Antiplasmodial and leishmanicidal activity of biflavonoids from Indian Selaginella bryopteris

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Antiplasmodial and leishmanicidal activity of biflavonoids from Indian *Selaginella bryopteris*

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ABSTRACT

A series of eleven biflavonoids containing amentoflavone and hinokiflavone derivatives from the Indian medicinal herb *Selaginella bryopteris* has been investigated for their antiprotozoal activity using *in vitro* assays against the K1 strain of *Plasmodium falciparum*, *Leishmania donovani*, *Trypanosoma brucei rhodesiense* and *Trypanosoma cruzi*. The highest antiprotozoal activity was displayed by 7,4′,7″-tri-*O*-methylamentoflavone which exhibited an IC_{50} of 0.26 μM. This compound showed no significant cytotoxicity ($IC_{50} > 150$ μM) evaluated using L-6 cells. The strongest activity against *Leishmania* was detected for 2,3-dihydrohinokiflavone ($IC_{50} = 1.6$ μM), whereas for *Trypanosoma* no significant activity was observed ($IC_{50} > 12.5$ μg/mL for the extract). To evaluate the *in vivo* activity against *Plasmodium* of the most active compound, trimethylated amentoflavones were obtained by partial synthesis starting from amentoflavone. The synthesized mixture of trimethylated amentoflavones did not show activity in the *Plasmodium berghei* mouse model against female NMRI mice at 50 mg/kg.

1. Introduction

Infectious protozoal diseases such as malaria, leishmaniasis, human African trypanosomiasis and Chagas disease are major causes of morbidity and mortality in developing countries and the need for the discovery of antiprotozoal drugs is high. Acquired resistance leads to a loss of treatment capacity for currently available drugs (Wellems and Plowe, 2001). Previously, antiprotozoal and antimalarial activity for eight biflavonoids has been reported by Weniger et al. (2006) and others (Ichino et al., 2006; Azebaze et al., 2007). The heterosporous fern genus *Selaginella* is a rich source of biflavonoids (Sun et al., 1997; Silva et al., 1995; Lin et al., 2000; Chen et al., 2005). From the more than 60 species of *Selaginella* occurring in India, a few species are used medicinally, for example *S. bryopteris* (L.) Bak. as a tonic and for the regeneration of vitality (Dixit, 1982, 1999). In a survey of Indian *Selaginella* species, extracts of *S. bryopteris* were screened against *Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi*, *Leishmania donovani* and *Plasmodium falciparum* and IC_{50} values of eleven biflavone derivatives were determined.

The highest antiprotozoal activity was found for 7,4′,7″-tri-*O*-methylamentoflavone (**11**), a minor compound in *S. bryopteris* (Swamy et al., 2006). To study the *in vivo* activity of **11** in a *Plasmodium berghei* NMRI mouse model, we used a semisynthetic approach to convert amentoflavone (**1**) into a mixture of predominantly tri-*O*-methylated derivatives. This is the first report on *in vivo* data of antimalarial biflavonoids.

2. Results and discussion

From the *S. bryopteris* ethanolic extract, fractions of different polarity were obtained using toluene, EtOAc and *n*-BuOH for liquid–liquid partitioning. These were tested against *T. b. rhodesiense* STIB 900, *T. cruzi* Tulahuen strain C2C4, *L. donovani* strain MHOM-ET-67 and *P. falciparum* K1 (Table 1). The EtOAc fraction showed the highest activity and was further selected for the isolation of twelve pure compounds described in Swamy et al. (2006). The modest activity of the EtOAc fraction against

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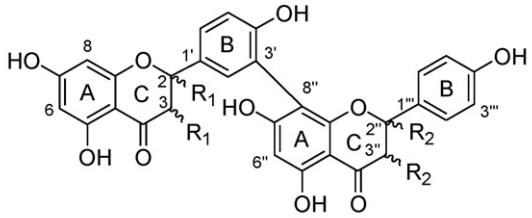
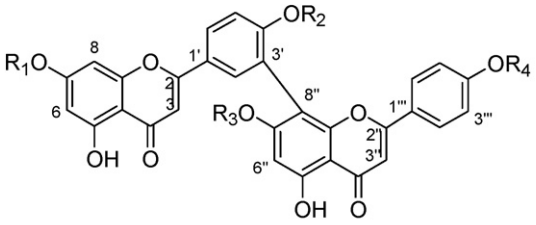
both *Trypanosoma* spp. did not justify further testing of the pure compounds against these pathogens. The activities of the isolated biflavonoids, with the exception of 2″,3″-dihydrohinokiflavone (**7**) of which the amount was too small for testing, are shown in Table 2. All isolated biflavonoidal compounds belong to two distinct subgroups. Compounds **1**–**4** and **9**–**11** are of the amentoflavone type, showing a C3′–C8″ interflavonyl linkage. The remaining compounds (**5**, **6**–**8** and **12**) belong to the hinokiflavone type, showing a C4′–*O*–C6″ linkage. Within each subgroup, the degree of saturation increases at positions C2–C3 and C2″–C3″, respectively. Three methylated derivatives of amentoflavone, together with one methylated hinokiflavone were also investigated. Hence, the presented data will help to complete the picture drawn by Weniger et al. (2006) for the antiplasmodial and leishmanicidal activity of biflavonoids. All non-methylated amentoflavones showed a lack of antiprotozoal and cytotoxic activity, regardless of the degree of saturation within the pyranone ring (C-ring).

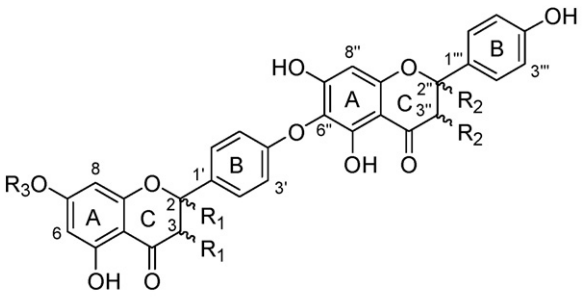
For the hinokiflavone series, a different behavior was observed. All three hinokiflavone derivatives (**5**, **6** and **8**) exhibited leishmanicidal activity in the range of 2–4 μM, and antiplasmodial activity within 2–9 μM, respectively. Interestingly, the increased degree of saturation in **6** and **8** led to a significant reduction in cytotoxicity as compared to **5**. Obviously, for non-methylated biflavones, a C–*O*–C linkage of the subunits promotes activity as shown for lanaroflavone (**13**) and hinokiflavone (**5**) (Weniger et al., 2006). It could be demonstrated in amentoflavones **9**–**11** that methylation had a strong influence on both antiprotozoal activities and cytotoxicity. Leishmanicidal and cytotoxic activity decreased as the degree of methylation for the compounds increased, whereas the antiplasmodial activity increased, leading to an IC_{50} of 0.26 μM and a selectivity index of >600 (=IC_{50} of cytotoxicity/IC_{50} of antiplasmodial activity) for the trimethylated amentoflavone (**11**). This general observation is also in accordance with Weniger et al. (2006). A further aspect lies very likely in the methylation pattern. Additionally, the



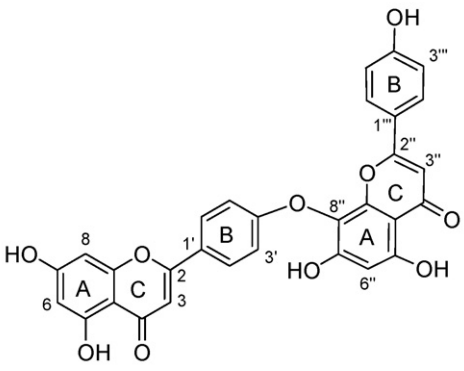
1 R_1 = Δ R_2 = Δ amentoflavone
2 R_1 = H R_2 = Δ 2,3-dihydroamentoflavone
3 R_1 = Δ R_2 = H 2″,3″-dihydroamentoflavone
4 R_1 = H R_2 = H tetrahydroamentoflavone



9 R_1 = H R_2 = Me R_3 = H R_4 = H bilobetin
10 R_1 = Me R_2 = H R_3 = H R_4 = H sequoiaflavone
11 R_1 = Me R_2 = H R_3 = Me R_4 = Me heveaflavone
14 R_1 = Me R_2 = Me R_3 = H R_4 = Me sciadopitysin



5 R_1 = Δ R_2 = Δ R_3 = H hinokiflavone
6 R_1 = H R_2 = Δ R_3 = H 2,3-dihydrohinokiflavone
7 R_1 = Δ R_2 = H R_3 = H 2″,3″-dihydrohinokiflavone
8 R_1 = H R_2 = H R_3 = H tetrahydrohinokiflavone
12 R_1 = Δ R_2 = Δ R_3 = Me 7-*O*-methylhinokiflavone



13 lanaroflavone

Table 1
Antiprotozoal activities of fractions of the ethanolic extract from *Selaginella bryopteris*

Fraction	*T. b. rhod.*	*T. cruzi*	*L. don.* Axen.	*P. falc.* K1	Cytotox L-6
Toluene	24.1	>30	13.0	4.6	>90
Ethyl acetate	12.4	20.5	9.3	1.0	32.6
Butanol	28.5	>30	>30	>5	>90
Standard[a]	0.003	0.5	0.085	0.09	0.003

IC_{50} values given in μg/mL. Values represent the average of four determinations (two determinations of two independent experiments); errors for individual measurements differed by less than 50%.

[a] *T. b. rhod.* = *Trypanosoma brucei rhodesiense*, standard: melarsoprol, *T. cruzi* = *Trypanosoma cruzi*, standard: benznidazole, *L. don.* Axen. = *Leishmania donovani* (axenic amastigotes assay), standard: miltefosine, *P. falc.* K1 = *Plasmodium falciparum* (K1 strain), standard: chloroquine, Cytotox L-6 = cytotoxicity evaluated in the L-6 cell line, standard: podophyllotoxin.

Table 2
Activities of pure biflavonoids from the EtOAc fraction of the ethanolic extract of *S. bryopteris*

Compound	*L. don.* Axen.	*P. falc.* K1	Cytotox L-6
Amentoflavone (**1**)	>55.6	>9.3	>100
2,3-Dihydroamentoflavone (**2**)	>55.6	>9.3	>90
2″, 3″-Dihydroamentoflavone (**3**)	>55.6	>9.3	>100
2,3,2″,3″-Tetrahydroamentoflavone (**4**)	51.3	>9.3	>100
Hinokiflavone (**5**)	2.9	2.3	42.4
2,3-Dihydrohinokiflavone (**6**)	1.6	4.5	6.2
2″,3″-Dihydrohinokiflavone (**7**)	n.d.[a]	n.d.	n.d.
2,3,2″,3″-Tetrahydrohinokiflavone (**8**)	4.2	9.0	>100
4′-*O*-Methylamentoflavone (**9**) bilobetin	16.5	0.3	32.0
7-*O*-Methylamentoflavone (**10**) sequoiaflavone	34.1	7.8	76.0
7,7″,4‴-Tri-*O*-methylamentoflavone (**11**) heveaflavone	>51.0	0.26	>150
7-*O*-Methylhinokiflavone (**12**)	1.7	2.2	4.0
Standard	0.5	0.13	0.02

IC_{50} values given in μM. For abbrevations and used standards see Table 1. Values represent the average of four determinations (two determinations of two independent experiments); errors for individual measurements differed by less than 50%.

[a] Not determined.

7,4′,4‴-trimethylated amentoflavone (**14**, sciadopitysin) in Weniger et al. showed an antiplasmodial activity about five times lower than the 7,7″,4‴-trimethylated amentoflavone (heveaflavone, **11**), indicating the importance of the methylation pattern. Since the most active compound (**11**) against *P. falciparum* turned out to be a minor component from the EtOAc fraction and amentoflavone was readily available, a semisynthetic approach was used to obtain methylated amentoflavone species for evaluation in an *in vivo P. berghei* mouse model (Peters, 1987). The derivatization process was optimized towards the synthesis of a mixture of predominantly trimethylated amentoflavones. The methylation grade was determined by HPLC–MS. The percentage of trimethylated amentoflavones after optimization of the methylation process was found to be 70% by HPLC–MS. Prior to *in vivo* testing, the *in vitro* antimalarial activity of this mixture was determined against *P. falciparum*. It showed an IC_{50} of about 1.2 μM and a cytotoxicity against L-6 cells of about 53 μM. However, the relatively high *in vitro* activity against *P. falciparum* was completely lost in the *P. berghei in vivo* mouse model using female NMRI mice when tested at a dose of 4× 50 mg/kg. For the interpretation of this finding, bioavailability studies are needed.

3. Experimental

3.1. General experimental procedures

S. bryopteris (L.) Bak. was collected in December 2003 from Warangal, India. The plants were identified by Dr. V.S. Raju, Department of Botany, Kakatiya University, Warangal, India. Isolation and structural determination of the eleven biflavones from *S. bryopteris* is described in Swamy et al. (2006).

3.2. Derivatization

The *O*-methylation of amentoflavone was performed with trimethylsilyldiazomethane ($TMSCHN_2$) (Presser and Hüfner, 2004; Aoyama and Terasawa, 1984). To a stirred solution of amentoflavone (100 mg, 0.186 mmol) in MeOH–MeCN (9:1, 10 mL), $TMSCHN_2$ (2 M etheric solution, 0.698 mL) and *N*,*N*-diisopropylethylamine (0.238 mL, 1.396 mmol) were added dropwise at RT in several portions over a period of 5 days. The mixture was concentrated *in vacuo* to give the mixture of methyl ethers.

The methylation grade was determined by HPLC–MS on a reversed phase Agilent Zorbax SB-C18 column (2.1 mm × 150 mm; 300 μL/min) using a gradient of MeCN in water (each with 0.1% of formic acid) as eluent. Mass spectrometry ESI-LC–MS (positive mode) was performed on a Thermo Finnigan Surveyor liquid chromatograph LCQ Deca XP combined with an LCQ Deca XP Plus mass detector.

3.3. Bioassays and determination of in vitro IC_{50}: P. falciparum

Antiplasmodial activity was determined using the K1 strain of *P. falciparum* (resistant to chloroquine and pyrimethamine). A modification of the [^{3}H]-hypoxanthine incorporation assay was used (Matile and Pink, 1990). Briefly, infected human red blood cells in RPMI 1640 medium with 5% Albumax were exposed to serial drug dilutions in microtiter plates. After 48 h at 37 °C in a reduced oxygen atmosphere, 0.5 μCi [^{3}H]-hypoxanthine was added to each well. Cultures were incubated for a further 24 h before they were harvested onto glass-fiber filters and washed with distilled water. The radioactivity was counted using a BetaplateTM liquid scintillation counter (Wallac, Zurich, Switzerland). The results were recorded as counts per minute (CPM) per well at each drug concentration and expressed as percentage of the untreated controls. From the sigmoidal inhibition curves, IC_{50} values were calculated. Assays were run in duplicate and repeated once.

3.3.1. T. b. rhodesiense

Minimum Essential Medium (50 μL) supplemented according to Baltz et al. (1985) with 2-mercaptoethanol and 15% heat-inactivated horse serum was added to each well of a 96-well microtiter plate (Räz et al., 1997). Serial drug dilutions were prepared covering a range from 90 to 0.123 μg/mL. 10^4 bloodstream forms of *T. b. rhodesiense* STIB 900 in 50 μL were added to each well and the plate incubated at 37 °C under a 5% CO_2 atmosphere for 72 h. 10 μL of resazurin solution (12.5 mg resazurin dissolved in 100 mL distilled water) was then added to each well and incubation continued for a further 2–4 h. The plate was then read in a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and emission wavelength of 588 nm (Brun and Schönernberger, 1979; Baltz et al., 1985). Fluorescence development was measured and expressed as percentage of the control. Data were transferred into the graphic programme Softmax Pro (Molecular Devices), which calculated IC_{50} values.

3.3.2. T. cruzi

Rat skeletal myoblasts (L-6 cells) were seeded in 96-well microtiter plates at 2000 cells/well in 100 μL RPMI 1640 medium with 10% FBS and 2 mM L-glutamine. After 24 h, the medium was removed and replaced by 100 μL per well containing 5000 trypomastigote forms of *T. cruzi* Tulahuen strain C2C4 containing the β-galactosidase (Lac Z) gene. 48 h later the medium was removed from the wells and replaced by 100 μL fresh medium with or without a serial drug dilution. Seven 3-fold dilutions were used covering a range from 90 to 0.123 μg/mL. Each drug was tested in duplicate. After 96 h of incubation, the plates were inspected under an inverted microscope to assure growth of the controls and sterility. Then, the substrate CPRG/Nonidet (50 μL) was added to all wells. A color reaction developed within 2–6 h and could be read photometrically at 540 nm. Data were transferred into the graphic programme Softmax Pro (Molecular Devices), which calculated IC_{50} values.

3.3.3. L. donovani (axenic amastigotes assay)

50 μL of culture medium, a 1:1 mixture of SM medium and SDM-79 medium at pH 5.4 supplemented with 10% heat-inactivated FBS, was added to each well of a 96-well microtiter plate (Costar, USA) (Cunningham, 1977; Brun and Schönernberger, 1979). Serial drug dilutions in duplicates were prepared covering a range from 30 to 0.041 μg/mL. Then, 105 axenically grown *L. donovani* amastigotes (strain MHOM/ET/67/L82) in 50 μL medium were added to each well and the plate incubated at 37 °C under a 5% CO_2 atmosphere for 72 h. 10 μL of resazurin solution (12.5 mg resazurin dissolved in 100 mL distilled water) was then added to each well and incubation continued for a further 2–4 h. The plate was then read in a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and emission wavelength of 588 nm. Fluorescence development was measured and expressed as percentage of the control. Data were transferred into the graphic programme Softmax Pro (Molecular Devices) which calculated IC_{50} values.

3.3.4. Cytotoxicity using L-6 cells

The rat skeletal myoblast cell line (L-6 cells) was used to assess cytotoxicity. The cells were grown in RPMI 1640 medium supplemented with 1% L-glutamine (200 nM) and 10% fetal bovine serum at 37 °C in 5% CO_2 in air. Assays were performed in 96-well microtiter plates, with each well receiving 100 μL of culture medium with 4×10^4 cells. After 24 h, the medium was removed from all wells and serial drug dilutions were prepared covering a range from 90 to 0.123 μg/mL. Each drug was tested in duplicate. After 72 h of incubation the plates were inspected under an inverted microscope to assure growth of the controls and sterile conditions. Then, 10 μL of Alamar blue (12.5 mg resazurin dissolved in 100 mL distilled water) was added to each well and the plates were incubated for another 2 h. Then, the plates were read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. EC_{50} values are determined using the microplate reader software Softmax Pro (Molecular Devices Cooperation, Sunnyvale, CA, USA).

3.3.5. NMRI mouse model

All efficacy studies were approved by the institutional animal experimentation ethics committee. *In vivo* antimalarial activity was assessed basically as previously described in Peters (1987). Groups of three female NMRI mice (20–22 g) intravenously infected with 2×10^7 parasitized erythrocytes on day 0 with GFP-transfected *P. berghei* strain ANKA (Franke-Fayard et al., 2004). Compounds ($4\times$ 50 mg/kg) were formulated in 100% DMSO, diluted 10-fold in distilled water and administered intraperitoneally in a volume of 10 mL/kg on four consecutive days (4, 24, 48 and 72 h post-infection). Parasitaemia was determined on day 4 post-infection (24 h after last treatment) by FACS analysis. Activity was calculated as the difference between the mean per cent parasitaemia for the control ($n = 5$ mice) and treated groups expressed as per cent relative to the control group. The survival time in days was also recorded up to 30 days after infection. A compound was considered curative if the animal survived to day 30 after infection with no detectable parasites. The positive control was treated with chloroquine ($4\times$ 10 mg/kg).

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