Betulinic Acid, The First Lupane-Type Triterpenoid Isolated from Both a Phomopsis sp. and Its Host Plant Diospyros carbonaria Benoist

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Abstract

Following a biological screening using a dengue replicon virus-cell-based assay, *Diospyros carbonaria* ethyl acetate extract was investigated, affording six known lupane-type triterpenoids endowed with anti-DENV-2 NS5 polymerase activity. The study of the associated microbial community of this species permitted us to identify 38 endophytes belonging to five different orders. Nine out of these 38 strains showed significant activity on the dengue replicon assay. The chemical investigation of the most active one, *Phomopsis* sp. SNB-LAP1-7-32, led to the isolation of betulinic acid, an anti-viral secondary metabolite isolated previously from the host plant. This result is the first example of a lupane-type triterpenoid isolated from both an endophyte and its host plant. Its presence in the *Phomopsis* strain may result from gene transfer and/or specific niche selection.

Keywords: *Diospyros carbonaria*, *Phomopsis* sp., Dengue polymerase inhibition, Triterpenoids, Plant-endophyte common metabolite

Introduction. - Dengue is ranked by the World Health Organization (WHO) as the most important mosquito-borne viral disease in the world. In 50 years, its incidence has increased 30-fold [1]. Five DENV serotypes (DENV1-DENV5) [2] can cause high fever, headache, nausea, vomiting, muscular pains, skin rash and in the worst case, a general bleeding leading to a shock-like state [3]. Recently, Sanofi Pasteur registered a first vaccine, Dengvaxia®, in Mexico, in Brazil and in the Philippines [4], but this vaccine offers a preventive treatment only. Currently, no effective anti-dengue therapy is available.

In an effort to identify new inhibitors of dengue virus replication, a systematic study with 3563 ethyl acetate extracts from 1500 tropical plants was performed with a dengue replicon virus-cell-based assay. Among them, extracts prepared from species of the genus *Diospyros* (Ebenaceae) exhibited particularly significant activities [5]. *Diospyros carbonaria*, one of the most active ones, was selected for a thorough chemical and biological study. We also paid particular attention to the associated endophytic fungal community of this species. Endophytes are microorganisms that grow intra- and/or inter-cellularly in the tissues of their host plants without causing symptoms [6]. In 1993, the discovery of a common taxane metabolite between the endophyte *Taxomyces andreanae* and its host plant *Taxus brevifolia* (from which paclitaxel had been isolated [7]), has led to a closer consideration of the metabolomic content of these microorganisms and especially the common metabolites with the host plant. Since then, many important plant metabolites such as podophyllotoxin, camptothecine, vinblastine, hypericin, and diosgenin have been found in endophytes [8]. These studies shown that endophytes are a rich source of bioactive compounds and also that they can produce the same compounds as those originated from their

host plants [9].

In this study, we embarked upon investigating on the origin of the antiviral potential of *D. carbonaria* ethyl acetate extract, and we screened *D. carbonaria* endophytic fungi hoping that we may find active plant metabolites or analogs in the endophytes.

Results and discussion. - Among 3563 ethyl acetate extracts from 1500 tropical plants evaluated for their inhibition of dengue virus replication, *D. carbonaria* bark ethyl acetate extract showed a significant activity (44% at 5 µg/ml). The phytochemical investigation of this extract afforded 6 known triterpenoids identified by comparison with spectroscopic data reported in the literature: 3*β*-*O*-*cis*-*p*-coumaroylalphitolic acid (**1**), 3*β*-*O*-*trans*-*p*-coumaroylalphitolic acid (**2**), eucalyptic acid (**3**) [10], betulinic acid (**4**) [11], betulin (**5**) [12], and betulinic aldehyde (**6**) [13].

All compounds isolated were evaluated for their antiviral activity in a dengue polymerase assay using the RNA-dependant RNA polymerase (RdRp) domain of the non-structural protein 5 (NS5). The cytotoxicity potential of these compounds was also evaluated on HCT-116 and MRC-5 cells (*Table 1*).

Please insert Table 1 here

Compounds **1-6** showed significant RdRp inhibiting activities with IC_{50}s between 2.2 and 7.5 µM. Among these, betulinic acid (**4**) and betulinic aldehyde (**6**) possessed the best selectivity indices, whereas compounds **2**, **3** and **5** were moderately cytotoxic, i.e., cytotoxic at 10 µg/ml on HCT-116 and MRC-5 cells, and not cytotoxic at 1 µg/ml. If the anti-dengue activities of compounds **4** and **6** were already reported in our previous work [14] [5], the antiviral properties of compounds **1-3** and **5** are reported for the first time. These results confirmed the anti-dengue potential of triterpenoids endowed with a lupane-type skeleton, as exemplified by betulinic acid 3*β*-caffeate, which significantly inhibited RdRp activity [15]. More generally, antiviral properties of betulinic acid (**4**), betulin (**5**) and betulinic aldehyde (**6**), the

three most common molecules of this chemical series, have been often reported against various types of viruses (13-21).

The discovery of 6 active compounds against dengue virus replication in *D. carbonaria* led us to investigate chemically and biologically the microbial community associated to this species. Thirty-eight fungal strains belonging to 5 different orders were isolated from the leaves (27 strains) and bark (11 strains) of this species: 8 *Phomopsis* sp. (Diaporthaceae, Diaporthales), 5 *Colletotrichum* sp. (Glomerellaceae, Glomerellales), 9 *Aspergillus* sp. (Trichocomaceae, Eurotiales), 11 *Syncephalastrum* sp. (Syncephalastraceae, Mucorales) and 5 Botryospheriales sp. (*Fig. 1*).

The fungi were cultivated on PDA and extracted with ethyl acetate. The crude extracts were tested in the dengue virus replication inhibition assay, in which cell viability was evaluated concomitantly (*Table 2*).

Please insert Table 2 here

On 38 strains evaluated, 9 showed a significant inhibition of dengue virus replication without cytotoxicity at 10 µg/ml: *Phomopsis* sp. (SNB-LAP1-7-32, SNB-LAP1-7-62, SNB-LAP1-79), *Syncephalastrum racemosum* (SNB-LAP1-7-50, SNB-LAP1-7-67, SNB-LAP1-7-81), *Botryosphaeriales* sp. (SNB-LAP1-7-78) and *Aspergillus niger* (SNB-LAP1-7-80, SNB-LAP1-7-82).

Phomopsis sp. SNB-LAP1-7-32 was the most active strain and was the strain inducing least cytotoxicity. Thus, this strain was cultivated on large scale and the culture was extracted with ethyl acetate. Flash chromatography generated eight fractions, of which fraction VIII showed 75% inhibiting activity against the NS5-RdRp at 10 µg/ml. The purification of this active fraction afforded betulinic acid (**4**) with a yield of 0.01% (*Fig. 2*). This compound, obtained in trace quantity, has been identified by comparison with literature data [11], and with our own data. This is the first time that betulinic acid was isolated from an endophyte.

Please insert Figure 2 here

To confirm that the presence of betulinic acid was not artifactual, the cryopreserved SNB-LAP1-7-32 strain was cultivated again and the culture was extracted using the same procedure. UHPLC-MS-Single Ion Recording (SIR) analysis of this second EtOAc extract indicated clearly the presence of betulinic acid in the crude extract. Our study showed that this active compound was not only produced by *D. carbonaria*, but also by one of its endophytes.

Previous studies have shown that endophytes were able to produce triterpenoids. For example, lanostane-type triterpenoids were isolated from the endophytes *Scleroderma* sp. [16], *Ceriporia lacerate* [17], and *Diaporthe* sp. [18]. Oleane-type triterpenoids were isolated from *Xylaria papulis* [19], *Pestalotiopsis clavispora* [20], and ursane-type triterpenoids were isolated from *Annulohypoxylon stygium* [21] and *Pestalotiopsis clavispora* [22]. In contrast, lupane-type triterpenoids, had never been isolated from any fungus until now.

Conclusions. - This work further demonstrates that host plant and its endophyte can produce the same active metabolites. Several different mechanisms could explain these common metabolites [23]. In some cases, the biosynthetic mechanism of the same compound evolved independently in plants and in their associated microorganisms (gibberellin) [24], in which case the presence of the endophyte in the plant could originate from a niche specific adaptation mechanism.
Horizontal gene transfer between the host plant and its associated microorganism have also been hypothesized, but this transfer has only been shown to occur between microbial endophytes [25]. In some other cases, interactions between host plant and their endophytes leads to the co-production of bioactive compounds [26].

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Experimental part

General. Optical rotation was measured in methanol using an Anton Paar MCP 300 polarimeter in a 100 mm long 350 µl cell. NMR spectra were recorded in $CDCl_3$ on a Bruker 600 MHz spectrometer equipped with a 1 mm inverse detection probe. UHPLC was performed with an Acquity Waters UHPLC system equipped with a Waters Acquity PDA detector. Wavelength range was between 210 to 410 nm. Separation was achieved on a Kromasil BEH C_{18} column (1.7 µm, 2.1 mm × 50 mm)

at a flow rate of 0.6 ml/min. Elution was conducted with a H_2O:acetonitrile (ACN) gradient as follow: 95/5 to 0/100 in 5.5 min. The UHPLC system was coupled to a Waters LCT Premier XE mass spectrometer equipped with an electrospray ionization source. The ionization was carried out in positive mode in the 80-1500 *m/z* range. ACN used for HPLC and UHPLC chromatography was LC-MS grade (Fisher Scientific, Illkirch, FR). Deionized water was obtained from a Millipore (Bedford, USA) Milli-Q purification system. For all HPLC and UHPLC analysis, 0.1% formic acid has been added to ACN and water.

Flash chromatography was performed on a Grace Reveleris system with dual UV and ELSD detection equipped with a Reveleris 40 g silica C_{18} cartridge. Effluents were monitored at 254 and 280 nm. H_2O, ACN, dichloromethane (DCM) and tetrahydrofuran (THF) were used for Flash chromatography. Thin-layer chromatography (TLC) were conducted on 60 A F254 Merck plates, visualized using UV detection, and sprayed with a 1% solution of vanillin in concentrated sulfuric acid. Thermo Kromasil analytical and preparative C_{18} column (250 × 4.6 mm and 250 × 21.2 mm, 5 µm) were used for HPLC separations using a Waters autopurification system equipped with a sample manager (Waters 2767), a column fluidics organizer, a binary pump (Waters 2525), a UV-vis diode array detector (190-600 nm, Waters 2996), and a Polymer Laboratory PL-ELS 1000 ELSD detector. The flow rate was 1 ml/min for analytical HPLC analysis and 17 ml/min for preparative HPLC analysis, using a gradient of H_2O mixed with an increasing proportion of ACN. Both solvents were modified with 0.1% formic acid. UHPLC-MS-SIR was used for the verification of the presence of betulinic acid in *Phomopsis* sp. extract. This analysis was performed using a Waters (Milford, USA) Acquity UHPLC-TQD

(Triple Quadrupole Detector) system controlled by the MassLynx 4.1 software. Separation was achieved on a Waters HSS T3 column (2.1 x 100mm) at a flow rate of 0.6 ml/min. Elution was conducted with a H_2O:ACN gradient (modified with 0.1% formic acid) as follows : 95/5 to 0/100 in 7 min and 100 ACN during 3 min. The retention time of betulinic acid (**4**) was 7.0 min. Ionisation was performed by negative-ion electrospray, a cone voltage of 65 Volts was applied and the quadrupole filter mass spectrometer was operated in the single ion recording mode (SIR) at *m*/*z* 455.7 (betulinic acid $[M - H]^-$) and at *m/z* 934.2 $[2M+Na-2H]^-$.

Plant material. The leaves and bark of *D. carbonaria* was collected in October 2013 in French Guiana (Saint Elie). The species was identified by Dr. V. Eparvier. Voucher specimen was deposited in the Cayenne Herbarium (CAYVE18).

Extraction of D. carbonaria*. D. carbonaria* was dried using a hot-air drying machine (temperature did not exceed 40 °C) or were air-dried for a period of 1 week at ambient temperature. The plant material (1.3 kg) was powdered using a blade miller (PX-MCF 90D Kinematica), and was extracted with EtOAc (3 x 300 ml) to yield a crude extract of 9 g after concentration *in vacuo* at 40 °C.

Isolation of compounds from D. carbonaria*.* The extract (9 g) was purified by flash chromatography with a gradient of H_2O:ACN (95:5 - 80:20 - 50:50 - 20:80 - 0:100) then, ACN:THF (50:50, and 100:0) at 40 ml/min. Seven fractions were gathered based on their TLC profiles. Upon further fractionation with prep-HPLC with H_2O:ACN (50:50, flow rate 21 ml/min), fraction V (52.7 mg) led to the isolation of 3*β*-*O*-*cis*-*p*-coumaroylalphitolic acid **(1)** (3.2 mg, Rt = 12.8 min), 3*β*-*O*-*trans*-*p*-

coumaroylalphitolic acid (**2**) (21.6 mg, Rt = 14.9 min), and eucalyptic acid (**3**) (3.01 mg, Rt = 15,9 min). Fraction VI (148.7 mg) led to the isolation of betulinic acid (**4**) (13.56 mg, Rt = 9.6 min), betulin (**5**) (2.87 mg, Rt = 10.6 min), and betulinic aldehyde (**6**) (1.37 mg, Rt = 17.6 min) with an isocratic H_2O:ACN ratio (5:95, flow rate 21 ml/min).

Isolation, identification and extraction of endophytes. The general procedures adopted for isolation and identification of the endophytic microorganisms followed the methodology described by Casella *et al.* [27]. Thirty-eight fungal strains were isolated from *D. carbonaria* leaves and bark. All strains were sequenced externally by BACTUP-France for identification. The fungal rDNA sequences obtained were aligned with DNA sequences from GenBank, NCBI (http://www.ncbi.nlm.nih.gov/genbank, consulted on July, 2015). The sequence of the most active endophyte *Phomopsis* sp. SNB-LAP1-7-32 was deposited in the GenBank with accession number KU977311.

Large-Scale Cultivation and Extraction of SNB-LAP1-7-32. Phomopsis sp. was cultivated on solid PDA medium at 26 °C for 15 days on 482-14 cm diameter Petri dishes (total 23.5 m^2). The contents of the Petri dishes were cut into small pieces, transferred into a large container, and macerated with EtOAc 3 times for 24 h. The organic solvent was collected via filtration, washed with water in a separating funnel, yielding 7.3 g of extract (0,31 g/m^2). To eliminate fatty acids, extract was diluted in heptane:H_2O:MeOH (2:1:1) and the H_2O:MeOH phase was evaporated to dryness under reduced pressure, yielding 3.4 g of extract.

Isolation of betulinic acid from Phomopsis sp. *SNB-LAP1-7-32 EtOAc extract.* The H_2O:MeOH fraction (3.4 g) was purified by flash chromatography with a gradient of H_2O:ACN (95:5 - 80:20 - 50:50 - 20:80 - 0:100) then, DCM:ACN (50:50, 100:0) at 40 ml/min. Eight fractions were gathered based on their TLC profiles. Upon further fractionation with prep-HPLC with H_2O:ACN (15:85, flow rate 21 ml/min), fraction VIII (23 .7 mg) led to the isolation of betulinic acid **(4)** (0.39 mg, Rt = 12.2 min).

Antiviral assay. D. carbonaria extracts and pure compounds were tested on a DENV-NS5 RNA-dependant RNA polymerase assay (DENV-NS5 RdRp assay). Endophytes from *D. carbonaria* were tested on dengue replicon assay. For biological assay, all samples were diluted in DMSO 100% at 1 mg/ml. DENV-NS5 RdRp assay has been described previously [28]. We will describe only dengue virus replication inhibition assay.

This test uses COS monkey cell lines for expression (chimeric replicon). In place of the structural genes, the replicon contained a gene construct encoding a fusion protein (EGFF-PAC) comprised of the Enhanced Green Fluorescent Protein (EGFP) and Puromycin *N*-Acetyltransferase (PAC). To validate the assay, a broad spectrum antiviral compound, Ribavirin, which inhibits DENV replication, was tested. Total EGFP fluorescence was then correlated to this cell number and shows viral replication [29].

Cells were grown in Dulbecco's modified eagle medium (DMEM) (PAA) supplemented with 1% penicillin, 1% streptomycin and 10% fetal calf serum (FCS). For COS-DV EGFP-replicon containing cells, puromycin (Sigma) was added at

2 µg/ml. Replicon replication inhibition tests were done using a DMEM medium without phenol red (Invitrogen), supplemented with penicillin, streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate and 10% FCS, without puromycin. Ribavirin (Sigma) used as a control at 10 µM was dissolved in DMSO at 20 mM, and was stored at -20 °C. Control cells and DV2-NS5 EGFP-replicon cells were seeded in black 96-well plates at density of 7.5×10^3 cells per well, in complete uncolored medium supplemented with 0.5% DMSO (v/v) and 10% fetal calf serum. After 24 h, the medium was removed and cells were incubated with 100 µl of the same medium containing natural extracts or pure compounds at the final concentration of 10, 5 or 1 µg/ml in DMSO in each well. In each 96-well plate, 6 control wells were also included. The media were renewed after 24 h, as cells were incubated with each compound for a total of 48 h. The inhibitory effect of the compounds was compared to their cytotoxic effects measured as follows. In addition to the treated and control cells, a range of cell numbers, from 1.5×10^4 to 6×10^4 were freshly seeded, into each 96 well plates, and incubated for 5 to 6 h. The EGFP fluorescence from each well was read with a Tecan SafireII® fluorimeter at 490 nm (excitation) and 510 nm (emission). To determine the cytotoxic effect, 10 µl of Celltiterblue® reagent (Promega) was added in each well, the plates were incubated for a further 90 minutes at 37 °C and 5% CO_2 and the fluorescence read at 560 nm (excitation) and 590 nm (emission). A 590 nm fluorescence curve produced using the range of cell numbers was then used to define an equation to calculate the number of cells present in each well. The 510 nm fluorescence values were divided by the calculated cell number and mean values for each point of concentration were reported as a percentage of the mean control value. Values for each compounds concentration were reported as a percentage of the control value. We estimate that an extract showing percentage

greater than 40% of inhibition and less than 80% cell death at 10 µg/ml is an active extract.

Cell Culture and Proliferation Assay. Human HCT-116 colorectal carcinoma and MRC-5 cell line derived from normal lung tissue were obtained from the American Type Culture Collection (Rockville, MD, USA) and were cultured according to the supplier's instructions. Human MRC-5 cells were grown in DMEM supplemented with 10% fetal calf serum (FCS) and 1% glutamine. Human HCT-116 colorectal carcinoma were grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 1% glutamine. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Cell growth inhibition was determined by an MTS assay according to the manufacturer's instructions (Promega, Madison, WI, USA). Briefly, the cells were seeded in 96-well plates (2.5×10^3 cells/well) containing 200 µl of growth medium. After 24 h of culture, the cells were treated with the tested compounds at 1 and 10 µM final concentrations. After 72 h of incubation, 40 µl of resazurin was added for 2 h before recording absorbance at 490 nm with a spectrophotometric plate reader. The percent cytotoxicity index [(OD490 treated/OD490 control) x 100] was calculated from three experiments.

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TABLE 1. *ANTIVIRAL ACTIVITY AND CYTOTOXICITY OF COMPOUNDS **1-6***

	DENV-NS5 RdRp[a])	**% inhib. HCT-116 cell prolif.[b])**	**% inhib. MRC-5 cell prolif.[b])**
	IC_{50} [μM]		
3*β-O-cis-p-* Coumaroylalphitolic acid **(1)**	3.0 ± 0.4	59.3 ± 0.2	6.2 ± 4.9
3*β-O-trans-p-* Coumaroylalphitolic acid **(2)**	2.2 ± 0.1	96.1 ± 0.8	32.7 ± 3.5
Eucalyptic acid **(3)**	2.3 ± 0.3	83.2 ± 1.2	28.7 ± 4.8
Betulinic acid **(4)**	4.3 ± 3.1	10.1 ± 0.9	0.1 ± 0.8
Betulin **(5)**	4.1 ± 0.4	50.7 ± 0.5	69.0 ± 3.5
Betulinic aldehyde **(6)**	7.5 ± 1.1	18.6 ± 4.5	0.4 ± 1.2
Ribavirin (control antiviral)	0.78 ± 0.12	-	-

[a]) IC_{50} values are mean values ± SD from three replicates.

[b]) Percentage inhibition of cell proliferation, at 10 μg/ml. None of the compounds were cytotoxic at 1 μg/ml.

TABLE 2. *ENDOPHYTE IDENTIFICATION, EXTRACT DENGUE REPLICATION INHIBITION, AND EXTRACT CYTOTOXICITY*

Plant part	Strain code	NCBI accession number	Closest species in NCBI with accession number	% Ident.	Possible identification	% inhibition on dengue replicon assay[a])			% viability on COS cells[a])		
						1	5	10	1	5	10
D. carbonaria leaves	SNB-LAP1-7-2	KU977303	Fungal endophyte strain MS202 (JX155873.1)	97	*Phomopsis* sp.	0	1	16	100	94	100
	SNB-LAP1-7-3	KU977304	Fungal endophyte strain MS202 (JX155873.1)	98	*Phomopsis* sp.	5	5	12	93	100	100
	SNB-LAP1-7-8	KU977305	Fungal endophyte strain C1316.19 (JX010280.1)	100	*Colletotrichum* sp.	0	0	5	90	92	89
	SNB-LAP1-7-17	KU977306	Fungal endophyte strain C1316.19 (JX010280.1)	100	*Colletotrichum* sp.	7	17	13	87	85	82
	SNB-LAP1-7-19	KU977307	Fungal endophyte strain CM2 (AY745986.1)	98	*Phomopsis* sp.	4	0	0	91	82	88
	SNB-LAP1-7-20	KU977308	Fungal endophyte strain C1316.19 (JX010280.1)	99	*Colletotrichum* sp.	15	5	3	97	89	94
	SNB-LAP1-7-21	KU977309	Fungal endophyte strain MS202 (JX155873.1)	98	*Phomopsis* sp.	0	14	18	100	92	87
	SNB-LAP1-7-31	KU977310	Fungal endophyte strain C1316.19 (JX010280.1)	99	*Colletotrichum* sp.	0	0	0	98	97	84
	SNB-LAP1-7-32	**KU977311**	**Fungal endophyte strain MS202 (JX155873.1)**	**98**	***Phomopsis* sp.**	**36**	**43**	**60**	**99**	**95**	**100**
	SNB-LAP1-7-36	KU977299	Fungal endophyte strain CNRMA 03.414 (DQ119016.1)	99	*Syncephalastrum racemosum*	0	0	3	99	86	93
	SNB-LAP1-7-37	KU977312	Fungal endophyte strain C1316.19 (EU605880.1)	99	*Colletotrichum* sp.	0	0	15	94	87	86
	SNB-LAP1-7-38	KU977313	Fungal endophyte strain CM 2 (AY745986.1)	97	*Diaporthales* sp.	0	0	0	91	85	86
	SNB-LAP1-7-59	KU977314	Fungal endophyte strain FQ-5-2 (KF438017.1)	100	*Aspergillus niger*	0	0	0	94	83	86
	SNB-LAP1-7-62	KU977315	Fungal endophyte strain STRI:ICBG-Panama:TK1537 (KF435623.1)	99	*Phomopsis* sp.	10	17	21	90	80	77
	SNB-LAP1-7-67	KU977316	Fungal endophyte strain CNRMA 03.414 (DQ119016.1)	99	*Syncephalastrum racemosum*	10	30	32	99	88	91
	SNB-LAP1-7-69	KU977317	Fungal endophyte strain P-ZG-4-1-2 (KT192419.1)	100	*Aspergillus niger*	0	0	0	99	86	89
	SNB-LAP1-7-70	KU977318	Fungal endophyte strain 7234 (KR016814.1)	99	*Botryospheriales* sp.	0	1	0	95	80	82
	SNB-LAP1-7-71	KU977319	Fungal endophyte strain ASM-06 (LC092112.1)	99	*Aspergillus niger*	0	0	0	92	80	82
	SNB-LAP1-7-72	KU977320	Fungal endophyte strain 290 (KR015353.1)	99	*Botryospheriales* sp.	0	0	0	91	80	75
	SNB-LAP1-7-73	KU977300	Fungal endophyte strain GSL1_4 (KF269211.1)	99	*Botryospheriales* sp.	0	0	8	97	76	67
	SNB-LAP1-7-74	KU977321	Fungal endophyte strain 7233 (KR016813.1)	99	*Botryospheriales* sp.	0	0	0	87	81	68
	SNB-LAP1-7-77	KU977322	Fungal endophyte strain SST10_RBF (KT254585.1)	100	*Aspergillus niger*	0	6	11	90	88	73
	SNB-LAP1-7-78	KU977323	Fungal endophyte strain 290 (KR015353.1)	99	*Botryospheriales* sp.	21	34	52	85	65	63
	SNB-LAP1-7-79	KU977324	Fungal endophyte strain STRI:ICBG-Panama:TK1537 (KF435623.1)	97	*Phomopsis* sp.	0	22	27	85	73	77

	SNB-LAP1-7-81	KU977325	Fungal endophyte strain CNRMA 03.414 (DQ119016.1)	98	*Syncephalastrum racemosum*	9	21	28	89	75	77
	SNB-LAP1-7-82	KU977326	Fungal endophyte strain FQ-5-2 (KF438017.1)	100	*Aspergillus niger*	16	29	50	81	74	66
D. carbonaria bark	SNB-LAP1-7-7	KU977327	Fungal endophyte strain CNRMA 03.414 (DQ119016.1)	100	*Syncephalastrum racemosum*	0	7	11	88	93	78
	SNB-LAP1-7-28	KU977328	Fungal endophyte strain CNRMA 03.414 (DQ119016.1)	99	*Syncephalastrum racemosum*	0	0	8	84	81	81
	SNB-LAP1-7-29	KU977329	Fungal endophyte strain CNRMA 03.414 (DQ119016.1)	99	*Syncephalastrum racemosum*	3	0	1	87	81	80
	SNB-LAP1-7-34	KU977330	Fungal endophyte strain CNRMA 03.414 (DQ119016.1)	100	*Syncephalastrum racemosum*	0	0	0	91	74	88
	SNB-LAP1-7-48	KU977331	Fungal endophyte strain CNRMA 03.414 (DQ119016.1)	99	*Syncephalastrum racemosum*	1	8	2	92	83	82
	SNB-LAP1-7-50	KU977332	Fungal endophyte strain AQGSS (KP721597.1)	100	*Syncephalastrum racemosum*	22	40	40	87	74	64
	SNB-LAP1-7-51	KU977333	Fungal endophyte strain NRRL 2393 (NR_131291.1)	99	*Aspergillus unguis*	0	6	7	82	71	65
	SNB-LAP1-7-52	KU977334	Fungal endophyte strain CNRMA 03.414 (DQ119016.1)	100	*Syncephalastrum racemosum*	6	7	0	92	81	87
	SNB-LAP1-7-61	KU977335	Fungal endophyte strain TUHT73 (LN482469.1)	99	*Aspergillus niger*	0	7	4	91	72	71
	SNB-LAP1-7-75	KU977301	Fungal endophyte strain DAOM 144847 (JN938906.1)	99	*Syncephalastrum racemosum*	5	0	6	86	82	70
	SNB-LAP1-7-76	KU977336	Fungal endophyte strain NRRL 2393 (NR_131291.1)	99	*Aspergillus unguis*	0	0	4	82	69	58
	SNB-LAP1-7-80	KX078452	Fungal endophyte strain P-ZG-4-1-2 (KT192419.1)	99	*Aspergillus niger*	3	25	57	81	74	66

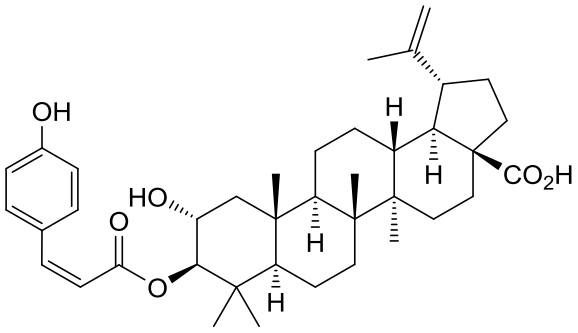
[a]) % inhibition and % viability at 1, 5, and 10 μg/ml. Control: Ribavirin (10 μM, 49% inh., 82% cell viability)

Titles to Figures

Fig. 1. *Endophytes isolated from* D. carbonaria *leaves and bark, by order*

Fig. 2. *DEDL trace of the prep-HPLC chromatogram of fraction VIII (H_2O:ACN 15:85, 21 ml/min). Betulinic acid (**4**) was the second major compound in this fraction. The major compound was isolated and was found to be a fatty acid.*

Chemical structure blocks



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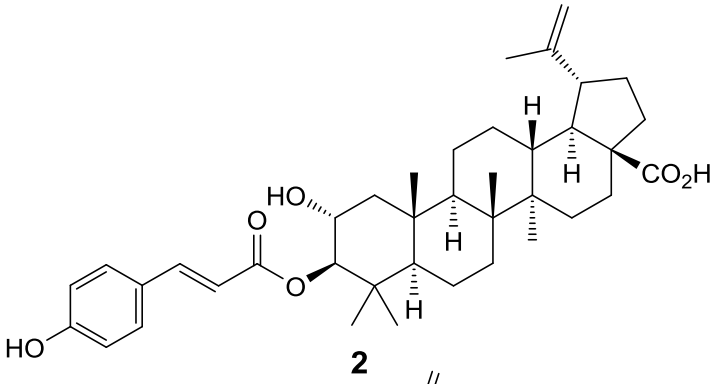

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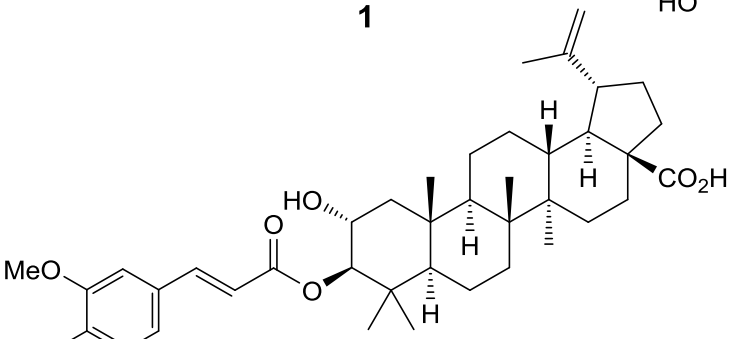

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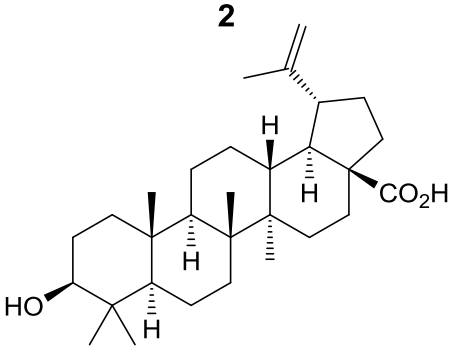

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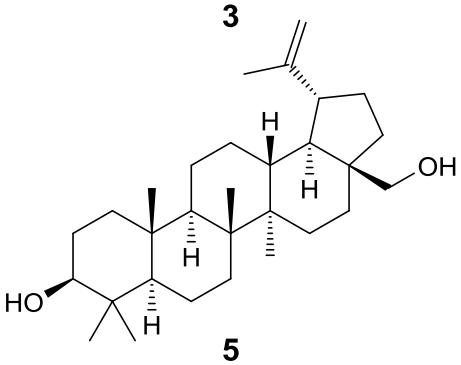

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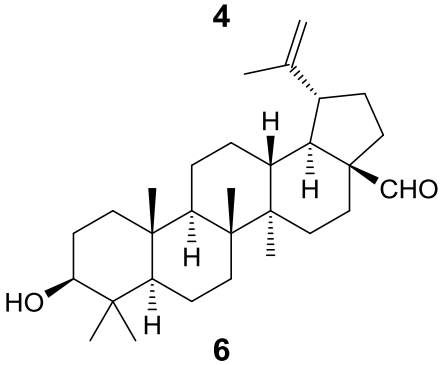


6

Figures

Fig. 1

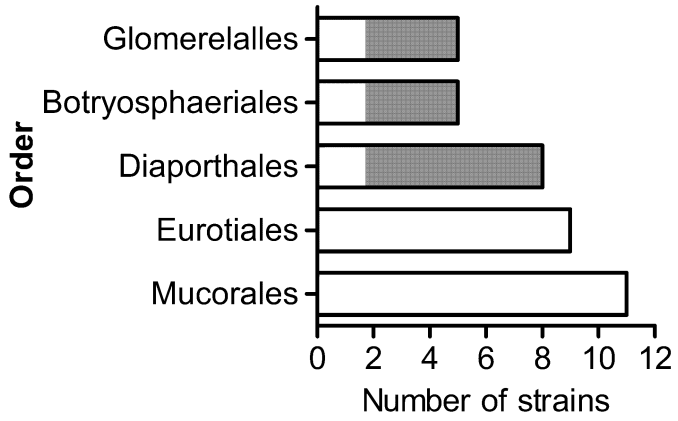
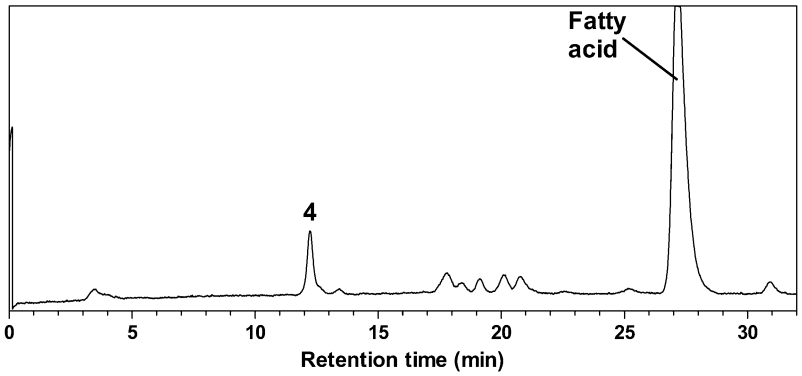


Fig. 2



Graphical abstract