HIV-1 Inhibitory Compounds from *Calophyllum brasiliense* Leaves

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The hexane, acetone and methanol extracts of *Calophyllum brasiliense* leaves were fractionated following a three bioassay guide: high HIV-1 RT inhibition, low cytotoxicity on MT2 cells and high inhibition of HIV-1 IIIb/LAV replication. This led to the isolation of three anti HIV-1 dipyranocoumarins: calanolides A and B and soulattrolide. In contrast, other isolated compounds such as apetalic acid, isoapetalic acid, a structural isomer of isoapetalic acid, friedelin, canophyllol and amentoflavone were devoid of HIV-1 RT inhibitory activity. Calanolide C was also obtained as a natural product and showed moderate inhibitory properties.

Key words *Calophyllum brasiliense*; HIV-1 RT; calanolide

The dipyranocoumarin (+)-calanolide A (**6**) has potent activity against human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT). This compound was first isolated from the tropical rainforest tree *Calophyllum lanigerum* var. *austrocoriaceum* (Clusiaceae) in 1992.[1)] The chemical constituents of *Calophyllum* species have been actively studied and yielded a number of dipyranocoumarins with strong anti HIV-1 RT activity, for instance: (−)-calanolide B (**7**) isolated from *C. lanigerum* var. *austrocoriaceum*;[1)] inophyllums B and P isolated from *C. inophyllum*;[2)] soulattrolide isolated from *C. inophyllum*[3)] and *C. teysmanii*.[4)]

The genus *Calophyllum* consists of 180 species with a pantropical distribution. Species studied for anti HIV activity have been collected mainly in Malaysia and Sri Lanka. In the American Continent, *Calophyllum* is represented by 8 species;[5)] among them, *Calophyllum brasiliense* is the widest distributed species from Brazil to Mexico.[6)] Previous chemical studies of *C. brasiliense* leaves from Brazil have reported hyperin, amentoflavone, quercetin, gallic and protocatechuic acids.[7)] We have previously reported that the organic extracts of *C. brasiliense* leaves collected in Los Tuxtlas, Mexico, showed significant inhibition on HIV-1 RT and HIV replication.[8)] We are now reporting the bioguided isolation of the active compounds of this species.

MATERIALS AND METHODS

Plant Materials and Extracts Preparation *C. brasiliense* leaves were collected at Río Chumiapan, in San Andrés Tuxtla, state of Veracruz, Mexico. A voucher was deposited with the number 14425 at Herbarium of Mexican Institute for Social Security (IMSSM) in Mexico City. Leaves were dried at room temperature and powdered (945.5 g). Extracts were prepared with hexane, acetone and methanol at room temperature. The solvent was concentrated *in vacuo*.

Isolation of the Active Constituents Bioguided chromatography was carried out with hexane, acetone and methanol extracts previously reported with anti HIV-1 properties.[8)] Identification of pure compounds was performed by spectroscopic methods (500 MHz for ^{1}H-NMR and 125 MHz for ^{13}C-NMR) and high resolution MS FAB. Bidimensional spectra and comparison with published data (HMQC and HMBC) were relevant for identification, as well as $[\alpha]_D$.

Hexane Extract: The extract was concentrated in a rotary evaporator (32 g). Two triterpens, friedelin[9)] (**1**, 1.82 g) and canophyllol[9)] (**2**, 1.64 g) precipitated spontaneously during solvent evaporation. Part of the extract (16 g) was fractionated by silica gel (70—230 mesh) column chromatography (CC) eluting with hexane–ethyl acetate (1 : 0 to 0 : 1). A total of 270 fractions were collected and monitored by TLC. Similar fractions were combined to give 27 pooled fractions which were tested for anti HIV-1 RT properties. Active fractions 18 and 19 were eluted with hexane–ethyl acetate (9 : 1). Fraction 18 was subjected to chromatography column (silica gel C-18) using acetone–water (7 : 3 to 1 : 0). Apetalic acid (**3**, 520 mg) was obtained in subfractions 5—6 eluted with acetone–water (7 : 3). The remaining subfractions 7—8 were rechromatographed on silica gel C-18 eluting with acetonitrile–water (9 : 1 to 1 : 0). Final purification was achieved by preparative HPLC (column: Shiseido Silica Gel AG 80 Å, 5 μm, size 10 mm i.d.×250 mm; flow: 3 ml/min) using an isocratic elution (hexane–ethyl acetate 5 : 5) obtaining successively soulattrolide (**9**, 10 mg), (−)-calanolide B (**7**, 90 mg), (+)-calanolide C (**8**, 30 mg) and (+)-calanolide A (**6**, 10 mg). The other active fraction, 19 was chromatographed by CC (silica gel C-18) using acetone–water (7 : 3 to 1 : 0). Fractions 4—9 eluted with acetone–water (7 : 3) were combined and subjected to CC (silica gel C-18) eluting with methanol–water, acidified with acetic acid 1% (8 : 2). The structural isomer of isoapetalic acid (**5**, 20 mg), and isoapetalic acid (**4**, 20 mg) were obtained.

Acetone Extract: After concentrating the extract (46 g), part of it (5 g) was subjected to CC with Diaion (HP20) eluting successively with methanol–water 6 : 4 (fr. 1), methanol (fr. 2) and ethyl acetate (fr. 3). Fractions 1 and 3 were inactive. From fraction 2 (245 mg), amentoflavone[10)] (**10**, 30 mg) precipitated spontaneously. The remaining material from this fraction inhibited HIV-1 RT. The analysis by TLC showed the presence of calanolides (Rf=0.42; acetone–water 8 : 2, deep blue spot; stained with 2% $Ce(SO_4)_2$ in 2 N H_2SO_4, reference (−)-calanolide B).

Methanol Extract: Part of the extract (500 mg) was passed through a polyamide column in order to remove tannins according with Tan *et al*., 1991.[11)] The column was first soaked

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in water overnight. Elution started with water, followed by 50% methanol, and finally, absolute methanol. The eluates were collected, combined and evaporated to dryness. The other part of the methanol extract was macerated with EtOAc obtaining an insoluble fraction. This material was positive for tannins with 1% gelatine–NaCl solution, and 3% ferric chloride–30% sulfuric acid.

Compound **3** (Apetalic Acid): Yellow oil. HR-FAB-MS *m/z*: 389.1963 (Calcd for $C_{22}H_{29}O_6$: 389.1965). FAB-MS *m/z*: 389 ($M^+ + 1$), 373, 329. $[\alpha]_D^{25} -53°$ ($c = 1.5$, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) and ^{13}C-NMR ($CDCl_3$) data were found to be in good agreement with the literature values.[12]

Compound **4** (Isoapetalic Acid): Yellow oil. HR-FAB-MS *m/z*: 389.1963 (Calcd for $C_{22}H_{29}O_6$: 389.1965). FAB-MS *m/z*: 389 ($M^+ + 1$), 373, 329. $[\alpha]_D^{25} -16.1°$ ($c = 1.0$, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) and ^{13}C-NMR ($CDCl_3$) data were found to be in good agreement with the literature values.[13]

Compound **5** (Structural Isomer of Isoapetalic Acid): Yellow oil. ^{1}H-NMR ($CDCl_3$) δ: 0.86 (3H, t, $J = 7.0$ Hz, 24-CH_3), 1.14 (2H, m, 23-CH_2), 1.2 (3H, d, $J = 7.0$ Hz, 16-CH_3), 1.44 (3H, s, 18-CH_3), 1.44 (3H, s, 17-CH_3), 1.49 (3H, d, $J = 6.1$ Hz, 15-CH_3), 1.88 (2H, m, 22-CH_2), 2.54 (1H, dq, $J =$ 11, 7 Hz, 3-H), 2.76 (1H, dd, $J = 15.0$, 7.0 Hz, 20-H), 2.86 (1H, dd, $J = 15.0$, 8.0 Hz, 20′-H), 3.74 (1H, m, 19-H), 4.16 (1H, m, 2-H), 5.45 (1H, d, $J = 10.0$ Hz, 10-H), 6.53 (1H, d, $J = 10.0$ Hz, 11-H), 12.70 (1H, s, 5-OH). ^{13}C-NMR ($CDCl_3$) δ: 10.1 (C-16), 14.0 (C-24), 19.6 (C-15), 20.9 (C-23), 28.2 (C-17), 28.3 (C-18), 30.3 (C-19), 35.3 (C-22), 45.7 (C-3), 78.0 (C-9), 78.8 (C-2), 101.0 (C-12), 101.6 (C-14), 115.8 (C-11), 125.7 (C-10), 155.1 (C-7, C-13), 198.7 (C-4). EI-MS *m/z*: 389 ($M^+ + 1$), 373, 271. $[\alpha]_D^{25} -28°$ ($c = 1.0$, $CHCl_3$).

Compound **6** ((+)-Calanolide A): Yellow oil. HR-FAB-MS *m/z*: 393.1680 (Calcd for $C_{22}H_{26}O_5Na$: 393.1678). FAB-MS *m/z*: 371 ($M^+ + 1$), 353, 299. $[\alpha]_D^{25} +48.2°$ ($c = 0.5$, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) and ^{13}C-NMR ($CDCl_3$) data were found to be in good agreement with the literature values.[1]

Compound **7** ((−)-Calanolide B): White needles, mp 176—179 °C. HR-FAB-MS *m/z*: 393.1680 (Calcd for $C_{22}H_{26}O_5Na$: 393.1678). FAB-MS *m/z*: 371 ($M^+ + 1$), 353, 299. $[\alpha]_D^{25} -38.6°$ ($c = 0.5$, acetone). ^{1}H-NMR ($CDCl_3$) and ^{13}C-NMR ($CDCl_3$) data were found to be in good agreement with the literature values.[1]

Compound **8** ((+)-Calanolide C): Yellow oil. HR-FAB-MS *m/z*: 393.1685 (Calcd for $C_{22}H_{26}O_5Na$: 393.1678). FAB-MS *m/z*: 371 ($M^+ + 1$), 353, 299. $[\alpha]_D^{25} +96.3°$ ($c = 1.0$, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) and ^{13}C-NMR ($CDCl_3$) data were found to be in good agreement with the literature values.[14]

Compound **9** (Soulattrolide): Light yellow needles, mp 177—181 °C. HR-FAB-MS *m/z*: 427.1526 (Calcd for $C_{25}H_{24}O_5Na$: 427.1521). FAB-MS *m/z*: 405 ($M^+ + 1$), 387, 371. $[\alpha]_D^{25} -14°$ ($c = 0.25$, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) and ^{13}C-NMR ($CDCl_3$) data were found to be in good agreement with the literature values.[3]

Biological Assays Antiviral activity of *C. brasiliense* fractions were evaluated by three successive assays. HIV-1 RT inhibition was first screened, and those fractions that showed inhibition over 70% were selected for the next bioassay. Cytotoxic effect of the fractions was examined on human lymphocytes cell lines. Non toxic fractions were selected for the last bioassay, which consisted on determining inhibition of HIV-1 IIIb/LAV replication. Nevirapine, a non nucleoside reverse transcriptase inhibitor (NNRTI) was used as positive control.

HIV-1 RT Inhibition Assay Fractions and pure compounds were evaluated by a non-radioactive immuno and colorimetric assay (Lenti RT Activity Assay, Cavidi Tech).[15] Assay was performed according to the protocol provided by manufacturers. Each fraction was dissolved in DMSO and tested at 50 μg/ml. Concentration tested for pure compounds was 1 mM. IC_{50} values were calculated for most active compounds (over 70%).

Toxic Effect on Human Lymphocytes Cell Line The cytotoxic effect of anti HIV-1 RT active fractions were determined on human lymphocytic MT2 cells. The assay was performed as follows: MT2 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 0.25 μg/ml of streptomycin and 100 μg/ml of penicillin G, in presence of the fractions. Culture was maintained at 37 °C under 5% CO_2 humidified atmosphere. Fractions were dissolved in DMSO and tested at 50 μg/ml. After 48 h, cellular death was assessed by direct microscopic examination of trypan blue stained cells. Results were compared with a control of MT2 free of extract.

Inhibition of HIV-1 IIIb/LAV Replication Inhibition of viral replication by fractions was measured in a coculture of IIIb/LAV-Molt4 cells and non-infected MT2 cells in the presence of the fractions. Concentration of fractions and conditions of culture were the same as those described for the toxicity assay. After 48 h inhibition of viral replication was measured by a p24 antigen enzyme immunoassay (Genetic Systems HIV-1Ag EIA, Bio-Rad) performed in the culture supernatant.

RESULTS

Most of the fractions obtained from the *C. brasiliense* hexane leaves extract showed low HIV-1 RT inhibition, however, fractions 18 and 19 showed high inhibition (over 70%) (Table 1). Both fractions were non toxic to MT2 human lymphocytes (Fig. 1A), and also strongly inhibited IIIb/LAV replication (Fig. 1B). Isolation of the pure compounds from these fractions was performed by chromatography, obtaining compounds **3** to **9** (Fig. 2). All of them were tested, but only compounds **6**, **7** and **9** showed a potent inhibition on HIV-1 RT. IC_{50} were calculated for these compounds, that were

Table 1. Evaluation of HIV-1 Inhibitory Properties of Fractions from *Calophyllum brasiliense* Leaves Extracts

Fraction/extract	HIV-1 RT inhibition (%)
Hexane	
1—17	<40
18	74.8±3.3
19	75.5±3.7
20—27	<40
Acetone	
1	12.7±3.1
2	59.5±3.5
3	28.2±1.7
Methanol	
EtOAc insoluble fraction	74.2±1.6
Extract after polyamide column	38.6±4.9

Values are means±S.D., n=3.

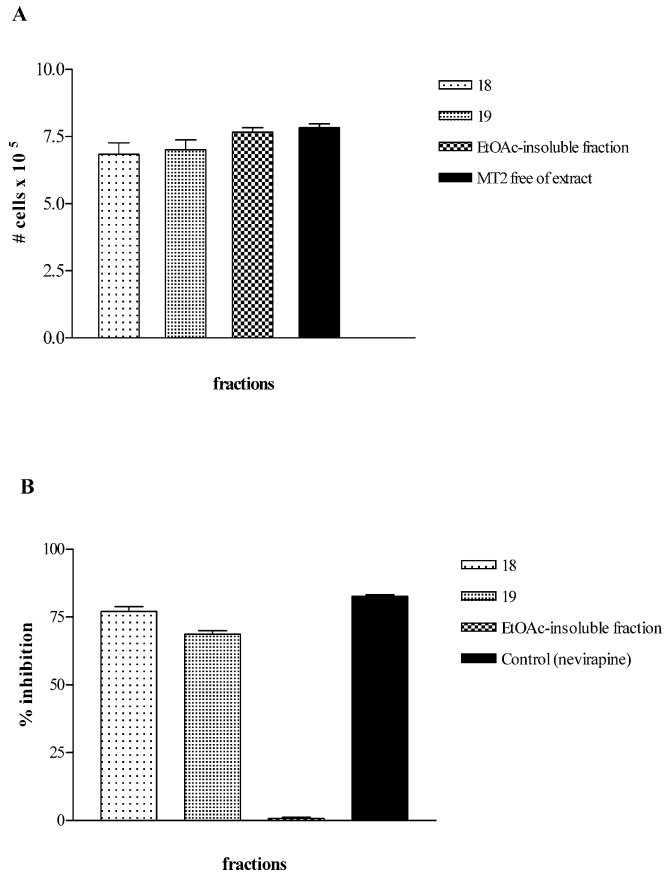

Fig. 1. Toxic and Antiviral Effect of Active Fractions from Hexane and Methanol Extracts from *Calophyllum brasiliense* Leaves

(A) Cell viability of lymphocytes MT2. (B) Inhibition of HIV-1 IIIb/LAV replication.

0.34, 0.5 and 0.66 μM/ml, respectively. Compound **8** was moderately active (50 to 70% inhibition), while compounds **4** and **5** exhibited low inhibition. Compound **3** was inactive (Table 2). Compounds **1** and **2** were also isolated from the hexane extract, but any of them was able to inhibit HIV-1 RT (Table 2).

Regarding to acetone extract, fractions 1, 2 and 3 were evaluated for HIV-1 RT inhibition but only fraction 2 showed moderate activity (Table 1). This fraction analyzed by TLC suggested that contains traces of calanolides. Amentoflavone (**10**) was also obtained from fraction 2 and tested, showing low HIV-1 RT inhibition (Table 2). This compound showed a very strong cytotoxic effect on MT2 cells ($<2.0\times10^5$ cells) when we compared with control free of substance.

The methanol extract showed high HIV-1 RT inhibitory activity (83.3%), however, this value decreased to 38.6% after tannin removal (Table 1). EtOAc insoluble fraction from methanol extract was able to inhibit HIV-1 RT activity and showed low cytotoxicity (Fig. 1A), nevertheless it was not able to inhibit IIIb/LAV replication (Fig. 1B).

DISCUSSION

The chemical analysis indicates that main compounds of *C. brasiliense* hexane extract from the leaves are two triterpenes, friedelin (**1**) and canophyllol (**2**), and three chromanone carboxylic acids, apetalic acid (**3**), isoapetalic acid (**4**) and a structural isomer of isoapetalic acid (**5**). The minority compounds are four dipyranocoumarins, (+)-calanolide A (**6**), (−)-calanolide B (**7**), (+)-calanolide C (**8**) and soulattrolide (**9**).

The apetalic acid (**3**) has been previously isolated as its methyl ester from the bark of *C. brasiliense* seed kernels.[12] Isoapetalic acid (**4**) and its structural isomer (**5**) have been isolated as their methyl esters from the bark of *C. bracteatum*, *C. calaba* var. *calaba*, *C. moonii* and *C. trapezifolium*.[13]

It is interesting to note that the main compounds, triterpenes and chromanone carboxylic acids, found in the hexane extract were inactive or showed a low inhibition on HIV-1 RT (Table 2). On the other hand, the minority compounds (+)-calanolide A (**6**), (−)-calanolide B (**7**) and soulattrolide (**9**) were identified as the active compounds against HIV-1 RT. These compounds have been previously isolated from the fruit, twigs, bark and latex of different *Calophyllum* Asiatic species.[1,3,4] Recently, (+)-calanolide A (**6**) and (−)-calanolide B (**7**) were isolated from the stem bark of *C. brasiliense* from Brazil,[16] however, this is the first time that these dipyranocoumarins are found in the leaves. Both calanolides are considered as non nucleoside reverse transcriptase inhibitors (NNRTI) that can inhibit primarily HIV-1 RT, but fail to inhibit HIV-2 RT. They interact allosterically with the RT, altering its structural conformation, resulting in a partial or total disruption of the RT catalityc complex, template-primer, and the substrate.[17] Specifically, (+)-calanolide A inhibits HIV-1 RT by binding two sites, one competitive and other noncompetitive.[18] It is considered as a unique inhibitor among NNRTIs,[19] since it is able to inhibit the AZT resistant variant which bears two mutations at codon 181, tyrosine →cysteine (Y181C), and codon 131, leucine→asparagine (L103N).[20] Most NNRTIs are not able to inhibit the variant bearing the Y181C amino acid mutation.[21] The importance of ring D (2,3-dimethylcroman-4-ol) for HIV-1 inhibitory properties of calanolides A and B, inophyllums and cordatolides has been thoroughly demonstrated.[22,23] Highest inhibitory activity of HIV-1 RT has been observed for calanolide A that posses *trans* configuration between methyl groups at C-10 and C-11, while other configurations led to a reduction in potency. An oxygenated function at C-12, and substituents at C-4 are also relevant for HIV-1 RT inhibitory activity following the series propyl>phenyl>methyl.[24]

Regarding compound **8**, Kashman *et al*., 1992[1] reported the isolation of (+)-calanolide C from the fruit and twigs of *C. lanigerum*. However, the proposed structure was reviewed and corrected by McKee *et al*., 1995 after obtaining the synthetic product.[14] The original assignments for (+)-calanolide C proposed by Kashman *et al*., 1992[1] are actually those of pseudocalanolide C.[14] The NMR chemical shifts observed for the compound **8** here isolated, led to its identification as (+)-calanolide C according to data reported by McKee *et al*., 1995.[14] Diagnostic signals were 6.62 for H-8 and 5.07 for H-12 as well as 116.6 for C-8, 106.5 for C-8a, 62.9 for C-12 and 105.8 for 12a. To our best knowledge, this is the first time that (+)-calanolide C (**8**) is obtained as a natural product, and evaluated against HIV-1 RT. Although compound **8** exhibits the pharmacophoric ring D, as well as a propyl group on C-4, it did not show potent inhibition on HIV-1 RT. This could be due to the β-*cis* orientation of methyl groups on C-10 and C-11 (Fig. 2). Therefore our results support

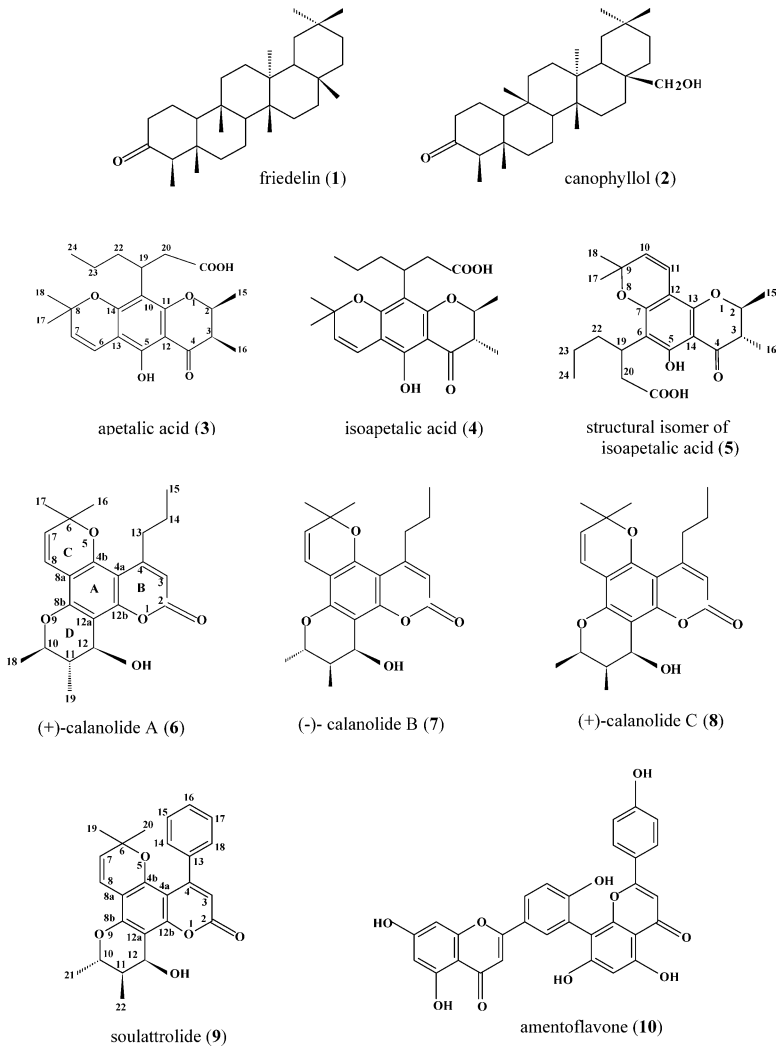

Fig. 2. Isolated Compounds from *Calophyllum brasiliense* Leaves

Table 2. Evaluation of Pure Compounds Isolated from *Calophyllum brasiliense* Leaves against HIV-1 RT

Compounds	HIV-1 RT inhibition (%)
1	20.2±1.6
2	7.6±2.1
3	2.0±0.6
4	20.6±3.2
5	29.7±1.7
6	81.5±0.9
7	76.2±2.2
8	50.7±2.0
9	77.7±1.6
10	17.5±2.4
Control (nevirapine)	82.6±3.1

Values are means±S.D., n=3.

the structure–activity relationships (SAR) of *Calophyllum* dipyranocoumarins proposed to date.[24,25]

Concerning to the acetone extract, we previously reported that this extract had an inhibitory effect on HIV-1 RT, but it was also cytotoxic to MT2 human lymphocytes.[8] The former activity could be probably due to traces of calanolides. Meanwhile, amentoflavone (**10**) which was devoid of inhibitory activity on HIV-1 RT, was cytotoxic to MT2 cells and therefore could be responsible of the overall acetone extract toxicity.

It is known that polar extracts may contain tannins and others like polyphenol compounds able to inhibit HIV-1 RT,[11,26] DNA polymerase,[27] DNA topoisomerase I and II,[28] and the binding of HIV-1 gp120/CD4 T-cell receptor.[11,29] Since tannins show high affinity for proteins and affect a number of biochemical reactions, they are generally considered as unselective inhibitors.[30] In our case, the methanol extract of *C. brasiliense* leaves showed HIV-1 RT inhibition[8] that could be attributed to tannins.

Our data indicate that anti-HIV properties of *C. brasiliense* leaves extracts are due to the presence of (+)-calanolide A (**6**), (+)-calanolide B (**7**), and soulattrolide (**9**). Interestingly, dipyranocoumarins were not detected in *C. brasiliense* in a previous study performed by other authors, using a TLC screening.[31] This could arise from the existence of chemotypes in the species. In Mexico, we have found two populations of *C. brasiliense* that differ in the chemistry of their leaves. One chemotype with chromanones and calanolides was object of this paper, while second chemotype that we have recently reported, contains mammea type coumarins such as mammea A/BA, A/BB, isomammeigin, mammea B/BA, B/BB, C/OA, C/OB, B/BA cyclo F and B/BB cyclo F,

all of them inactive against HIV-1 RT.[32)]

CONCLUSION

The chemical analysis of the extracts of *Calophyllum brasiliense* leaves collected in San Andres Tuxtla, state of Veracruz, showed that the minority compounds dipyranocoumarins, (+)-calanolide A, (−)-calanolide B, and soulattrolide are the responsible of its anti-HIV properties. On the other hand, the majority compounds, the chromanone carboxylic acids, triterpens and biflavonoid were inactive against HIV-1 RT.

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