungal effects against *Alternaria alternata*, *Botrytis cinerea* and *Fusarium oxysporum*. Compound **2** appeared during the exponential phase of fungal growth while neohydroxyaspergillic acid **3** appeared during the stationary phase, suggesting that **2** is the biosynthetic precursor of **3**.

Additional molecular ions compatible with pyrazynes that were detected during the exponential phase included flavacol, and aspergilliamide while ochramide A was mostly detected during the stationary phase of the fermentation. Additional polyketides (dihydroaspyrone, aspyrone, asperlactone) and the alkaloid circumdatin H were detected during the stationary phase of the fermentation curve. The toxin ochratoxin A has not been detected. Therefore, the fungal endophyte SPH2 could be a biotechnological tool for the production of ixodocidal mellein.

Supplementary Materials: Material and Methods Table S1: Bioactivity screening of SPH2 microextract, Table S2: Nematicidal activity of SPH2.

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