Antivascular and anti-parasite activities of natural and hemisynthetic flavonoids from New Caledonian Gardenia species (Rubiaceae)

ARTICLE *in* EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY · MARCH 2015

Impact Factor: 3.45 · DOI: 10.1016/j.ejmech.2015.01.012

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European Journal of Medicinal Chemistry 93 (2015) 93–100

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Original article

Antivascular and anti-parasite activities of natural and hemisynthetic flavonoids from New Caledonian *Gardenia* species (Rubiaceae)

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ARTICLE INFO

Article history:
Received 15 September 2014
Received in revised form
6 January 2015
Accepted 7 January 2015
Available online 8 January 2015

Dedicated to the memory of Prof. François Tillequin.

Keywords:
Flavonoids
Antivascular agents
Endothelial cells
Antiplasmodial
Trypanocidal
Antileishmanial

ABSTRACT

A series of 16 flavonoids were isolated and prepared from bud exudate of *Gardenia urvillei* and *Gardenia oudiepe*, endemic to New Caledonia. Most of them are rare polymethoxylated flavones. Some of these compounds showed noticeable activity against *Leishmania (Leishmania) amazonensis*, *Plasmodium falciparum* and *Trypanosoma brucei gambiense*, in addition to tubulin polymerization inhibition at low micromolar concentration. We also provide a full set of NMR data as some of the flavones were incompletely described.

1. Introduction

Microtubules are composed of α- and β-tubulin heterodimers which are in constant dynamic interaction. They are key components of the cytoskeleton and are deeply involved in cell division and mitosis. Over the last two decades, several anticancer agents were developed targeting the tubulin polymerization of endothelial cells of neovessels formed during the tumoral process [1]. They can act as antiangiogenics by preventing the formation of tumor vasculature, or as vascular disrupting agents, such as combretastatin A4 and analogs, able to compromise selectively established tumor vasculature [2]. In this case, exploitation of the differences between normal and immature tumor blood vessels leads to hemorrhagic necrosis within the tumors.

The protozoal cytoskeleton is also deeply involved in the successive steps of the life cycle of parasite species [3–5]. The high mortality and morbidity rate caused by malaria, sleeping sickness and leishmaniasis in developing countries makes the fight against these parasitic infections a priority in public health policy.

Some natural flavonoids have previously been shown to possess antiangiogenic, antivascular, anti-proliferative, anti-plasmodial, trypanocidal and leishmanicidal properties [6–16]. In this context, the aim of this study was to further explore some of these biological activities of flavone derivatives exhibiting rare substitution

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http://dx.doi.org/10.1016/j.ejmech.2015.01.012
0223-5234/

patterns on A ring, isolated from the exudate of *Gardenia urvillei* and *Gardenia oudiepe* (Rubiaceae), both endemic from New Caledonia and considered as non-endangered species. Flavonoids were extracted from concentrated plant exudate using a minimum of organic solvent. This extraction methodology is efficient and respectful to both the phytoresource and the environment. Methylated and acetylated derivatives were then semi-synthetized in order to establish structure–activity relationships within this series of compounds.

2. Results and discussion

2.1. Preparation of the flavonoids library

The exudate covering the flowering buds and leaf bases of *G. urvillei* or *G. oudiepe* (Rubiaceae) was dissolved in a minimum volume of dichloromethane. The solution obtained was filtered to remove all small plant pieces and evaporated to dryness under reduced pressure. The use of exudate is highly appreciated for studies working with endemic species because the material collection does not cause damage to the tree. Moreover, vegetal extraction is not required because the exudate, sometimes referred to as 'gum' or 'resin', is a concentrate of secondary metabolites [17–19] that allows us to save extraction time and to avoid the use of large volumes of organic solvents.

Chromatographic separations on silica gel permitted the isolation of the following flavonoids: 5,7-dihydroxy-3,3′,4′,6-tetramethoxyflavone (**1**) and 3′,5,7-trihydroxy-3,4′,5′,6-tetramethoxyflavone (**2**) from *G. oudiepe*; 5,7-dihydroxy-3,3′,4′,5′,6-pentamethoxyflavone (**3**), 4′,5,7-trihydroxy-3,6,8-trimethoxyflavone (**6**), 4′,5,7-trihydroxy-3,6-dimethoxyflavone (**7**), santin (5,7-dihydroxy-3,4′,6-trimethoxyflavone) (**8**), and 3-methoxy-kaempferol (kaempferol-3-monomethylether) (**12**), from *G. urvillei* (Scheme 1). Some of these natural products were rarely mentioned in the literature, sometimes without or with incomplete NMR data (See Supporting Information). Moreover, noticeable anti-tubulin and anti-parasitic activities were reported for closely related flavones [16].

This led us to proceed, in a second step, to methylation and acetylation in order to perform a screening of bioactivities on a library of 3-methoxykaempferol derivatives which were never evaluated together on such targets. Thus, compounds **3**, **8** and **12** were subsequently methylated (Scheme 2). **4** and **5** were generated from **3**, **9** and **10** from **8**, and **13** and **14** from **12**. Two acetylated derivatives **15** and **16** were also semi-synthetized from commercial kaempferol (**11**) (Scheme 3).

We then submitted this library of polymethoxylated and polyacetylated flavonoids (cf. Schemes 1–3) to various *in vitro* assays aimed at detecting cytotoxic, antivascular, anti-tubulin and anti-parasitic activities, as described hereafter.

2.2. Biological evaluation

2.2.1. Cytotoxic activity on L6 and B16 cell lines

The flavonoids were initially tested for their cytotoxicity against the L6 rat skeletal myoblast cell line and the B16 murine melanoma cell line. The results presented in Table 1 show that most compounds were relatively non-cytotoxic to L6 cells with 13 out of the 16 compounds showing less than 50% of inhibition of cell proliferation (IC_{50}) at a concentration of 10 μM.

For the B16 murine melanoma cell line, most compounds also demonstrated relatively high IC_{50} values with the exception of **4** (IC_{50} = 11.5 μM). There was no apparent correlation between the two cell lines in terms of cytotoxicity.

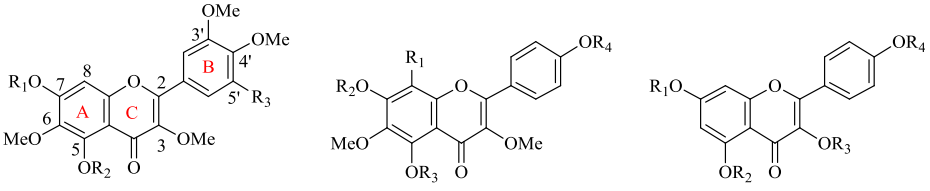
2.2.2. Morphological activity on EA.hy926 endothelial cells

The structural stilbene similarity of methoxylated flavonoids with the antivascular agent combretastatin A4 (Fig. 1) makes them promising candidates as potential inhibitors of tumor blood vessels. We therefore tested our library of compounds on endothelial cells using immortalized HUVEC (EA.hy926), and evaluated their rounding-up effect, which is considered predictive of a potential *in vivo* antivascular activity [20,21].

Four compounds (**1**, **2**, **3** and **8**) presented low micromolar rounding up activity on EA.hy926 endothelial cells (Table 1), ranging from 1.56 to 25 μM. Fig. 2 depicts the typical rounding up activity of compound **2** on EA.hy926 endothelial cells after a 2 h exposure time. As stated above, this alteration in endothelial cell morphology is an indicative feature of potential antivascular activity. Despite several publications concerning the antiangiogenic and antivascular properties of flavonoids and their modified analogs [7,8,22–25], this study is, to the best of our knowledge, the first to explore the 3-methoxy-flavones bearing quite rare substitution patterns on A ring, i.e. 5-OH, 6-OMe, 7-OH, 8-H, along with their methylated derivatives in the last 15 years [26–28].

2.2.3. Inhibition of tubulin polymerization (ITP)

Some flavones were reported to inhibit tubulin polymerization [26–28]. This prompted us to test our library of compounds for this activity. We initially performed a screening test at the concentration of 30 μM to compounds **1**, **2**, **3** and **8**, previously selected for their morphological effects on EA.hy926 cells. All samples exhibited potential inhibitory activity at this concentration (Table 1). The IC_{50} values of these compounds were then determined in the low micromolar concentration range and therefore presented significant inhibitory activity on the tubulin polymerization. Santin (**8**) showed the most promising profile (5.7 μM), while **1**, **2** and **3** exhibited a similar activity with IC_{50} of 19.3, 17.8 and 21.4 μM,

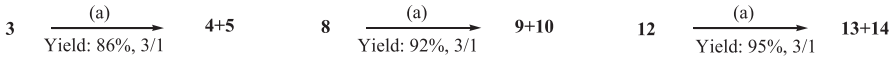


1: R1=R2=R3=H
2: R1=R2=H; R3=OH
3: R1=R2=H; R3=OMe
4: R1=Me; R2=H; R3=OMe
5: R1=R2=Me; R3=OMe

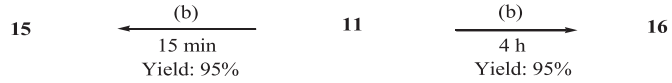
6: R1=OMe; R2=R3=H; R4=H
7: R1=R2=R3=R4=H
8: R1=R2=R3=H; R4=Me
9: R1=H; R2=Me; R3=H; R4=Me
10: R1=H; R2=R3=R4=Me

11: R1=R2=R3=R4=H
12: R1=R2=H; R3=Me; R4=H
13: R1=Me; R2=H; R3=Me; R4=H
14: R1=Me; R2=H; R3=R4=Me
15: R1=Ac; R2=H; R3=R4=Ac
16: R1=R2=R3=R4=Ac

Scheme 1. Flavonoids from *G. urvillei* and *G. oudiepe* and modified analogues.



Scheme 2. Methylation of natural compounds **3**, **8** and **12**. (a) Reagents and conditions: Me_2SO_4 4 equiv., DBU 2 equiv. in dried acetone, room temperature, 1 h. Neutralization with HCl 10% to pH7, precipitation with ice water.



Scheme 3. Acetylation of kaempferol (**11**). (b) Reagents and conditions: Ac_2O 4 equiv. in dried pyridine, room temperature, 15 min or 4 h, precipitation with ice water.

respectively.

2.2.4. Structure – activity ITP and morphological effect relationships

Following the determination of inhibition of tubulin polymerization (ITP) and morphological effects induced by several compounds, structural requirements could be established. The good activity of 5,7-dihydroxy-3,3′,4′,6-tetramethoxyflavone (**1**) and santin (**8**) was also presented by centaureidin (3′,5,7-trihydroxy-3,4′,6-trimethoxy-flavone) [26–28] the only 3-methoxy-flavone bearing this kind of substitution on the A ring evaluated for ITP. The structural differences are in 3′ on the B ring: the lack of a hydroxyl group for **1** and a methoxyl group instead of a hydroxyl one for **8**. This underlines the importance of a 4′-methoxyl substitution on the B ring and is supported by the fact that **7**, which bears a phenol on B ring at the 4′ position, did not show any rounding-up activity. Similarly, product with phenol on 4′ and methoxyl group on 3′ was shown a weak inhibitor of tubulin polymerization [28]. The substitution pattern presented by **8**, with only one methoxyl group on 4′ was shown to express the strongest biological effect in our study. Further substitution by additional methoxyl and/or hydroxyl group as in the case of **1**, **2** and **3** led to a 3-fold less active metabolite in the ITP assay. These structure–activity relationships are illustrated in Fig. 3.

The ITP observed for **8** and **1** may also be considered in comparison with the results published for calycopterin-4′-O-methyl ether [29] and for 5-hydroxy-3,3′,4′,6,7,8-hexamethoxyflavone [28], respectively. Surprisingly, these last two compounds were not active, despite bearing the same substitution pattern on the A ring, similarly to the very strong inhibitors of tubulin polymerization, e.g., 5,3′-dihydroxy-3,6,7,8,4′-pentamethoxy-flavone [28,30] and 3′-amino-5-hydroxy-3,6,7,8,4′-pentamethoxyflavone [29]. In this case, for a same B ring, active compounds were those with a santin-type A ring. This statement attracted our attention and let us hypothesize that the mode and/or site of action is not the same as for 3-methoxy-flavones with 5-hydroxy-6,7,8-trimethoxyl substitution. Indeed, even if flavonoids seem to bind closely to the colchicine binding site [31], the exact position remains to be determined, data furnished by the literature are sometimes controversial for related compounds [32–34].

Natural product **6** did not show any rounding up activity. However, this could arise from the presence on the B ring of a single phenol only on 4′. This kind of substitution appears to be smaller than the minimum hindrance required, at least a methoxyl group on 4′, as in the case of **7** compared to compound **8,** as described above.

As a kind of illustration, the analogs of **6** with a 3′,4′-dihydroxy or 4′-hydroxy-3′-methoxy [28] showed no ITP activity or cytotoxicity. Nevertheless, this ability to inhibit tubulin polymerization has already been observed in the case of flavones with a 5,7-dihydroxy-6,8-dimethoxyl pattern on the A ring and a 3′-hydroxy-4′-methoxy or 3′-amino-4′-methoxy substituted B ring [29,35]. A too bulky substitution on the B ring seems to lead to a slight decrease of ITP as observed in the case of flavones isolated from *G. urvillei* and *G. oudiepe*, exemplified by a 3′-hydroxy-4′,5′-dimethoxyl **2** or 3′,4′,5′-trimethoxyl substitution **3** [28].

Interestingly, the role of the methoxyl group on 4′ in this flavone series seems similar of the one played by the 4′ methoxyl group of the B ring in combretastatin series and like in this series the ITP activity is well correlated with the morphologic effect on EA.hy926 cells [20,21].

Furthermore, the role of a methoxyl group on position 3 is also predominant as illustrated by the lack of activity of compounds exactly corresponding to **1** and **8** but bearing a single proton on 3. These compounds are inactive (compounds **45** and **73** of ref. [30]).

Generally, the methylated semi-synthetic derivatives on position 7 (compounds **4**, **9** and **13**) and on position 7 and 5 (compounds **5**, **10** and **14**) were all less active on EA.hy926 cells than the natural products used as starting material, and were therefore not evaluated for ITP.

2.2.5. Anti-parasitic activity

Noticeable anti-parasitic activity has been reported for flavones and related secondary metabolites such as flavanones, chalcones or catechins [22,24] and for semisynthetic analogs [28]. Nevertheless, with the exception of santin (**8**) [12], 5,7-dihydroxy-3,6-dimethoxyflavones have never been screened against this type of target.

This led us to perform an evaluation of natural and semi-synthetic flavones against the promastigote form of *Leishmania* (L.) *amazonensis*, the FcB1-Colombia strain of *Plasmodium falciparum* and *Trypanosoma brucei gambiense*.

2.2.5.1. Leishmania (L.) amazonensis. The leishmanicidal activity results in Table 2 report inhibitory activity for some of the flavones: natural products **1**, **2**, **3** and **7** were active with an IC_{50} value lower than 10 μM, while **9**, **12** and **15** presented an IC_{50} lower than 15 μM. Moreover, some structure–activity relationships emerge from this screening. The flavonoids isolated from both exudates all exhibit inhibitory activity, with the exception of **6** and **8**. Products **6**, **7**, **12** and **13** bear a single phenol on the B ring, a methoxyle group at position 3, and a phenol on position 5. Compound **7**, which also possesses an additional phenol at position 7 and a methoxyle at position 6 is the most potent within this series. The lack of the methoxyle group at position 6, together with an additional methoxyle on position 8, leads to a 3–4 fold decrease in the activity, as observed for flavones **12** and **6** respectively. These data may also be compared to those published for hispidulin [12], which differs from **7** by the presence of a proton at position 3, and was found to be very active against *Leishmania mexicana*. Santin (**8**) demonstrated a moderate activity against *Leishmania* (L.) *amazonensis*, as against *L. mexicana* [12], confirming that a single methoxyle group at position 4′ is detrimental to leishmanicidal activity. However, natural compounds **1**, **2** and **3**, with a bulkier B ring, were found to possess the same anti-leishmanial properties as **7**.

In the case of flavones trimethoxylated at 3′,4′,5′, further methoxylation at position 7 and 5 led to less active derivatives, while methoxylation of santine at position 7 increased the anti-leishmanial effect.

Table 1
Flavonoids **1**-**16**: cytotoxicity against L6 and B16 cell lines, rounding-up activity on endothelial cells (EA.hy926), and inhibition of tubulin polymerization (ITP).

Compound	Cytotoxicity			EA.hy926[c] cells rounding-up (μM)	ITP 30 μM[d]	ITP IC_{50}[e] μM)
	L6 cells[a]		B16 cells[b] IC_{50} μM			
	10 μM % viable cells	1 μM % viable cells				
1	94.7	99.7	28.9	25.0	75.1	19.3
2	44.2	97.6	33.9	1.6	99.0	17.8
3	78.2	96.1	91.2	12.5	69.9	21.4
4	53.5	99.2	11.5	>100	–	–
5	81.2	93.8	>100	>100	–	–
6	80.4	88.4	>100	>100	–	–
7	73.5	86.2	74.1	>100	–	–
8	40.8	90.9	45.7	12.5	100.0	5.7
9	96.5	85.3	>100	>100	–	–
10	93.6	88.3	>100	50–100	–	–
11	100.0	93.8	53.7	>100	–	–
12	61.0	85.0	75.8	>100	–	–
13	38.7	81.3	57.5	50.0	–	–
14	100.0	99.1	>100	100.0	–	–
15	94.8	90.2	39.8	>100	–	–
16	99.7	91.3	40.7	>100	–	–
Combrestatin A4	–	–	–	0.27	–	–
Colchicine	–	–	–	–	–	0.36

[a] Percentage of viable L6 cells at 10 μM (rat skeletal myoblast cell line).
[b] Concentration required to cause 50% cytotoxicity to murine B16 melanoma cells (IC_{50} μM).
[c] Concentration (μM) required for the endothelial cells to adopt rounding shape within 2 h.
[d] Percentage of inhibition of tubulin polymerization (ITP) at 30 μM.
[e] Concentration required to cause 50% inhibition (IC_{50} μM) of tubulin polymerization.

For kaempferol (**11**), the moderate activity is in agreement with previous reports on *Leishmania donovani* [36], *Leishmania peruviana* and *Leishmania braziliensis* [37]. Regarding its methylated and acetylated derivatives, 3,7-kaempferoldimethylether (**12**) and tri-

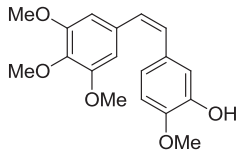


Fig. 1. Combretastatin A4.

2.2.5.2. P. falciparum and T. b. gambiense. Some of the flavones demonstrated noticeable activity against *Plasmodium* and *Trypanosoma*. The screening for anti-plasmodial activity against the FcB1-Colombia strain of *P. falciparum* resulted in detectable inhibition at 10 μM for compounds **1**, **3**, **4** and **5**. They possess a 3′,4′-dimethoxy or a 3′,4′,5′-trimethoxy substitution on the B ring. For these latter, on the A ring, the activity increased with the degree of methoxylation.

After this first step of selection, the most active derivative **5**, semi-synthesized from **3**, was found to be active against *P. falciparum* with an IC_{50} of 5.8 μM and an IC_{90} of 10.8 μM by using the [^{3}H]-hypoxanthine assay. These data firmly support those pub-

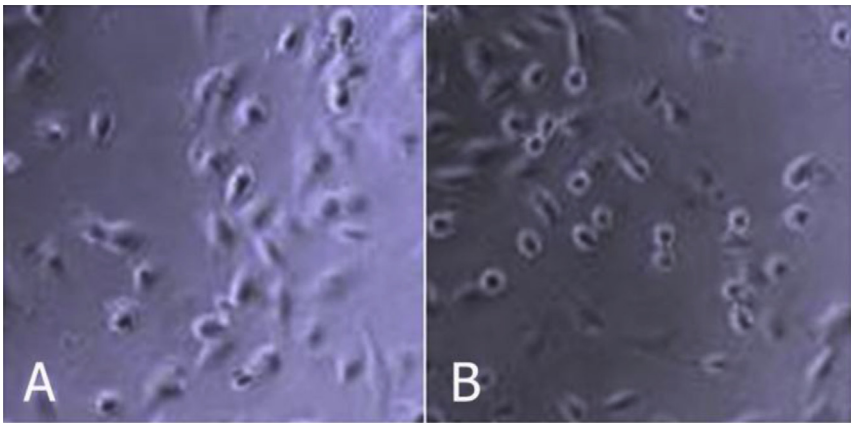


Fig. 2. A) Control endothelial cells EA.hy926 incubated with 1% DMSO (solvent), B) Endothelial cells exposed to compound **2** at 6.25 μM for 2 h.

acetylkaempferol (**15**) are the most promising compounds, while the high IC_{50} of **16** agrees with former reports whereby the latter did not show any strong effect against *L. peruviana* and *L. braziliensis* [37].

lished for 3′,4′,5′,5,6,7-hexamethoxyflavone isolated from *Ageratum conyzoides* [11], which bears an hydrogen on position 3. However, this natural product exhibited cytotoxicity against L6 rat skeletal myoblast cell line, while **5** was non-toxic when tested

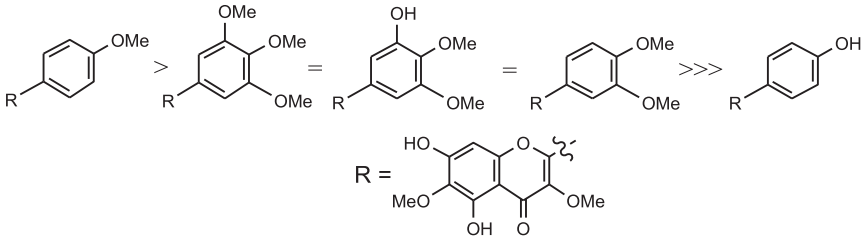


Fig. 3. Comparison of the substitutions needed for activity in the inhibition of tubulin polymerization assay on the B ring for the 5,7-dihydroxy-3,6-methoxy-flavone derivatives.

against the L6 and B16 melanoma cell lines. This leads to the conclusion that the 3-methoxyl substitution does not change the antiplasmodial activity, but affords improved target selectivity which could permit to avoid detrimental side effects.

When screened against *T. b. gambiense*, good inhibition levels were observed at 50 μM for **1**, **2**, **7**, **8**, **11** and **12**. All of the aforementioned compounds are naturally occurring, have relatively low degree of methylation and, with the exception of **1** and **2**, share a B ring substituted only at 4′. At 10 μM, only **1** continued to demonstrate a strong effect against parasite growth. The IC_{50} of **1** was determined as 3.7 μM.

3. Conclusion

Our work once again demonstrates the importance of natural products as a source of leader compounds for antiangiogenesis or vascular disruption [28,29,34,35]. It is noteworthy that this series of naturally occurring compounds should be regarded as a model for future development and as valuable starting materials for pharmacomodulation. These promising data are also enhanced by the fact that the most active flavonoids were found to be specific for different targets, without any acute cytotoxicity on mammalian cells (B16 and L6 cells). Moreover, santin (**8**), the strongest inhibitor of tubulin polymerization from our library, was also found nontoxic at high concentrations on human peripheral mononuclear blood cells [38] and on lymphoid mice cells [12] and exhibited strong cytotoxicity against several cancer cell lines [38], while 5,7-dihydroxy-3,3′,4′,5′,6-pentamethoxyflavone (**3**) showed a high selectivity index when comparing its high rounding-up activity with its very low cytotoxicity on B16 and L6 cells.

Regarding the anti-parasite evaluation, the antileishmanial activity of some derivatives is noticeable and may also constitute promising alternatives for a highly incapacitating disease currently treated by drugs with serious side effects.

As some compounds were incompletely described, we also provide a complete set of ^{1}H and ^{13}C NMR data (supporting information).

4. Experimental section

4.1. Plant material

Bud exudates of *G. urvillei* were collected by two of us (V.D. and C.P.) in August 2007 in the dry forest at the surroundings Noumea, South Province of New Caledonia, and bud exudates of *G. oudiepe* were collected in October 2008 in Forêt Plate, North Province of New Caledonia. Voucher specimens (POU-0143 and POU-0290, respectively) were deposited at the Herbarium of the Botanical and Tropical Ecology Department of the IRD Center, Noumea, New Caledonia.

4.2. Chemistry

4.2.1. Isolation of flavones *1-3, 6-8, 12*

30.0 g of the exudate covering the flowering buds and leaf bases of *G. urvillei* were dissolved in 200 mL of dichloromethane. The solution was filtered under suction through a Büchner funnel and evaporated to dryness under reduced pressure to furnish 27.0 g of exudate free from buds and leaf pieces.

400.0 g of flowering buds and leaf bases of *G. oudiepe* covered with exudate was dissolved in 1L of dichloromethane. The solution was filtered under suction through a Buchner funnel and evaporated to dryness under reduced pressure to furnish 102.0 g of exudate free from buds and leaf pieces.

Repeated chromatographic separations on silica gel column using cyclohexane-dichloromethane and dichloromethane-methanol mixtures of increasing polarity as eluent permitted the isolation of 5,7-dihydroxy-3,3′,4′,6-tetramethoxyflavone (**1**) (210 mg) and 3′,5,7-trihydroxy-3,4′,5′,6-tetramethoxyflavone (**2**) (12 mg) from *G. oudiepe* exudate; 5,7-dihydroxy-3,3′,4′,5′,6-pentamethoxyflavone (**3**) (170 mg), 4′,5,7-trihydroxy-3,6,8-trimethoxyflavone (**6**) (10 mg), 4′,5,7-trihydroxy-3,6-dimethoxyflavone (**7**) (150 mg), santin (5,7-dihydroxy-3,4′,6-trimethoxyflavone) (**8**) (320 mg), and 3-methoxy-kaempferol (isokaempferide) (**12**) (155 mg), from *G. urvillei* exudate.

4.2.2. Semi-synthesis of flavones *4, 5, 9, 10, 13, 14*

The methylation reactions were performed in one step by stirring the substrate with dimethyl sulfate (Me_2SO_4, 4 equiv.) and 1,8-diazabicyclo[5.4.0]undec-7-en (DBU, 2 equiv.) in dried acetone at room temperature for 1 h. The crudes were precipitated and washed with iced water. The resulting residue were solubilized with ethyl acetate (15 mL) and treated with a solution of 1N HCl (3 mL). The final products were extracted with ethyl acetate (3 × 10 mL); the organic phases were washed with a saturated solution of NaCl and dried over Na_2SO_4. After filtration and solvent evaporation, methylated compounds were purified by chromatography in a silica gel column, using a mixture of CH_2Cl_2/CH_3OH (95/5, v/v) as eluent.

Methylation of 125 mg (0.39 mmol) of 5,7-dihydroxy-3,3′,4′,5′,6-pentamethoxyflavone (**3**) gave **4** and **5** with a 3/1 ratio and an overall yield of 86%.

Methylation of 150 mg (0.44 mmol) of santin (**8**) gave **9** and **10** with a 3/1 ratio and an overall yield of 92%.

Methylation of 120 mg (0.40 mmol) of 3-methoxykaempferol (**12**) gave **13** and **14** with a 3/1 ratio and an overall yield of 95%.

4.2.3. Semi-synthesis of flavone *15*

80 mg (0.28 mmol) of commercial kaempferol (**11**) were acetylated by stirring with acetic anhydride in dry pyridine at room temperature for 15 min. The crudes were precipitated and washed in iced water. The resulting was recrystallized in CH_2Cl_2/CH_3OH (9/1, v/v) to furnish 108 mg (0.26 mmol) of triacetyl derivative **15** (yield: 95%).

Table 2
Activity of compounds 1-16 against *L. (L.) amazonensis*, *P. falciparum*, and *T. brucei gambiense*.[a]

Compound	*L. (L.) amazonensis*	*L. (L.) amazonensis*	*P. falciparum* FcB1	*T. b. gambiense*	
	IC_{50} (μg/mL)	IC_{50} (μM)	At 10 μM[b]	At 50 μM[b]	At 10 μM[b]
1	3.05	8.15	21.3	99.8	76.2
2	3.99	10.23	0.0	82.6	37.8
3	3.9	9.65	13.2	38.1	42.9
4	9.1	21.77	21.7	40.5	17.6
5	13.66	31.61	35.0	72.4	25.9
6	9.84	27.33	0.6	50.7	14.6
7	2.67	8.07	0.0	88.8	40.7
8	21.73	63.15	0.0	83.1	33.2
9	5.3	14.80	0.0	8.0	18.3
10	13.83	37.17	0.0	24.9	29.4
11	8.27	27.56	0.0	86.5	55.1
12	4.49	14.29	2.9	93.4	36.6
13	7.53	22.95	7.2	26.4	25.2
14	13.6	33.00	3.9	19.9	4.6
15	5.57	12.27	7.6	5.6	0.0
16	14.56	48.52	3.6	13.7	0.0
Amphotericin B	0.067	0.0725	–	–	–
Chloroquine (IC_{50} nM)	–	–	0.110	–	–
Pentamidine (IC_{50} μM)	–	–	–	–	0.007

[a] Each sample was tested in three assays.
[b] Percentage of parasite growth inhibition.

4.2.4. Semi-synthesis of flavone **16**

80 mg (0.28 mmol) of commercial kaempferol (**11**) were acetylated by stirring with acetic anhydride in anhydrous pyridine at room temperature for 4 h. The crudes were precipitated and washed in ice water. The resulting was recrystallized in CH_2Cl_2/CH_3OH (9/1, v/v) to furnish 120 mg (0.26 mmol) of tetraacetyl derivative **16** (yield: 95%).

4.3. Cytotoxicity evaluation on L6 cells

The cytotoxicity analysis in the rat skeletal myoblast cell line (L6 cells) was conducted in 96-well microtiter plates, each well receiving 100 μL of culture medium with approximately 4×10^4 cells. After 24 h, the medium was removed from all wells, and serial drug dilutions prepared at 10 μM and 1 μM. Each compound at each concentration was tested in triplicate. After 72 h of incubation, plates were inspected under an inverted microscope to ensure growth of the controls and sterile conditions, and then 10 μL of MTT added to each well for 2 h. After formazan crystal formation, the culture medium supernatant was removed from the wells by aspiration without disturbing the formazan precipitate. 100 μL of detergent reagents were then added to each well, and plates left at room temperature in the dark for 2 h. Absorbance was measured at 570 nm in order to determine the cell proliferation rate.

4.4. Cytotoxicity evaluation on B16 cells

Murine B16 melanoma cells were grown in DMEM medium containing 2 mM L-glutamine, 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin (37 °C, 5% CO_2). All compounds were initially dissolved in DMSO at a stock concentration of 2.5 mg/mL and were further diluted in cell culture medium. Exponentially growing cells were plated onto 96-well plates at 5000 cells per well in 100 μL of culture medium. 24 h after plating, 100 μL of medium containing the compound at final concentrations ranging from 0.01 to 100 μM were added to the wells containing the cells (in triplicate), and incubated for 48 h at 37 °C with 5% CO_2. After the 48 h exposure period to the test compounds, cell viability was assayed using the MTT test and absorbance read at 562 nm in a microplate reader (BioKinetics Reader, EL340). Appropriate controls with DMEM only and MTT were run to subtract background absorbance. The compound concentration that inhibited cell viability by 50% (inhibitory concentration for 50% of cells, or IC_{50}) was determined using the GraphPad Prism software.

4.5. Endothelial cell morphology assay

To assess the effects of the compounds on the morphology of endothelial cells, we used the EA.hy926 endothelial cell line, which is derived from the fusion of human umbilical vein endothelial cells (HUVEC) with the permanent human cell line A549 [39]. The EA.hy926 cell line is considered as one of the best immortalized HUVEC cell lines because these cells express most of the biochemical markers of parental HUVEC cells [40]. EA.hy926 cells were grown in DMEM containing 2 mM L-glutamine, 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere (37 °C, 5% CO_2). Exponentially growing EA.hy926 cells were plated onto 96 well plates at 5000 cells/100 μL/well. 24 h after plating, the medium was aspirated, and 100 μL of medium containing the test compound added to the well containing the cells (in triplicate) and incubated for 2 h (37 °C, 5% CO_2). The highest concentration used was 100 μM followed by serial two-fold dilutions down to low nanomolar concentrations, if needed for the most morphologically active compounds. After the 2 h-incubation period, digital photographs were taken of representative center areas of each well at a magnification of 100X and 200X. The results are presented as the minimum concentration that could elicit the rounding up of more than 15% of cells in a field in order to exclude normal mitotic cells which is less that 15% in control 1% DMSO treated cells. Triplicate concentrations on the same plate were routinely performed with combretastatin A4 included in the experiments as a positive internal standard.

4.6. Inhibition of tubulin polymerization assay (ITP assay)

Tubulin assembly in microtubules was carried out using the fluorescent dye DAPI (4′,6-diamidino-2-phenylindole) [41] in a 96-well plate format as described previously [42,43]. The standard assay was performed as follows: wells were charged with purified tubulin (Cytoskeleton, 97% pure, final concentration 1 mg/mL) in

PME buffer (100 mM PIPES (1,4-piperazinebis(ethanesulfonic acid); 1 mM $MgSO_4$; 2 mM EGTA) with 10 μM DAPI and varying concentrations of the test compounds using colchicine at 30 μM as a positive internal standard control. The final concentrations used for the test compounds were started at 30 μM and diluted in 3-fold decrements until no inhibition was observed. Triplicate wells were run for each concentration. After pre-incubation at room temperature for 45 min, 1 mM GTP (5 μL) was added to each well to initiate tubulin polymerization, and the plate was then transferred to a thermostatically-controlled Victor plate reader at 37 °C for an additional 2 h. Fluorescence was then read at the excitation wavelength of 360 nm and emission wavelength of 450 nm. The percentage of inhibition was determined as follows: 1 − (ΔF(sample/ΔF(control) X 100, where ΔF control = F(no inhibition) − F(complete inhibition), and ΔF sample = F(sample) − F(complete inhibition with colchicine). The IC_{50} for compound-induced inhibition of tubulin polymerization is the concentration at which the extent of inhibition of polymerization is 50% of the maximum value, as determined from the semi-logarithmic plot of percentage inhibition as a function of the logarithm of compound concentration fitted to a sigmoidal model with variable slope using the nonlinear regression program SigmaPlot (Jandel Scientific). Under these conditions the IC_{50} value for colchicine inhibition of tubulin polymerization was 0.36 μM.

4.7. P. falciparum in vitro assay

Antiplasmodial activity was determined on the FCB1 strain from Colombia *P. falciparum*. A modified [^{3}H]-hypoxanthine incorporation assay was used. Briefly, infected human red blood cells in culture were exposed to serial drug dilutions (50 and 10 μM) in 96-well microtiter plates. Each compound at each concentration was tested in triplicate. After 48 h of incubation at 37 °C in a reduced oxygen atmosphere, 25 μL of [^{3}H]-hypoxanthine was added to each well. Cultures were incubated for a further 24 h prior to harvesting on glass-fiber filters and washing with distilled water. Radioactivity was counted using a 1450 Microbeta trilux (Wallac) (Liquid scintillation and luminescence counter). The results were recorded as counts per minute per well at each drug concentration and expressed as a percentage of the untreated controls. Some compounds that revealed moderate activity were then selected to determine IC_{50} values. Repeated assays with decreasing concentrations (from 100 to 0.195 μM) were conducted. IC_{50} values were calculated using the sigmoidal inhibition curves. All assays were performed in triplicate. The antimalarial drug chloroquine was used as positive control.

4.8. T. b. gambiense in vitro assay

Antitrypanosomal activity was determined by using the bloodstream form of *T. b. gambiense* Feo strain [44,45]. Briefly, bloodstream forms (10^5 parasites/mL) were exposed to 50 and 10 μM drug concentrations in 96-well microtiter plates. Each compound and each concentration was tested in triplicate. Plates were then incubated for 72 h at 37 °C under an atmosphere of 5% CO_2. 20 μL of resazurin (45 μM final concentration) was then added to each well. Incubation was continued for 4 h at 37 °C under 5% CO_2, then at room temperature for 22 h. Wells were read at excitation (535 nm), emission (590 nm) on an FL 600 Microplate Fluorescence Reader (Biotek) and results expressed as a percentage of the untreated controls. Repeated assays with decreasing concentrations (from 10 to 0.005 μM) were conducted. IC_{50} values were calculated using the sigmoidal inhibition curves. All assays were performed in triplicate. The trypanocide drug pentamidine was used as positive control.

4.9. Leishmania (Leishmania) amazonensis in vitro assay

The compounds were tested against promastigotes of *Leishmania* (*Leishmania*) *amazonensis* (L(L)a)-MHOM/BR/PH8) maintained in Schneider's culture medium (Sigma) with 20% heat-inactivated fetal calf serum, at 22–25 °C. Compounds were dissolved in DMSO (Sigma) or DMSO/Tween 80 (Sigma) (50/50) mixture, and diluted in Schneider's medium (Sigma). The final DMSO concentration for each experiment did not exceed 0.1% and growth controls with/without DMSO were performed. Experiments were performed in 96-well plates with compounds at concentrations ranging from 100.00 to 1.56 μg/mL. The inoculum consisted of 10^5 parasites per well in logarithmic growth phase. The cells were incubated at 22–25 °C for 48 h. Amphotericin B (Sigma) was used as the reference drug. Negative controls were performed with 10^5 parasites in Schneider's and culture medium only. Promastigote viability was based on cellular conversion of the soluble tetrazolium salt MTT (3-[4.5-dimethylthiazol-2-yl]-2.5-diphenyltetrazolium bromide, Sigma) into insoluble formazan by mitochondrial enzymes. MTT (5 mg/mL) was dissolved in Schneider's medium, 33 μL/well, for 4 h at 37 °C. Formazan was dissolved in DMSO (50 μL/well). The number of viable promastigotes was determined spectrophotometrically at 570 nm. All assays were performed in triplicate.

Conflict of interest

The authors have no conflicts of interest to disclose.

Acknowledgments

This work was financially supported by the Agence Universitaire de la Francophonie (AUF, grant Hoang Linh Mai), by the Centre National de la Recherche Scientifique (CNRS), the Institut National de la Santé et de la Recherche Médicale (INSERM) and by a grant from the Institut National du Cancer (INCa, Boulogne-Billancourt, France). We thank Dr. Alexandre Maciuk (Paris Sud University) for providing some NMR facilities. The authors are very grateful to the South Province and the North Province of New Caledonia who facilitated our field investigations.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.01.012.

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