analytica chimica acta 624 (2008) 147–153

available at www.sciencedirect.com

journal homepage: www.elsevier.com/locate/aca

Screening of anti-HIV-1 inophyllums by HPLC–DAD of *Calophyllum inophyllum* leaf extracts from French Polynesia Islands

Frédéric Laure[a], *Phila Raharivelomanana*[a,*], *Jean-François Butaud*[a], *Jean-Pierre Bianchini*[a], *Emile M. Gaydou*[b]

[a] *Biodiversité Terrestre et Marine, Université de la Polynésie Française, BP 6570, 98702 Faa'a, Tahiti, French Polynesia*
[b] *UMR CNRS 6263, Equipe AD2M (Phytochimie), Case 461, Faculté des Sciences et Techniques de Saint-Jérôme, Université Paul Cézanne (Aix-Marseille III), Avenue Escadrille Normandie-Niemen, 13397 Marseille Cedex 20, France*

ARTICLE INFO

Article history:
Received 29 March 2008
Received in revised form 4 June 2008
Accepted 5 June 2008
Published on line 9 July 2008

Keywords:
Calophyllum inophyllum
Clusiaceae
Tamanu
Inophyllums
Coumarins
Neoflavonoids
Anti-HIV-1 agents
High pressure liquid chromatography-UV-diode array detection
Chemodiversity
Multivariate analyses

ABSTRACT

Various pyranocoumarins, calophyllolide, inophyllums B, C, G_1, G_2 and P, from *Calophyllum inophyllum* (Clusiaceae) leaves of French Polynesia (Austral, Marquesas, Society and Tuamotu archipelagos) have been determined in 136 leaf extracts using a high pressure liquid chromatography-UV-diode array detection (HPLC–UV-DAD) technique. Results show a wide range in chemical composition within trees growing on eighteen islands. The use of multivariate statistical analyses (PCA) shows geographical distribution of inophyllums and indicate those rich in HIV-1 active (+)-inophyllums. Inophyllum B and P contents (0.0–39.0 and 0.0–21.8 mg kg^{-1}, respectively) confirm the chemodiversity of this species within the large area of French Polynesia. The study suggests the presence of interesting chemotypes which could be used as plant source for anti-HIV-1 drugs.

1. Introduction

The genus *Calophyllum* belonging to the Clusiaceae family, is a source of secondary metabolites such as triterpenes and steroids, benzopyrans, xanthones, coumarins and neoflavonoids [1]. *Callophyllum inophyllum*, the most abundant species of this genus, is an evergreen tree in the tropical area of Africa, America and Asia [2]. This tree is also widespread in French Polynesia (locally called as *Tamanu*) and used in folk medicine [3]. We reported the structures of new secofriedelane and friedelane acids, and neoflavonoids from *C. inophyllum* [4,5]. Some dipyranocoumarins isolated from the *Calophyllum* genus, show anti-HIV-1 activity [1]. In 1992, Kashman et al. [6,7] isolated from the Malaysian tree *C. lanigerum*

* *Corresponding author*. Tel.: +689 803 822; fax: +689 803 804.
E-mail address: phila.raharivelomanana@upf.pf (P. Raharivelomanana).
0003-2670/$ – see front matter
doi:10.1016/j.aca.2008.06.046

var. *austrocoriaceum*, (+)-calanolide A, which was shown to be a strong anti-HIV-1 active coumarin. (+)-Calanolide A is a 4-propyl-dipyranocoumarin structurally related to 4-phenyl-coumarins. One year later, Patil et al. [8] isolated from the Malaysian tree *C. inophyllum*, (+)-inophyllum B, and other new compounds. Compounds containing 4-methyl-pyranocoumarins have been reported in *C. cordato-oblongum*, an endemic species of Sri Lanka [9] which were found to inhibit HIV-1 reverse transcriptase [10]. Other HIV-1 inhibitory dipyranocoumarins from *C. brasiliense* leaves have been isolated [11]. During a chemotaxonomic survey of *Calophyllum* extracts present in the National Cancer Institute (NCI), four new pyranocoumarins were isolated from *C. lanigerum* var. *austrocoriaceum* and *C. teysmannii* var. *inophylloide*. The structure and anti-HIV activities of these compounds were described [12]. The investigation of 315 organic extracts from 31 taxa of *Calophyllum*, present in the NCI was analyzed for related pyranocoumarins using a simple thin layer chromatography system [13]. These obtained results suggest that there may be distinct various chemotypes for *Calophyllum* species in particular in *C. teysmanii* var. *inophylloide* [13].

Although a large number of leaf and latex samples of species have been investigated by the NCI, leaves coming from French Polynesia have not been examined for their coumarin contents. Since this large part of the World contain several hundred of islands where numerous *C. inophyllum* are growing, it should be interesting to control any change of coumarin content in leaf within some of these islands. Therefore we undertook a chemotaxonomic study to follow the biodiversity of this species throughout 136 trees located on the main 4 archipelagos of French Polynesia: Society, Tuamotu, Marquesas and Austral islands. Leaf extracts were analyzed for related pyranocoumarins (Fig. 1) using a HPLC (high pressure liquid chromatography) method and UV-DAD detection. The data obtained were then examined using multivariate statistical analyses such as principal component analysis (PCA).

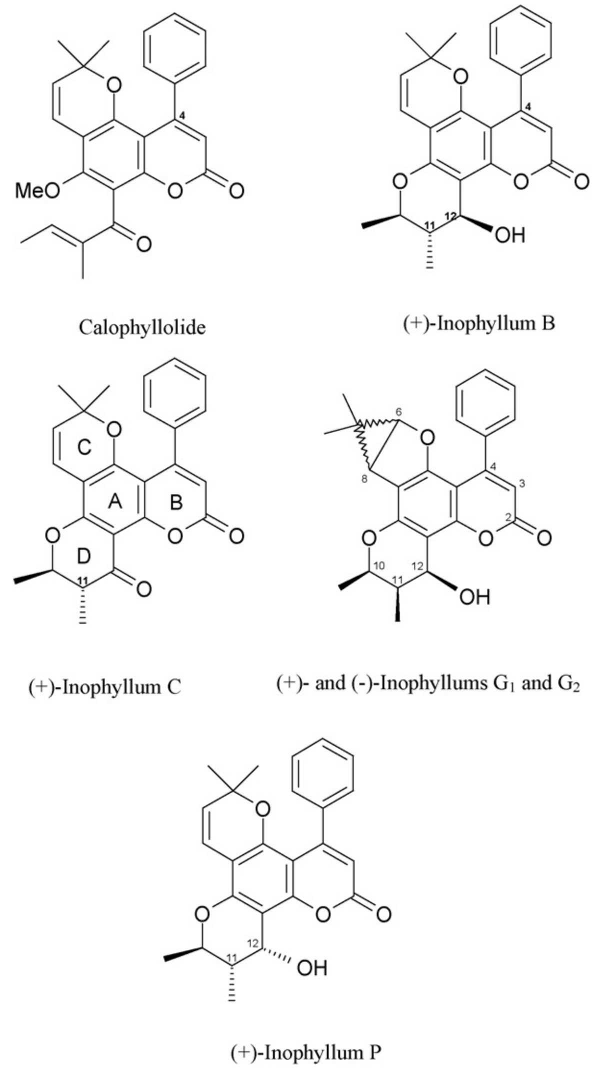

Fig. 1 – Structures of calophyllolide and inophyllums investigated.

2. Materials and methods

2.1. Plant material

Samples were collected in a large number of islands as possible, in the four main archipelagos of French Polynesia, in order to be representative of the biodiversity of the *C. inophyllum* trees growing in this part of the world. We collected about 10 different samples distributed around each islands investigated. Each sample was composed of different growth age leaves, collected on various tree branches. This small amount of leaf (10–12 g) was used for extraction and HPLC analyses. *C. inophyllum* leaves were collected on eighteen islands belonging to the four main archipelagos: Society Islands (Tahiti (9 samples), Moorea (12), Tetiaroa (11), Raiatea (7), Tahaa (7), Bora Bora (3), and Maupiti (6)), Tuamotu Islands (angiroa (16), Hao (4), and Manihi (2)), Marquesas Islands: Nuku Hiva (13), Hiva Oa (14), Motane (6), Ua Huka (4), and Tahuata (10)) and Austral Islands (aivavae (4), Rurutu (5), and Tubuai(3)). Therefore 136 leaf samples of *C. inophyllum* were collected from the French Polynesia islands as shown in Fig. 3.

2.2. Leaf extractions

Extraction at room temperature of crushed and dried leaves (10 g) with cold ethyl acetate (100 mL) during 1 week yielded a dark green oil (600 mg of crude extract) which was purified for HPLC analyses on a silica Sep pak.

2.3. Compound identifications

Structures of compounds were identified by means of nuclear magnetic resonance (NMR) (500 MHz: Bruker Avance DRX-500 apparatus equipped with a cryosonde) and a mass spectrometry (MS) (Sciew API III Plus spectrometer equipped with an electrospray ionisation (ESI) atmospheric ion source). Data were then checked by comparison of their spectral data with literature values for calophyllolide [14], inophyllums B and C [8,15–17], for inophyllums P, G_1 and G_2 [8]. These standards and some samples were also analyzed using MS by a HPLC–ESI-MS-MS [5] to confirm the attributions made by HPLC–UV/DAD

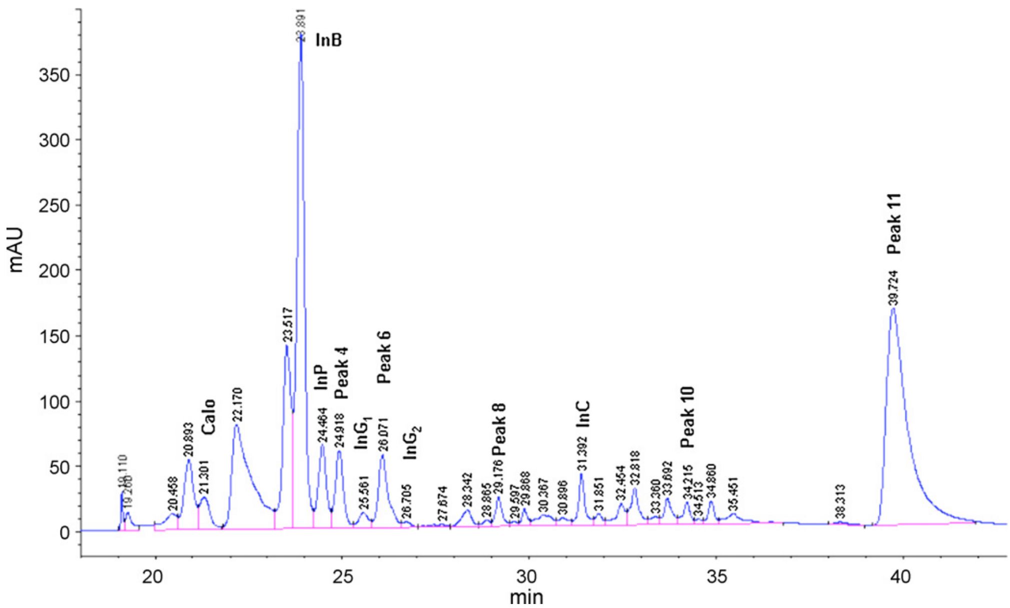

Fig. 2 – Typical HPLC–UV-DAD chromatogram of a leaf extract sample. Calo, calophyllolide; InB, InC, InP, InG_1 and InG_2, inophyllums B, C, P, G_1 and G_2. Peaks 4 and 6 are unidentified coumarins and peaks 8, 10 and 11 are unidentified compounds.

(diode array detector) analyses. Some peaks remained unidentified.

2.4. HPLC–UV/DAD analyses

Three chromatographic silica columns (silica uptisphere type from Interchrom, porosity 120 Å, granulometry 5 mm, size 250 mm × 4.6 mm) were used serially to separate the different coumarins contained in each leaf extract using a HP-1100 HPLC system with an auto sampler and an UV-DAD detector. Each extract (15 ml) was dissolved in *i*-propanol–i-octane (5/95, v/v) and injected for quantification at 360 nm. The eluent (1 mL min^{-1}) was a gradient of *i*-propanol–i-octane from 1 to 20% (v/v) during 25 min, *i*-propanol–i-octane (20/80, v/v during 25 min) followed by a stabilization period of 15 min. Eleven peaks were kept for the statistical analysis; their retention times were calophyllolide, 21.3 min; inophyllum B, 23.9 min; inophyllum P, 24.5 min; peak 4, 24.9 min; inophyllum G_1, 25.6 min; peak 6, 26.1 min; inophyllum G_2, 26.7 min; peak 8, 29.2 min; inophyllum C, 31.4 min; peak 10, 34.2 min and peak 11, 39.7 min (Fig. 2). The coumarin names were attributed by comparison of the peak UV spectra with those of purified molecules, analyzed in the same HPLC conditions. For content determination, we determined the mass of known compounds using area unity (AU) of the corresponding peaks at 360 nm given by isolated identified compounds: for inophyllums B and P, 2.5 ng AU^{-1}; inophyllums G_1 and G_2, 2.5 ng AU^{-1}; for inophyllum C, 9.5 ng AU^{-1} and for calophyllolide, 15 ng AU^{-1}. For unidentified coumarins and unidentified compounds, 5 ng AU^{-1} were taken.

Table 1 – Mean content (mg kg^{-1}) of main components in *Calophyllum inophyllum* leaf extracts from French Polynesia

	Samples	Calo[a]	InB[a]	InP[a]	Peak 4[b]	InG_1[a]	Peak 6[b]	InG_2[a]	Peak 8[c]	InC[a]	Peak 10[c]	Peak 11[c]
Each archipelago (mean)												
Austral	12	2.10	15.05	4.03	0.78	0.10	0.38	0.00	1.88	2.01	0.76	17.8
Marquesas	47	2.64	2.73	6.32	0.36	0.10	0.33	0.00	2.05	1.63	0.61	28.5
Society	55	1.19	10.08	2.39	0.99	0.39	1.17	0.04	1.45	1.32	0.58	19.3
Tuamotu	22	1.55	20.84	4.69	1.40	0.20	1.20	0.00	2.03	1.94	0.89	13.1
All archipelago												
Minimum		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.84
Maximum		7.45	39.0	21.8	4.94	2.21	5.39	1.57	8.27	7.23	5.72	77.9
Mean	136	1.93	9.45	4.58	0.81	0.22	0.80	0.02	1.75	1.66	0.69	21.1
S.D.		1.70	10.51	5.44	1.02	0.44	1.13	0.14	2.11	1.26	0.74	15.2

[a] See Figs. 1 and 2 for formula and abbreviations. For content determination, see Section 2.4. HPLC–UV/DAD analyses.
[b] Unidentified coumarin.
[c] Unidentified compound.

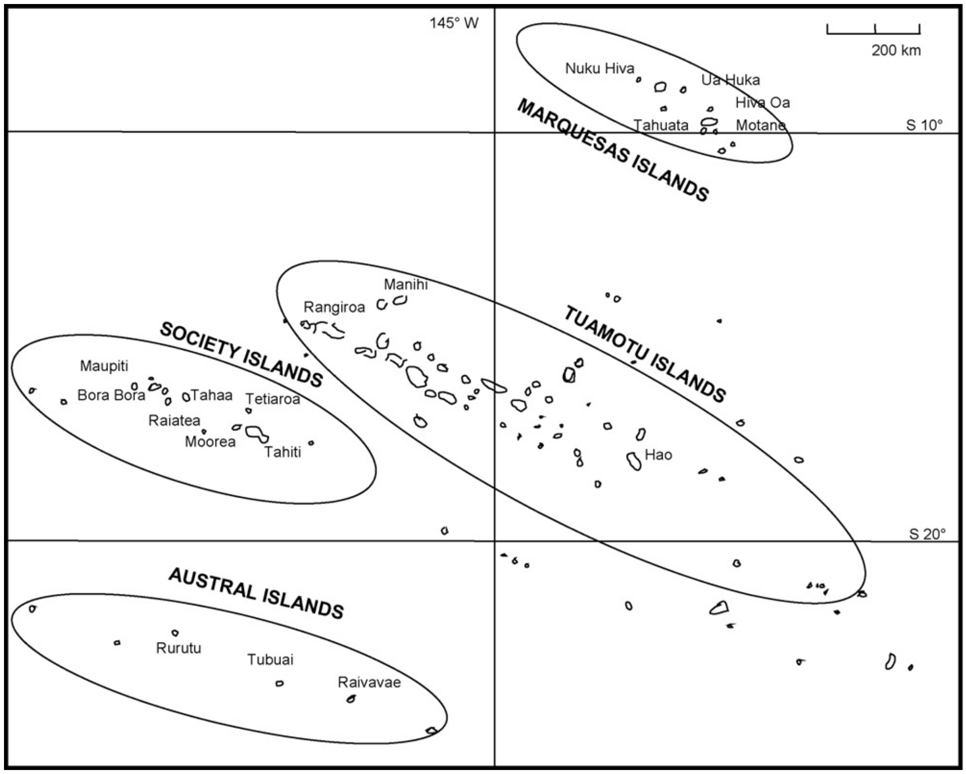

Fig. 3 – Map of the different French Polynesia archipelagos showing the various area of collect.

2.5. *Statistical analyses*

Multivariate statistical analyses were applied for geographical distribution using coumarin compositions of the 136 *C. inophyllum* leaf samples. Principal component analysis has been performed by using a data set composed of 136 samples and 11 independent variables (corresponding to the identified coumarins and the better separated peaks: inophyllums C, B, P, G_1, G_2, calophyllolide, 2 unidentified coumarins and 3 unidentified compounds). The data set was transformed into centered and reduced variables (standardized PCA). Data were processed with Addinsoft XLSTAT program version 6.1.

3. Results and discussion

3.1. *Coumarin content*

C. inophyllum (Tamanu) fresh leaves were randomly chosen to follow their chemodiversity on 18 islands of the four main archipelagos of French Polynesia. Isolation of the pure compounds from the various fractions for their identification was performed by chromatography as previously described [5]. Compounds identified are given in Fig. 1. As it can be seen, they are all belonging to the 4-phenyl series of pyranocoumarins:

Table 2 – Correlation matrix of main components[a] in *C. inophyllum* leaf extracts from French Polynesia

	Calo	InB	InP	Peak 4[b]	InG_1	Peak 6[b]	InG_2	Peak 8[c]	InC	Peak 10[c]	Peak 11[c]
Calophyllolide	1	0.163	**0.681**	0.125	0.070	0.068	0.008	−0.258	**0.688**	0.464	−0.444
Inophyllum B		1	0.107	0.543	0.352	0.424	0.159	−0.175	0.391	0.363	−0.589
Inophyllum P			1	0.164	0.020	0.106	−0.024	−0.174	0.586	0.390	−0.425
Peak 4[b]				1	**0.681**	**0.752**	0.242	−0.367	0.362	0.335	−0.309
Inophyllum G_1					1	**0.681**	0.401	−0.294	0.279	0.236	−0.249
Peak 6[b]						1	0.379	−0.349	0.274	0.235	−0.229
Inophyllum G_2							1	−0.079	0.045	0.039	−0.065
Peak 8[c]								1	−0.404	−0.324	−0.005
Inophyllum C									1	**0.759**	−0.561
Peak 10[c]										1	−0.452
Peak 11[c]											1

[a] See Figs. 1 and 2 for formula and abbreviations. For content determination, see Section 2.4. HPLC–UV/DAD analyses.
[b] Unidentified coumarin.
[c] Unidentified compound.

- calophyllolide which was first identified in *C. bracteatum* [14],
- the HIV-1 active (+)-inophyllum B having a (10*R*, 11*S*, 12*S*)-10, 11-dimethyl 12 chromanol ring,
- (+)-inophyllum P, the 12-epimer of inophyllum B having also a *trans* 10, 11-dimethylchromanol, but less active, and
- two less HIV-1 active inophyllums G_1 and G_2, first characterized by Patil et al. [8] which were identified in low amount in some leaves, together with inophyllum C having a 12-chromanone ring.

Finally, peaks 4 and 6 with a coumarin structure remained unidentified and peaks 8, 10 and 11, unidentified compounds were not coumarins.

Each *C. inophyllum* leaf extract was analyzed using three serial chromatographic silica columns to separate the different coumarins using an UV-DAD detector (Fig. 2). Table 1 lists the mean and range contents of main components and peaks for the sample extracts from the four archipelagos. Results obtained were submitted to statistical analysis considering each islands within each archipelago. As shown in Table 1, calophyllolide was found in all archipelago at a mean content of 1.93 mg kg^{-1}, but was not detected in the case of various islands of Marquesas, Austral and Tuamotu. The higher content was observed on a tree of Tubuai (Austral) islands. The range of the HIV-1 (+)-inophyllum B was very large (0.0 up to 39.0 with a mean of 9.45 mg kg^{-1}). (+)-Inophyllum P was found in lower amount than (+)-inophyllum B with a mean of 4.6 mg kg^{-1}, the maximum was found for a Tubuai tree (21.8 mg kg^{-1}, Austral archipelago, Fig. 3). (+)-Inophyllum C with a mean of 1.66 mg kg^{-1} was the third inophyllum from a weight point of view and the main content was observed with a tree growing on Tahiti (7.23 mg kg^{-1}). If G_1 was detected in most leaves at a lower amount (0.22 mg kg^{-1}), inophyllum G_2 was detected only on one Tetiaroa (Society archipelago) tree.

Table 2 gives the correlation matrix of leaf extract main components and Table 3 gives the mean content (mg kg^{-1}) for islands investigated, within the various archipelagos.

3.2. Chemodiversity of French Polynesia *C. inophyllum*

Since no clear chemical composition change between islands and archipelagos was observed, multivariate statistical analysis was undertaken. The data set was composed of 136 leaf samples, the 6 identified and the 5 unidentified remaining peaks. As shown in Table 2, poor positive or negative correla-

Table 3 – Mean content (mg kg^{-1}) of main components in *C. inophyllum* leaf extracts for islands investigated within the various archipelagos of French Polynesia

Islands	Samples	Calo[a]	InB[a]	InP[a]	Peak4[b]	InG_1[a]	Peak 6[b]	InG_2[a]	Peak 8[c]	InC[a]	Peak 10[c]	Peak 11[c]
Society archipelago												
Bora Bora	3	1.02	2.40	0.507	0.00	0.00	0.00	0.00	2.56	0.65	0.00	32.5
Maupiti	6	0.46	2.09	0.372	0.14	0.00	0.20	0.00	1.00	0.38	0.13	38.6
Moorea	12	2.02	15.2	4.822	1.42	0.67	1.73	0.00	0.67	1.69	0.74	12.8
Raiatea	7	0.55	8.68	2.123	0.17	0.00	0.54	0.00	4.42	0.94	0.42	18.3
Tahaa	7	0.43	6.36	1.243	0.47	0.13	0.34	0.00	1.91	0.68	0.27	15.2
Tahiti	9	1.73	12.0	2.613	1.08	0.43	0.84	0.00	0.75	2.50	1.35	11.4
Tetiaroa	11	1.15	12.6	2.049	2.03	0.79	2.60	0.20	0.65	1.27	0.46	22.2

Islands	Samples	Calo[a]	InB[a]	InP[a]	Peak4[b]	InG_1[a]	Peak 6[b]	InG_2[a]	Peak 8[c]	InC[a]	Peak 10[c]
Tuamotu archipelago											
Hao	4	1.26	21.51	3.04	1.73	0.00	2.53	0.00	1.92	0.87	22.2
Manihi	2	0.00	4.72	0.92	0.00	0.00	0.70	2.97	0.64	0.00	28.0
Rangiroa	16	1.81	22.69	5.57	1.49	0.28	0.92	2.42	2.10	1.01	8.9

Islands	Samples	Calo[a]	InB[a]	InP[a]	Peak4[b]	InG_1[a]	Peak 6[b]	InG_2[a]	Peak 8[c]	InC[a]	Peak 10[c]
Marquesas archipelago											
Hiva Oa	14	2.82	2.21	10.4	0.47	0.00	0.52	1.80	1.68	0.69	26.40
Motane	6	2.95	7.91	0.00	0.12	0.00	0.00	4.77	0.95	0.31	22.42
Nuku Hiva	13	3.37	2.44	6.86	0.62	0.37	0.62	0.00	2.51	0.93	33.75
Tahuata	10	0.94	0.17	2.57	0.18	0.00	0.00	3.90	0.66	0.15	30.59
Ua Huka	4	3.44	4.16	9.09	0.00	0.00	0.00	0.83	2.09	0.84	23.48

Islands	Samples	Calo[a]	InB[a]	InP[a]	Peak4[b]	InG_1[a]	Peak 6[b]	InG_2[a]	Peak 8[c]	InC[a]	Peak 10[c]
Austral archipelago											
Raivavae	4	2.12	8.61	1.46	0.83	0.00	0.40	0.00	2.97	0.90	20.56
Rurutu	5	1.47	22.95	3.38	0.70	0.09	0.12	2.81	1.30	0.60	13.89
Tubuai	3	3.13	10.46	8.56	0.84	0.27	0.57	2.87	1.91	0.83	20.84

[a] See Figs. 1 and 2 for formula and abbreviations. For content determination, see Section 2.4. HPLC–UV/DAD analyses.
[b] Unidentified coumarin.
[c] Unidentified compound.

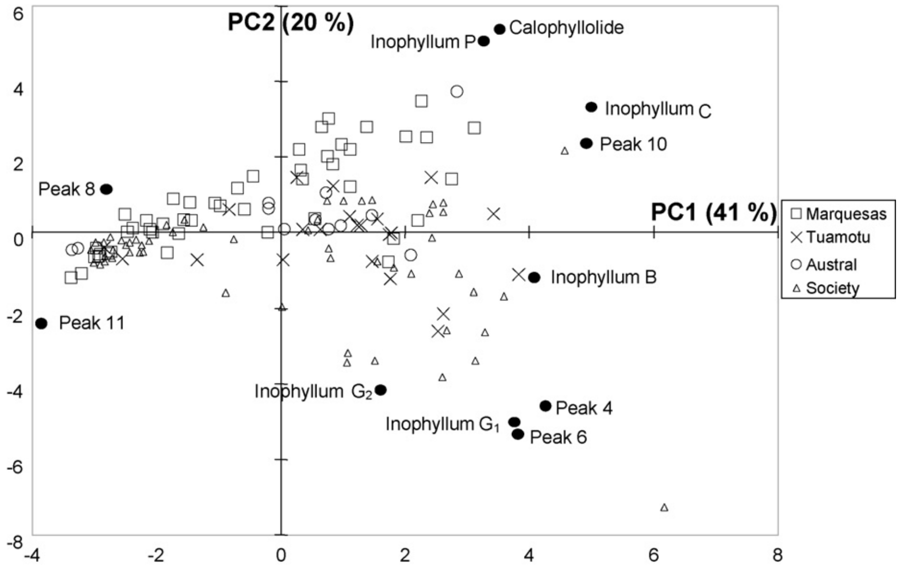

Fig. 4 – Two-dimensional plot of the various leaf *Calophyllum inophyllum* extract samples investigated by PCA for the four archipelagos from French Polynesia.

tion coefficients were observed between variables. A graphic representation of the projection of variables and samples onto the two first principal components is given in Fig. 4 using PCA. Axis 1, which represents 41% of the total information is positively loaded with the most inophyllums and negatively loaded with peaks 8 and 11. Some differentiation of Marquesas archipelago samples occurred with axis 2 (20% of the total information) since these samples are rich in inophyllum P and calophyllolide (6.32 and 2.64 mg kg^{-1}, respectively). Austral and Tuamotu archipelago trees are richer in the more active (+)-inophyllum B. As shown in Table 3, trees from Rurutu islands (Austral archipelago) were richer in (+)-inophyllum B compared to the other islands of this archipelago. All samples from Hao islands were rich in (+)-inophyllum B (16.8–24.6 mg kg^{-1}, mean 21.5 mg kg^{-1}, Table 3) but it was in Rangiroa islands that trees contained high content of this compound with in one case 39.0 mg kg^{-1}, Table 1, with a mean for this islands of 22.7 mg kg^{-1} (Table 3). In the case of (+)-inophyllum P, the methyl-12-epimer of (+)-inophyllum B, the best trees are growing on Moorea islands (4.8 mg kg^{-1}). Tentative to obtain better differentiation between archipelagos failed either using other PCA axes or factorial discriminant analyses.

4. Conclusion

The wide range of the more active (+)-inophyllums B and P (0–39.9 mg kg^{-1} for B, 0–21.8 mg kg^{-1} for P) show the chemodiversity of *C. inophyllum* in French Polynesia. These chemotypes are not closely correlated to islands, although Tuamotu and Austral archipelagos contained trees with high amount of the interesting HIV-1 active compounds. Since (+)-inophyllum B, in the inophyllum series, is the most promising candidates for anti-HIV-1 drug among *Calophyllum* coumarins [1], it should be necessary to focus investigations on these Tuamotu and Austral archipelago trees, to identify richer chemotypes which could be used as plant source for anti-HIV-1 drugs.

Acknowledgments

Financial support by the French Government and the French Polynesia Government Territory (grant for the study and the development of Tamanu oil) is greatly acknowledged.

REFERENCES

[1] T. Ishikawa, Heterocycles 53 (2000) 453.
[2] P.F. Stevens, J. Arnold Arboretum 61 (1980) 117.
[3] P. Pétard, Les plantes utiles de la Polynésie-Raau Tahiti, Haere pono Tahiti, Papeete, 1986, p. 225.
[4] F. Laure, G. Herbette, R. Faure, J.-P. Bianchini, P. Raharivelomanana, B. Fogliani, Magn. Reson. Chem. 43 (2005) 65.
[5] L. Charles, F. Laure, P. Raharivelomanana, J.-P. Bianchini, J. Mass Spectrom. 40 (2005) 75.
[6] Y. Kashman, K.R. Gustafson, R.W. Fuller, J.H. Cardellina III, J.B. McMahon, M.J. Currens, R.W. Buckeit Jr., S.H. Hughes, G.M. Cragg, M.R. Boyd, J. Med. Chem. 35 (1992) 2735.
[7] Y. Kashman, K.R. Gustafson, R.W. Fuller, J.H. Cardellina II, J.B. McMahon, M.J. Currens, R.W. Buckeit Jr., S.H. Hughes, G.M. Cragg, M.R. Boyd, J. Med. Chem. 36 (1993) 1110.
[8] A.D. Patil, A.J. Freyer, D.S. Eggleston, R.C. Haltiwanger, M.F. Bean, P.B. Taylor, M.J. Caranfa, A.L. Breen, H.R. Bartus, R.K. Johnson, R.P. Hertzberg, J.W. Westley, J. Med. Chem. 36 (1993) 4131.
[9] H.R. Dharmaratne, J.R. Mayuri Sajeevani, G.P. Marasinghe, E.M. Shantha Ekanayake, Phytochemistry 49 (1998) 995.
[10] H.R. Dharmaratne, W.M. Wanigasekera, E. Mata-Greenwood, J.M. Pezzuto, Plant. Med. 64 (1998) 64460.
[11] M. Huerta-Reyes, M.C. Basualdo, F. Abe, M. Jimenez-Estrada, C. Soler, R. Reyes-Chilpa, Biol. Pharm. Bull. 27 (2004) 1471.
[12] T.C. McKee, R.W. Fuller, C.D. Covington, J.H. Cardellina II, R.J. Gulakowski, B.L. Krepps, J.B. McMahon, M.R. Boyd, J. Nat. Prod. 59 (1996) 754.

[13] T.C. McKee, C.D. Covington, R.W. Fuller, H.R. Bokesch, S. Young, J.H. Cardellina II, M.R. Kadushin, D.D. Soejarto, P.F. Stevens, G.M. Cragg, M.R. Boyd, J. Nat. Prod. 61 (1998) 1252.
[14] R. Somanathan, M.U.S. Sultanbawa, J. Chem. Soc. Perkin Trans. 1 (1972) 1935.
[15] K. Kawazu, H. Ohigashi, T. Mitsui, Tetrahedron Lett. 19 (1968) 2383.
[16] K. Kawazu, H. Ohigashi, N. Takahashi, T. Mitsui, Bull. Inst. Chem. Res., Kyoto Univ. 50 (1972) 160.
[17] C. Spino, M. Dodier, S. Sotheeswaran, Bioorg. Med. Chem. Lett. 8 (1998) 3475.