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Chemical diversity and antiviral potential in the pantropical *Diospyros* genus

Laure-Anne Peyrat[a], Véronique Eparvier[a], Cécilia Eydoux[b], Jean-Claude Guillemot[b], Didier Stien[a,c*], Marc Litaudon[a*]

[a] *Institut de Chimie des Substances Naturelles, CNRS, UPR2301, University Paris-Saclay, 91198-Gif-sur-Yvette Cedex, France*
[b] *Centre de Recherche Architecture et Fonction des Macromolécules Biologiques, UMR 7257 CNRS, Aix-Marseille Univ., 163 Avenue de Luminy, 13288-Marseille Cedex 09, France*
[c] *Sorbonne Universités, UPMC Univ Paris 06, CNRS, Laboratoire de Biodiversité et Biotechnologies Microbiennes (LBBM), Observatoire Océanologique, 66650-Banyuls-sur-mer, France*

Abbreviations: COS, CV-1 (simian) in Origin and carrying the SV40 genetic material; DENV, Dengue viruses; DMEM, Dulbecco's Modified Eagle Medium; EGFP, Enhanced Green Fluorescent Protein; FCS, Fetal Calf Serum; NS, Non-Structural protein; ORF, Open Reading Frame; PAC, Puromycin *N*-Acetyltransferase; RdRp, RNA-dependant RNA polymerase.

* Corresponding author: Tel:/fax: +33430192476, *E-mail address:* didier.stien@cnrs.fr (D. Stien)
* Corresponding author: Tel:/fax: +33169823085, *E-mail address:* marc.litaudon@cnrs.fr (M. Litaudon)

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ABSTRACT

A screening using a dengue replicon virus-cell-based assay was performed on 3563 ethyl acetate (EtOAc) extracts from different parts of 1500 plants. The screening led to the selection of species from the genus *Diospyros* (Ebenaceae), among which 25 species distributed in tropical areas showed significant inhibitory activity on dengue virus replication. A metabolic analysis was conducted from the UPLC-HRMS profiles of 33 biologically active and inactive plant extracts, and their metabolic proximity is presented in the form of a dendrogram. The results of the study showed that chemical similarity is not related to plant species or organ. Overall, metabolomic profiling allowed us to define large groups of extracts, comprising both active and inactive ones. Closely related profiles from active extracts might indicate that the common major components of these extracts were responsible for the antiviral activity, while the comparison of chemically similar active and inactive extracts, will permit to find compounds of interest. Eventually, the phytochemical investigation of *D. glans* bark EtOAc extract afforded usnic acid and 7 known ursane- and lupane-type triterpenoids, among which 5 were found significantly active against dengue virus replication. The inhibitory potency of these compounds was also evaluated on a DENV-NS5 RNA-dependant RNA polymerase assay.

Keywords: *Diospyros* spp., *Diospyros glans*, Ebenaceae, Dengue virus, Triterpenoids, MZmine

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1. Introduction

Dengue viruses (DENV) are members of the *Flavivirus* genus, related to other medically important arboviruses such as yellow fever and Zika viruses. Due to *Aedes aegypti*, dengue is the most prevalent mosquito-borne viral disease of humans, affecting more than 50 million people annually. There are five phylogenetically and antigenically distinct serotypes of dengue viruses, DENV1 to DENV 5 [1], associated with high fever, headache, nausea, vomiting, muscular pains, skin rash and in the worst case, a general bleeding leading to a shock-like state. In 50 years, the incidence of dengue has increased 30-fold, and the WHO has estimated the annual number of deaths caused by dengue at 22,000 [2]. Global phenomena such as urbanization and international travels are key factors facilitating the spread of dengue. Efforts to decrease transmission by vector control have failed. Recently, a first vaccine, Dengvaxia®, was registered by Sanofi Pasteur in Mexico, in Brazil and in the Philippines [3]. This vaccine safely protects pre-adolescents to adults, but currently, no effective antiviral therapy is available to treat this disease.

Dengue virus has a capped positive-sense single stranded RNA genome containing a single open reading frame (ORF). The ORF encodes a polyprotein precursor, which is subsequently cleaved by cellular and viral proteases into three structural proteins (capsid, premembrane and envelope) as well as seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). The non-structural proteins assemble with cellular proteins to form a replication complex, where the viral RNA is synthesized [4].

In the present study, two bioassays have been used to detect and evaluate compounds of interest: a dengue polymerase assay using the RNA-dependant RNA polymerase (RdRp) domain of the non-structural protein 5 (NS5), and a dengue virus type 2 subgenomic replicon assay [5]. A systematic screening of EtOAc extracts from 1500 tropical plants was performed using the dengue replicon virus-cell-based assay. A large number of *Diospyros* spp. extracts showed significant antiviral potency, which led us to further investigate species of this genus. The metabolic profiling of some selected *Diospyros* extracts was therefore established, and *D. glans* bark extract was chemically investigated, leading to the isolation of usnic acid (**1**) and 7 known ursane- (**2**, **3** and **6**) and lupane-type triterpenoids (**4**, **5**, **7** and **8**).

Diospyros is the largest genus within the Ebenaceae family with over 350 species. This genus consists of trees and shrubs chiefly tropical and widely distributed. *Diospyros* is of great economic importance with species producing edible fruits, or used as wood or timbers [6]. Many *Diospyros* species have been reported to exhibit interesting pharmacological properties like antitumoral [7], antibacterial, antifungal [8], antipyretic and anti-inflammatory [9]. Moreover, antiviral properties against HIV [10], human noroviruses [11], influenza virus H3N2, H5N3, herpes simplex virus-1 [12], and against cowpea mosaic viruses [13] have been reported for various *Diospyros* species. So far, no *Diospyros* species were studied for their anti-dengue activity.

2. Experimental

2.1 Generals methods

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. ESI TOF MS measurements were performed using the Waters Acquity UPLC/HRMS system described above. Acetonitrile (ACN) used for UHPLC and HPLC chromatography was LC-MS grade (Fisher Scientific, Illkirch, FR). Deionized water was purified by Milli-Q water Millipore (Bedford, USA)

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purification system. For all HPLC and UHPLC analysis, 0.1% formic acid has been added to ACN and water. UHPLC was performed with a Waters Acquity UPLC system equipped with a Waters Acquity PDA detector. Wavelength range was between 210 to 410 nm. Separation was achieved on a Kinetex C_{18} column (1.7 µm, 2.1 mm × 150 mm) with a flow rate of 0.6 mL/min. The UHPLC system was coupled to a Waters LCT Premier XE mass spectrometer equipped with an electrospray ionisation source. The ionisation was carried out in positive mode in the 80-1500-*m/z* range. Flash chromatography was performed on a Grace Reveleris system with dual UV and ELSD detection equipped with a Reveleris 40 g silica cartridge. Effluents were monitored at 254 and 280 nm. Thin-layer chromatography (TLC) were conducted on 60 A F254 Merck plates and visualized using UV detection, and sprayed with a 1% solution of vanillin in concentrated sulfuric acid. Kromasil analytical and preparative C_{18} column (250 × 4.6 mm and 250 × 21.2 mm, 5 µm Thermo) 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 PL-ELS 1000 ELSD Polymer Laboratory 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.

2.2 Plant material

Stem, bark and leaves of various *Diospyros* species were collected between 1998 and 2013 in French Guiana, Madagascar, Vietnam, Malaysia and New Caledonia. The *Diospyros* species found bioactive on the Dengue replicon assay are listed in Table S1 (Supplementary material). The species were identified by Dr. V. Eparvier (French Guiana), Mr. A. Rakotozafy (Madagascar), Dr. Nguyen Quoc Binh (Vietnam), Mr. T. Leong Eng (Malaysia), and Dr. M. Litaudon (New Caledonia). Voucher specimens were deposited in their respective National Herbaria.

2.3 Extraction of Diospyros spp.

Various plant parts of *Diospyros* spp. were dried using a hot-air drying machine (temperature did not exceed 40°C) or were air-dried for a period of one week at ambient temperature. The plant material was powdered using a blade miller (PX-MCF 90D Kinematica), and a small amount of each sample (ca. 100 g) was extracted with EtOAc (3 x 300 mL) to yield a crude extract after concentration in vacuo at 40 °C. The EtOAc extracts (100 mg) have then been submitted to a small polyamide filtration to remove tannins. These extracts, once filtered through polyamide cartridges, were evaluated for their antiviral activities using dengue virus type 2 subgenomic replicon assay. *D. glans* bark extract was selected for further investigation.

2.4 Isolation of compounds from Diospyros glans

D. glans bark (680 g) was extracted with EtOAc. The extract (1.5 g) was purified by flash chromatography with a step gradient of H_2O:ACN (20:80 - 30:70 - 40:60 - 50:50 - 60:40 - 100:0) at 40 mL/min. Six fractions were gathered based on their TLC profiles. Upon further fractionation with prep-HPLC with H_2O:ACN (50:50 to 0:100 in 10 min, 0:100 during 15 min, flow rate 21 mL/min), fraction III (75.3mg) led to the isolation of usnic acid **(1)** (0.75 mg, Rt = 9.8 min), 11-oxo-acetyl ursolic acid (**2**) (1.35 mg, Rt= 10.4 min), and 13,28-epoxyurs-11-ene-3,28-dione (**3**) (1 mg, Rt = 13,8 min). Fraction IV (42.4 mg) led to the isolation of betulinic acid (**4**) (7.65 mg, Rt = 9.05 min) with a prep-HPLC gradient H_2O:ACN

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(30:70 to 0:100 in 10 min, 0:100 during 15 min, flow rate 21 mL/min). Fraction V (80.9 mg) led to the isolation of (3*β*)-3,23-dihydroxylup-20(29)-en-28-oic acid (**5**) (0.47 mg, Rt = 11.0 min), (3*β*)-3-(acetyloxy)-urs-12-en-28-oic acid (**6**) (0.54 mg, Rt= 14.4 min), betulinic aldehyde (**7**) (0.35 mg, Rt = 15.90 min), and (3*β*)-3,23-dihydroxylup-12,20(29)-dien-28-oic acid (**8**) (0.47 mg, Rt = 16.3 min), with a prep-HPLC gradient H_2O:ACN (50:50 to 0:100 in 10 min, 0:100 during 15 min, flow rate 21 mL/min).

2.5 Biological assay

Diospyros EtOAc extracts were tested against a subgenomic dengue virus type 2 replicon system. Pure compounds isolated from *D. glans* bark extract were tested against subgenomic DENV2 system, and on a DENV-NS5 RNA-dependant RNA polymerase assay (DENV-NS5 RdRp assay). For biological assay, extracts and pure compounds were diluted in DMSO 100% at 1 mg/mL. DENV-NS5 RdRp assay has been described previously [14]. The compound concentration leading to 50% inhibition of NS5-mediated RNA synthesis was determined in the same buffer as previously described, using various concentrations (0; 0.5; 1; 2; 5; 10; 20; 50 µg/mL). IC_{50} values are mean values ± SD from three replicates.

The DENV replication assay 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 was tested. Total EGFP fluorescence was then correlated to this cell number and shows viral replication [15]. 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-DENV EGFP-replicon containing cells, puromycin (Sigma) was added at 2 µg/mL. Assays were done in 96 well plate using a medium without phenol red (Invitrogen), supplemented with penicillin, streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate and 10% FCS, without puromycin. Control cells and DENV2-NS5 EGFP-replicon cells were seeded in black 96-well plates at density of 7,5 x 10^3 cells per well, in complete colourless 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 x 10^4 to 6 x 10^4 were freshly seeded, into 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 compound concentration were reported as a percentage of the control value. We estimate that an extract showing percentage greater than 10 % of inhibition and less than 55 % cell death at 5 µg/mL is an active extract. Ribavirin (Sigma) was used as control at 10 µM final concentration. It was resuspended in 100% DMSO at 20 mM and stored at -20 °C.

2.6 Profiling analysis

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MZmine 2 is a software for mass-spectrometry data processing, with the main focus on LC-MS data [16]. This software allows comparing UPLC profiles to define clusters of chemical similarity analysis. It is based on generation of ion chromatograms. The samples used for LC-MS analyse were prepared by dissolving the extracts in MeOH at a concentration of 3 mg/mL, then filtering on Sep-Pak® C_{18} cartridge (Ref: WAT023590) and evaporating. The samples were adjusted at the final concentration of 1 mg/mL in methanol and were filtered on 0.2 µm PTFE filter. Elution was conducted with H_2O:ACN gradient as follows: 95:5 - 40:60 in 20 min, 40:60 - 0:100 in 5 min, 0:100 for 5 more min. The flow rate was 0.6 mL/min. DataBridge software (Version 3.5) was used to convert the data in the ".cdf" format legible by MZmine. Data extraction and alignment was achieved with MZmine 2 (Version 2.10) with the following parameters: Mass detection centroid: 100 for positive ionization, and 250 for negative ionization, FTMS Shoulder masse 0.005, Chromatogram builder 100 for negative ionization, and 250 for positive ionization, Tolerance 0.005 or 5 ppm. XL stat (2014) was used to generate a hierarchical clustering tree based on chemical similarity.

3. Results and discussion

One hundred thirty three EtOAc extracts, out of 3563 representative of 182 plant families, were found to exhibit a significant inhibiting activity against dengue virus replication (greater than 10% inhibition and less than 55% cell death at 5 µg/mL). The results were examined from a phylogenetic perspective by using a regression residual analysis according to Moerman [17],[18]. This analysis is used to reduce the relative impact of a group poorly represented and to correlate active extracts proportion to the population of each group. This analysis is shown in Figure 1. Figure 1A represents the linear regression analysis of active extracts against tested ones, per family. The average relative proportion of active extracts is 4.03%, and the regression line represents the predicted number of antiviral species. For each family, the residual value was calculated measuring the deviation of the mean based on regression analysis as follows: $r = NAE - (0.0403 \times NE)$, where NAE is the number of active extracts per family, and NE the total number of extracts tested per family. The Ebenaceae family is represented by 26 active out of 80 extracts tested (Supplementary material, Table S1.). The 4 families with the highest residual values, along with the two families with the lowest residual values are shown in figure 1B. The full data that allowed for construction of Figure 1 is given in supplementary material (Table S2.). Figure 1B shows unambiguously that the family Ebenaceae had a much higher residue than all others (21.78 vs. 4.06 for the Celastraceae showing the second best residual score). It was therefore relevant to focus clear attention on the origin of the antiviral potential of this plant family.

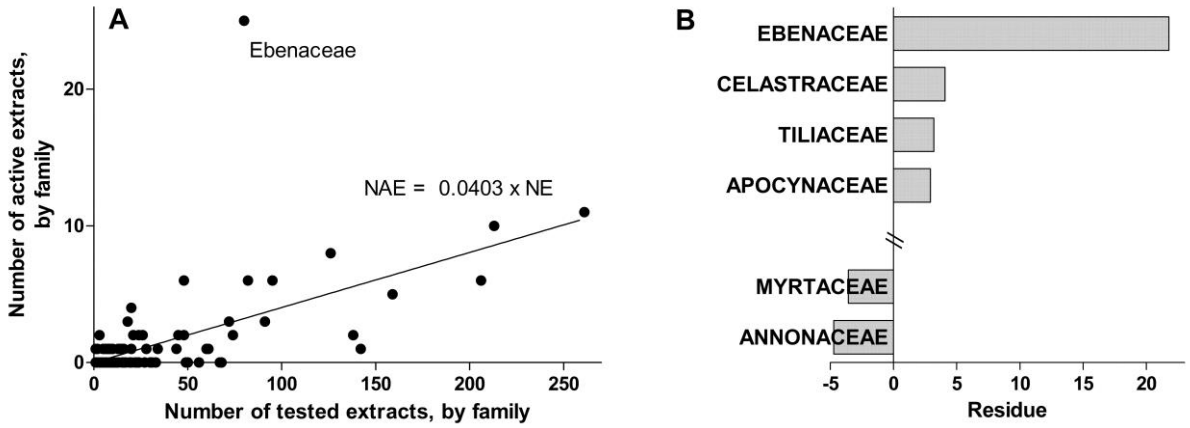

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Fig. 1 (A) Regression analysis showing the relative proportion of active extracts (dengue replication inhibition) per botanical family. Each dot is representative of one family.
(B) Botanical families with the 4 best and 2 lowest residual values, respectively.

A second regression analysis was performed taking into account the different plant parts. This analysis is presented in supplementary material (Table S3.). The results showed unambiguously that the bark extracts have the highest residual value (7.1266), suggesting that the study of this plant part should give best chances of success to find antiviral secondary metabolites. In contrast, the residual value calculated for leave extracts was the lowest.

All Ebenaceae species tested were from the *Diospyros* genus. To compare the chemical profiles of *Diospyros* spp. extracts, UPLC-HRMS profiles of the 26 active extracts along with 7 inactive extracts were recorded. For each peak of UPLC-HRMS profiles, retention time and major ion were compared with the software MZmine. A hierarchical clustering graph has been built with XLSTAT based on Pearson chemical similarities (Fig. 2). Extracts in clusters are chemically similar.

This dendrogram shows that extracts from the same species, but from different plant parts are usually not similar. For example, extracts from *D. apiculata* leaves and stem (DC-7665 and DC-7667, respectively) or from *D. vieillardii* leaves and bark (NC-31-18 and NC-31-20, respectively) were not part of the same cluster. This is also the case for extracts prepared from the same species but collected in different localities (*D. lancifolia* bark extracts, ZK-0109 vs. ZK-0126, and *D. apiculata* leaves extracts, ZK-0419 vs. DC-7665). In other cases, extracts from 2 different collections of the same species (*D. carbonaria* leaves, EV-0034 and EV-0050) or from different plant parts (*D. blancoi* leaves and fruits, LAP2-2-22 and LAP2-2-19) can be very similar. Interestingly, a partial geographical clustering can be observed, which is likely linked to phylogenetic proximity (clusters A and B, Fig. 2) [19],[20]. For example, New Caledonian extracts from *D. pancheri* (NC-28-16) and *D. pustulata* (NC-62-18) are chemically and phylogenetically close.

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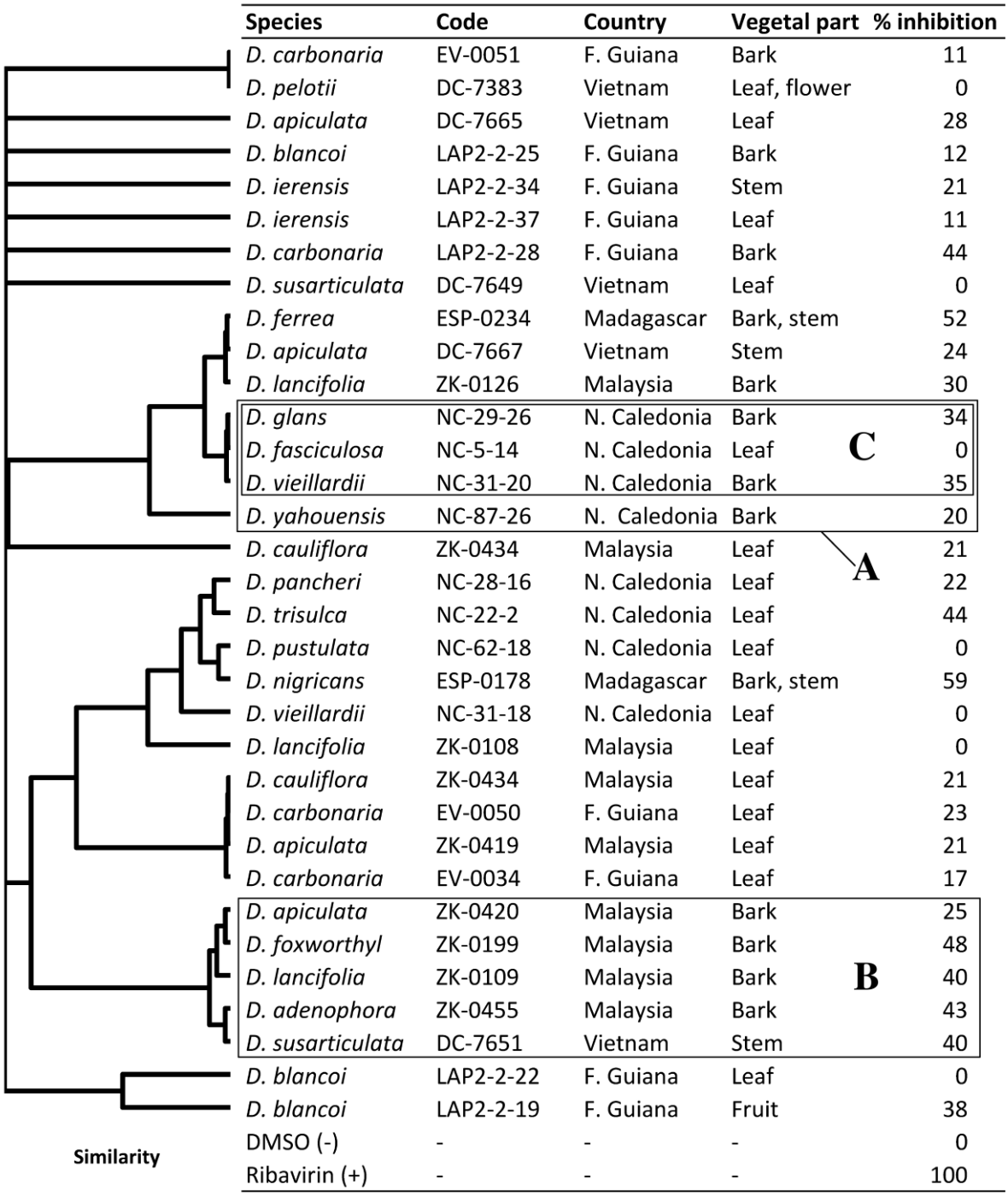

Species	Code	Country	Vegetal part	% inhibition	
D. carbonaria	EV-0051	F. Guiana	Bark	11	
D. pelotii	DC-7383	Vietnam	Leaf, flower	0	
D. apiculata	DC-7665	Vietnam	Leaf	28	
D. blancoi	LAP2-2-25	F. Guiana	Bark	12	
D. ierensis	LAP2-2-34	F. Guiana	Stem	21	
D. ierensis	LAP2-2-37	F. Guiana	Leaf	11	
D. carbonaria	LAP2-2-28	F. Guiana	Bark	44	
D. susarticulata	DC-7649	Vietnam	Leaf	0	
D. ferrea	ESP-0234	Madagascar	Bark, stem	52	
D. apiculata	DC-7667	Vietnam	Stem	24	
D. lancifolia	ZK-0126	Malaysia	Bark	30	
D. glans	NC-29-26	N. Caledonia	Bark	34	
D. fasciculosa	NC-5-14	N. Caledonia	Leaf	0	**C**
D. vieillardii	NC-31-20	N. Caledonia	Bark	35	
D. yahouensis	NC-87-26	N. Caledonia	Bark	20	
D. cauliflora	ZK-0434	Malaysia	Leaf	21	
D. pancheri	NC-28-16	N. Caledonia	Leaf	22	**A**
D. trisulca	NC-22-2	N. Caledonia	Leaf	44	
D. pustulata	NC-62-18	N. Caledonia	Leaf	0	
D. nigricans	ESP-0178	Madagascar	Bark, stem	59	
D. vieillardii	NC-31-18	N. Caledonia	Leaf	0	
D. lancifolia	ZK-0108	Malaysia	Leaf	0	
D. cauliflora	ZK-0434	Malaysia	Leaf	21	
D. carbonaria	EV-0050	F. Guiana	Leaf	23	
D. apiculata	ZK-0419	Malaysia	Leaf	21	
D. carbonaria	EV-0034	F. Guiana	Leaf	17	
D. apiculata	ZK-0420	Malaysia	Bark	25	
D. foxworthyl	ZK-0199	Malaysia	Bark	48	
D. lancifolia	ZK-0109	Malaysia	Bark	40	**B**
D. adenophora	ZK-0455	Malaysia	Bark	43	
D. susarticulata	DC-7651	Vietnam	Stem	40	
D. blancoi	LAP2-2-22	F. Guiana	Leaf	0	
D. blancoi	LAP2-2-19	F. Guiana	Fruit	38	
DMSO (-)	-	-	-	0	
Ribavirin (+)	-	-	-	100	

Fig. 2. Dendrogram showing chemical similarities of 33 *Diospyros* extracts. *Diospyros* species, extract code, country of origin, plant part and percentage of inhibition at 5 µg/mL of dengue virus replication. Three clusters A, B and C are framed within a rectangle.

Cluster C consists of 2 active (*D. glans* and *D. vieillardii*) and 1 inactive (*D. fasciculosa*) extracts (Fig. 2). Since these extracts are supposed to be chemically close to each other, we postulated that *D. glans* and *D. vieillardii* might contain active compounds missing in *D. fasciculosa.* The UHPLC-HRMS profiles of the 3 samples constituting cluster C are shown in Figure 4. Upon examination of these UHPLC traces, it was found that 2 secondary metabolites (corresponding to compounds 5 and 6, at Rt 22.2 and 23.5 min, respectively) were absent in the inactive extract. We assumed that these metabolites might be responsible for the

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biological activity found in these 2 extracts, and embarked upon isolation and characterization of these compounds from *D. glans* bark extract.

Overall, 8 compounds were isolated and identified by comparison with literature data: usnic acid (**1**) [21], 11-oxo-acetyl ursolic acid (**2**) [22], 13,28-epoxyurs-11-ene-3,28-dione (**3**) [23], betulinic acid (**4**) [24], (3*β*)-3,23-dihydroxylup-20(29)-en-28-oic acid (**5**) [25], (3*β*)-3-(acetyloxy)-urs-12-en-28-oic acid (**6**) [26], betulinic aldehyde (**7**) [27], and (3*β*)-3,23-dihydroxylup-12,20(29)-dien-28-oic acid (**8**) [28] (Fig. 3). Usnic acid (**1**) has never been isolated from higher plants to date. Although the outer bark of *D. glans* was cleaned carefully before sampling, its lichen origin is probable. An endophytic origin cannot also be excluded.

On the chromatographic profile of *D. glans* are indicated the correspondence between some peaks and compounds **2**, and **4**-**8** (Fig. 4). Compound **3** was not detected in the LC-HRMS profile of the crude extract and compound **1** can be seen at Rt = 18.28 min in negative ionization mode only. According to our hypothesis, (3*β*)-3,23-dihydroxylup-20(29)-en-28-oic acid (**5**) and (3*β*)-3-(acetyloxy)-urs-12-en-28-oic acid (**6**), which were not detected in the inactive *D. fasciculosa* extract, should be responsible of the antiviral activity of *D. glans* and *D. vieillardii* extracts. All isolated compounds from *D. glans* were evaluated using the dengue replicon virus-cell-based assay and the DENV-NS5 RNA-dependant RNA polymerase assay (Table 1).

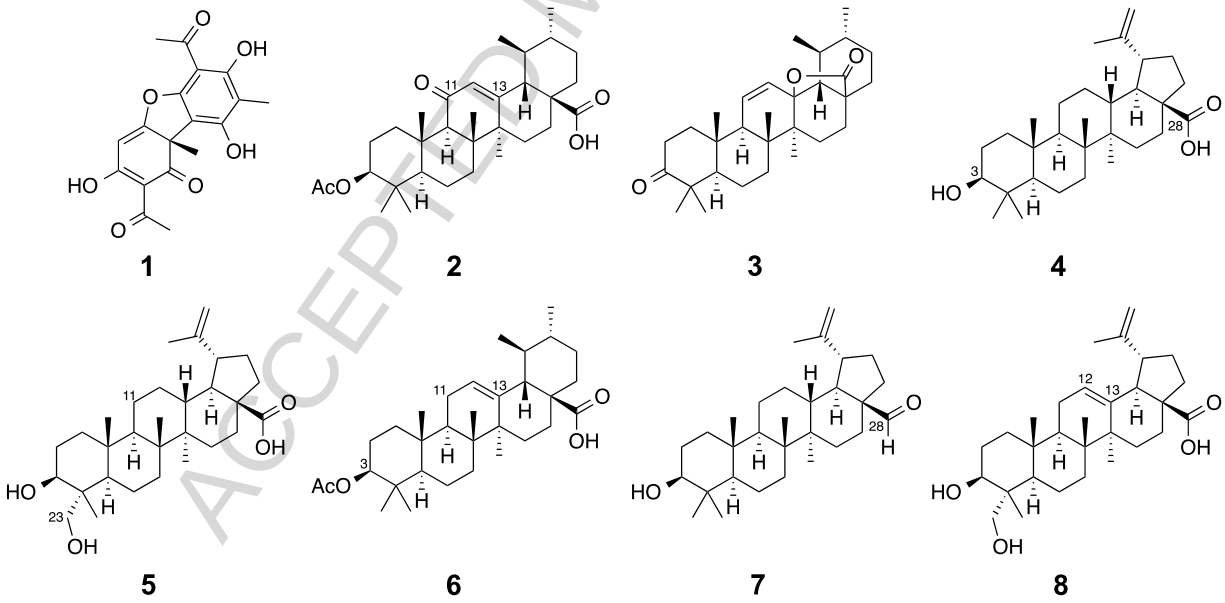

Fig. 3. Structures of compounds **1**-**8** isolated from *D. glans* bark extract.

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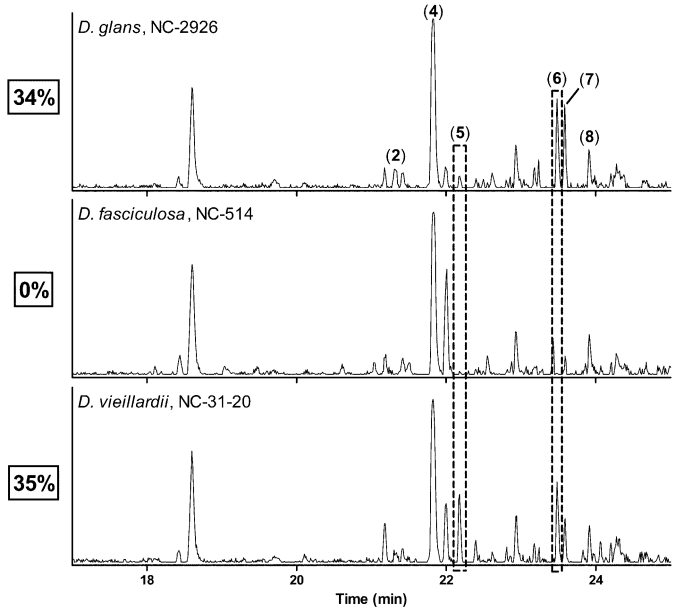

Fig. 4. Partial UHPLC-HRMS (17 to 25 min) in positive mode of *D. glans*, *D. fasciculosa* and *D. vieillardii* extracts from Cluster C. The viral inhibition rates at 5 µg/mL are indicated on the left of the chromatogram. In *D. glans* profile, the correspondance between the isolated compounds and some peaks is indicated. Compounds **5** and **6**, which are only seen in active *D. glans* and *D. vieillardii* extracts, are framed with doted rectangles at Rt 22.2 and 23.5 min, respectively.

Table 1
DENV-NS5 RNA-dependant RNA polymerase inhibitory, dengue virus replication activity, and cytotoxicity of compounds **1**-**8**.

Compounds	DENV-NS5 RdRp[a]	Dengue replicon assay[b] (% of inh.)			% of viability on COS cells[b]		
	IC_{50} (µM)	1	5	10	1	5	10
Usnic acid (**1**)	4.7 ± 0.3	0	90	91	62	15	0
11-Oxo-acetyl ursolic acid (**2**)	32.8 ± 7.2	0	0	0	86	99	75
13,28-Epoxyurs-11-ene-3,28-dione (**3**)	25.6 ± 4.4	0	0	0	99	100	82
Betulinic acid (**4**)	6.6 ± 0.6	0	35	57	100	96	50
(3*β*)-3,23-Dihydroxylup-20(29)-en-28-oic acid (**5**)	25.6 ± 4.2	0	39	45	100	100	71
(3*β*)-3-(Acetyloxy)-urs-12-en-28-oic acid (**6**)	7.0 ± 1.4	0	22	44	100	100	79
Betulinic aldehyde (**7**)	6.1 ± 0.7	0	20	55	100	100	70
(3*β*)-3,23-Dihydroxylup-12,20(29)-dien-28-oic acid (**8**)	5.3 ± 0.6	7	40	52	98	100	75

[a] IC_{50} values are mean values ± SD from three replicates. Control: 3'-dATP at 10 µM (93,7% ± 0,5)
[b] % of inhibition or % of viability at 1, 5, and 10 µg/mL. Control: Ribavirin at 10 µM (49% of inh., 82% of cell viability)

With the exception of usnic acid (**1**), the isolated compounds were not found cytotoxic on COS monkey cells. From the 7 triterpenoids isolated, betulinic acid (**4**), (3*β*)-3,23-dihydroxylup-20(29)-en-28-oic acid (**5**), (3*β*)-3-(acetyloxy)-urs-12-en-28-oic acid (**6**), betulinic aldehyde (**7**), and (3*β*)-3,23-dihydroxylup-12,20(29)-dien-28-oic acid (**8**) significantly inhibited dengue replication at 5 and 10 µg/mL. In the DENV-NS5 RdRp assay, compounds **4**, **6**, **7**, and **8** also exhibited a significant enzyme inhibition with IC_{50} in the 5.0 - 7.0 µM range, while the other isolated triterpenoids were found less active. The inhibitory potency found for betulinic acid on DENV-NS5 RdRp is similar to that reported previously (IC_{50} = 1.7 µM) [29]. Based on the analysis of chromatographic profiles, we expected that only (3*β*)-3,23-dihydroxylup-20(29)-en-28-oic acid (**5**) and (3*β*)-3-(acetyloxy)-urs-12-en-28-oic acid (**6**) would have been active. Clearly, this is not the case, and the reason why *D.*

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fasciculosa leaves extract, in which betulinic acid (**4**) is present in high relative proportion, was found not active, remains uncertain. Some interesting structure-activity relationships can be drawn from these results. In the ursane series (**2**, **3** and **6**), since compound **2** was found less active in the RdRp assay than compound **6**, and not active in the dengue replicon assay, it can be deduced that the keto function at C-11 is deleterious for the antiviral activity. In the lupane series (**4**, **5**, **7** and **8**), it can be deduced that the $\Delta^{12,13}$ double bond increases enzyme inhibition (comparing **5** vs. **8**, with IC_{50} of 25.6 and 5.3 µM, respectively), while the C-23 hydroxy group is deleterious (**5** vs. **4**, with IC_{50} 25.6 and 6.6 µM, respectively). In addition, it can be noticed that an acidic function or an aldehyde function at C-28 (**4** and **7**) plays an equivalent role in the antiviral activities. Previous studies have shown that betulinic acid and betulinic acid 3β-caffeate significantly inhibited the polymerase activity [29], [30] while other triterpenoids, such as lupenone and β-amyrone [31] and steroids, such β-sitosterol and stigmastenone derivatives [32] were found poorly active. Betulinic acid (**4**) and (3*β*)-3,23-dihydroxylup-12,20(29)-dien-28-oic acid (**8**) are well-known as antitumoral agents [33]. Betulinic acid (**4**) is also known for its antiplasmodial activity [34], and its antiviral properties against hepatitis C virus [35], influenza virus [36] and HIV [37]. Anti-inflammatory activity has been reported for compound **5** [38], while antibacterial and antiviral properties against influenza virus were reported for betulinic aldehyde (**7**) [39].

Ursane- and lupane-type triterpenoids are secondary plant metabolites widely distributed in Angiosperms and Gymnosperms. For example, ursolic acid derivatives are frequently found in species belonging to Ericaceae, Lamiaceae and Rosaceae [40], while betulinic acid is widespread in Ebenaceae, Betulaceae, Platanaceae, and Rhamnaceae [41]. It is of particular interest to know that lupane and ursane skeletons are the most common triterpene skeletons found in the genus *Diospyros* [5]. Lupeol, betulin and betulinic acid on the one hand, and α-amyrin, bauerenol and ursolic acid on the other hand were the major metabolites of these 2 classes, respectively. In addition, betulinic acid and other members of the lupane class are known to accumulate significantly in bark and heartwood [5]. These data are in full accordance with the results of our study.

4. Conclusion

We have shown that *Diospyros* species from Ebenaceae family have a high probability of producing secondary metabolites having antiviral activity against dengue virus. The chemical study of *D. glans* bark EtOAc extract led to the isolation of 8 compounds, of which betulinic acid (**4**), (3*β*)-3,23-dihydroxylup-20(29)-en-28-oic acid (**5**), (3*β*)-3-(acetyloxy)-urs-12-en-28-oic acid **(6),** betulinic aldehyde (**7**) and (3*β*)-3,23-dihydroxylup-12,20(29)-dien-28-oic acid (**8**), showed significant inhibition of the RdRp, and inhibited dengue replicon virus *in cellulo*. Considering other species of the *Diospyros* genus, these biological activities are apparently linked to the presence of ursane- and lupane-type triterpenoids, and in particular to betulinic acid, which was found in high relative proportion in most of the active extracts.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Antiviral activities of *Diospyros* spp. extracts and residue calculation of families and of plant parts can be found as supplementary data. Supplementary data to this article can be found in the on-line version, at doi:

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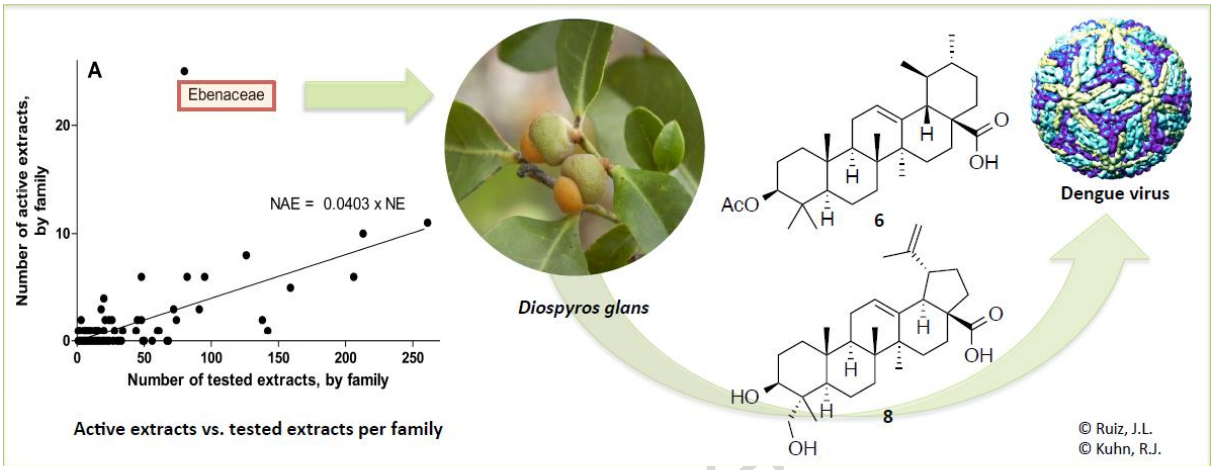

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