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NOTE

Antileishmanial amides and lignans from *Piper cubeba* and *Piper retrofractum*

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Abstract The *n*-hexane, ethyl acetate, methanol, and acetone extracts of *Piper cubeba* Linn. and *P. retrofractum* Vahl. (Piperaceae) were evaluated in vitro against promastigotes of *Leishmania donovani*, and all exhibited significant in vitro activity at 100 μg/ml. Two lignans, cubebin and hinokinin, were isolated from the hexane extract of *P. cubeba*; and one bis-epoxy lignan, (–)-sesamin, and two amides, pellitorine and piplartine, were isolated from the hexane and methanol extracts of *P. retrofractum*. Cubebin and piplartine showed significant antileishmanial activity in vitro at 100 μM and were further tested in vivo in a hamster model of visceral leishmaniasis. Piplartine showed activity at 30 mg/kg dose. This is the first report of antileishmanial activity of these two plants and their isolated constituents.

Keywords *Piper cubeba* · *Piper retrofractum* · Antileishmanial · Promastigotes · Lignan · Amide

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Introduction

Leishmaniasis is a group of tropical diseases caused by a number of species of protozoan parasites belonging to the genus *Leishmania*. Leishmaniasis, along with the parasitic diseases malaria and trypanosomiasis, has a major impact on human populations all over the world, particularly in Africa, Asia, and Latin America [1]. The World Health Organization (WHO) estimates that 350 million people live at risk of infection with *Leishmania* parasites. There are three main manifestations of leishmaniasis caused by different species of *Leishmania*. Visceral leishmaniasis, which is also known as kala-azar, is caused by *L. donovani.* More than 90% of the world's cases of visceral leishmaniasis (VL) are in India, Bangladesh, Nepal, Sudan, and Brazil. If untreated, it often leads to death [2]. Cutaneous and mucocutaneous leishmaniases are more prevalent in Afghanistan, Saudi Arabia, and some Latin American countries. Leishmaniasis is one of the opportunistic infections that attack HIV-infected individuals, and in the recent years, the coexistence of HIV and *Leishmania* species causing visceral disease has resulted in several thousand cases of dually infected individuals. Leishmaniasis patients are highly susceptible to HIV infection, and leishmaniasis accelerates the onset of AIDS by cumulative immunosuppression and by stimulation of the replication of the virus in HIV-infected patients [2]. The drugs of choice for the treatment of leishmaniasis are pentavalent antimonials such as sodium stibogluconate and meglumine antimonates. However, these present renal and cardiac toxicity. The second choice for treatment, pentamidine, is also associated with serious side effects. So there is an urgent need to discover new antileishmanial agents that are safe and cheap.

In our research program directed toward discovery of newer chemotherapeutic agents for leishmaniasis, we have

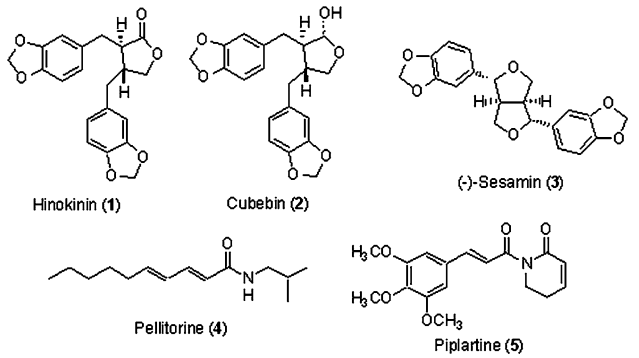

Fig. 1 Chemical structures of amides and lignans **1–5**

selected plants that are frequently encountered in Ayurvedic polyherbal formulations that were used to treat symptoms similar to kala-azar in medieval times, as leishmaniasis was unknown during those times. We have observed strong activity in the fruits of *Piper cubeba* Linn. (syn. *Cubeba officinalis* Raf.) and in the stem bark of *P. retrofractum* Vahl. (syn. *P. chaba* Hunter, *Chavica officinarum* Miq., *P. officinarum* DC.) (Piperaceae). Piperine, a major constituent of several Piper species, is reported to show antileishmanial activity both in vitro and in vivo [3–5] (Fig. 1).

Here we describe the isolation of three lignans, hinokinin, cubebin, and (–)-sesamin; and two amides, pellitorine and piplartine, from *P. cubeba* and *P. retrofractum* and their antileishmanial activity against *L. donovani* promastigotes in vitro by cell cytotoxicity assay. Active compounds were evaluated in vivo in *L. donovani*-infected golden hamster for VL. Antileishmanial activity of these plants and compounds is reported for the first time.

Materials and methods

Plant materials were extracted using Soxhlet extractor (Perfit India Ltd., India). Extracts were concentrated using vacuum rotary evaporator (Buchi R-200, Switzerland). The melting points were determined on a melting point apparatus (Mettler Toledo FP-72, Switzerland). IR spectra were taken on a Fourier transform infrared (FTIR) spectrometer (Nicolet, USA). MS were recorded on low-resolution gas MS (GCMS) (QS-5000, Shimadzu, Japan) or LCMS (Micromass, Waters, U.S.A.). NMR was recorded on 300 MHz spectrometer (Avance DPX 300, Bruker, Germany). Precoated TLC plates having silica gel 60 F_{254}, 0.2-mm thick (Merck, Germany), were used for TLC. Silica gel 60–120 mesh (CDH Laboratory Reagents, India) was used for column chromatography. Lignans were detected by methanolic sulphuric acid or anisaldehyde sulphuric acid reagent, and amides were detected by Dragendorff's reagent.

Extraction of plant material

Fruits of *P. cubeba* and stems of *P. retrofractum* were purchased from the local market, and the materials were identified and authenticated by Arvind Saklani, Department of Natural Products. A voucher specimen is kept in our laboratory for future reference. Plant materials were extracted sequentially with *n*-hexane, ethyl acetate, and methanol. The extracts were concentrated under vacuum on rotary evaporator to yield 10.6% of *n*-hexane, 3.7% of ethyl acetate, and 2.2% of methanolic extract of *P. cubeba* fruits. Similarly, *P. retrofractum* stems yielded 0.6% of *n*-hexane, 0.9% of ethyl acetate, and 0.9% of methanolic extract. Each plant material was separately extracted with acetone. The yields were 15.2% and 2.0% for *P. cubeba* and *P. retrofractum*, respectively.

In vitro antileishmanial evaluation

In vitro promastigote cell cytotoxicity assay using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide] cell proliferation assay was used to test the antileishmanial activity in vitro. *L. donovani* (0.125×10^6) promastigotes from logarithmic phase culture was allowed to grow for 48 h before the treatment of samples. After addition of samples, the cells were further allowed to grow for 48 h. MTT to a final concentration of 400 μg/ml was added and incubated for 3 h at 24°C. The cells were centrifuged at 6,000×*g*, and pellets were dissolved in dimethyl sulfoxide before taking the absorbance at 540 nm. The mean percentage of posttreatment viable cells was calculated relative to control, and results were expressed as the concentration inhibiting the parasite growth [6]. The standards miltefosine and pentamidine were used at reported IC_{50} values [6, 7].

In vivo antileishmanial evaluation

A hamster model of VL was used for in vivo screening. VL was produced by intracardiac injection of *L. donovani* amastigotes (2×10^7) in golden hamsters. Twenty days after infection, hamsters were administered the compound for a period of 10 days, with an interval of 5 days. Hamsters were sacrificed after 2 days of the last dose to examine spleen weight and spleen parasitic burden. Spleen parasitic burden was assessed from Giemsa-stained impression smears [8].

Statistical analysis

All the results are expressed as mean ± standard error of mean (SEM). One-way analysis of variance (ANOVA) was used for statistical analysis. When ANOVA showed

significant difference, post hoc analysis was performed with Tukey's test. $P < 0.05$ was considered statistically significant. Statistical analysis was carried out using Jandel Sigma Stat Version 2, software.

Results and discussion

Preliminary investigation on various extracts prepared from *P. cubeba* and *P. retrofractum* revealed that *n*-hexane extract of *P. cubeba* showed more than 90% inhibition of promastigotes of *L. donovani* in vitro at a concentration of 100 μg/ml, whereas methanol as well as acetone extracts from *P. retrofractum* showed more than 75% inhibition at a concentration 20 μg/ml. The IC_{50}s of all the extracts were determined, and three from *P. retrofractum* were found to show high activity, their IC_{50}s being less than 7.5 μg/ml. Extracts from *P. cubeba* showed comparatively weaker activity. The results are shown in Table 1.

n-Hexane extract of *P. cubeba* (10 g) was subjected to column chromatography on silica gel using hexane–ethyl acetate. Based on the thin-layer chromatography (TLC) pattern, fractions were pooled into four main fractions (1–4). Concentration of fraction 2 (25% ethyl acetate) yielded viscous oil (385 mg), which was identified as a lignan, hinokinin (**1**) [9–12]. Solvent evaporation of fraction 3 (30% ethyl acetate) yielded a colorless crystalline compound (780 mg) that was identified from the spectral data as cubebin (**2**) [9–11].

Chromatographic separation of the *n*-hexane extract of *P. retrofractum* (6 g) with the hexane–ethyl acetate gradient resulted in the isolation of the two compounds. Compound **3** (20% ethyl acetate, 22 mg) was characterized as (–)-sesamin [12, 13]. Compound **4** (25% ethyl acetate, 12 mg) gave a positive test with Dragendorff's reagent. The infrared (IR) spectrum indicated the presence of an amide group (3,372, 3,019, 1,670, and 1,522 cm^{-1}) as well as an olefinic double bond (1,630 and 927 cm^{-1}). The ultraviolet (UV) spectrum showed λ_{max} at 257 nm. This, along with the IR spectrum, suggested the presence of conjugated dienoic acid amide derivative. Compound **4** was identified as pellitorine by comparison of spectral data with literature values [14, 15].

Methanol extract (40 g) of *P. retrofractum* was subjected to vacuum liquid chromatography with the hexane–ethyl acetate gradient to yield five major fractions (Fr. 1–5). Chromatographic separation of fraction 4 (80% ethyl acetate, 3.5 g) with the hexane–ethyl acetate gradient on silica gel resulted in the isolation of compound **5** (50% ethyl acetate, 1.4 g), which gave a positive test with Dragendorff's reagent. The IR spectrum showed strong absorption at 1,684 and 1,620 cm^{-1}, which indicated the presence of conjugated amide. The ^{1}H-NMR spectra showed characteristic peak of cinnamoyl protons at δ 7.68 (d, J = 15 *Hz*) and 7.42 (d, J = 15 *Hz*). The doublet at δ 6.04 indicated an α–β unsaturated carbonyl moiety. Liter-

Table 1 In vitro antileishmanial evaluation of extracts and pure compounds against promastigotes of *Leishmania donovani*

Drug/extract	Concentration (μg/ml or μM)[a]	Inhibition of promastigotes[b] (%)	IC_{50} (μg/ml or μM)[a]
n-Hexane *P. cubeba*	10	27.0 ± 8.3	22.5
	25	53.1 ± 3.7	
	50	71.8 ± 5.2	
	100	92.5 ± 1.6	
EtOAc *P. cubeba*	10	25.9 ± 1.0	35.0
	25	43.2 ± 6.4	
	50	55.6 ± 2.7	
	100	70.8 ± 9.0	
MeOH *P. cubeba*	50	27.0 ± 0.0	100.0
	100	49.7 ± 0.2	
	150	59.2 ± 3.9	
Acetone *P. cubeba*	10	22.8 ± 8.1	70.0
	25	38.8 ± 4.9	
	50	42.5 ± 1.1	
	100	68.7 ± 4.0	
n-Hexane *P. retrofractum*	5	49.9 ± 1.7	ca. 5.0
	10	75.3 ± 1.1	
	25	86.7 ± 2.9	
	50	89.2 ± 4.4	
EtOAc *P. retrofractum*	10	28.0 ± 2.9	45.0
	25	41.0 ± 6.3	
	50	50.7 ± 2.1	
	100	54.2 ± 0.4	
MeOH *P. retrofractum*	5	20.0 ± 0.1	7.5
	10	63.3 ± 0.1	
	20	74.3 ± 0.0	
	100	95.8 ± 1.9	
Acetone *P. retrofractum*	1	25.8 ± 0.4	3.5
	5	60.0 ± 0.1	
	10	88.5 ± 0.0	
Cubebin (**2**)	10	30.7 ± 4.0	28.0
	25	45.8 ± 2.2	
	50	70.5 ± 5.1	
	100	79.4 ± 5.5	
Piplartine (**5**)	5	31.0 ± 8.2	7.5
	7.5	49.8 ± 1.1	
	10	57.0 ± 1.5	
Miltefosin	25	45.3 ± 6.4	ca. 25.0
Pentamidine	1.75	47.0 ± 2.5	ca. 1.8
Control	NA	0.0 ± 0.0	

[a] μg/ml for extracts and μM for pure compounds

[b] Results are expressed as percentage inhibition [mean ± standard error of mean (SEM); n = 3]

Table 2 In vivo antileishmanial evaluation of isolated compounds (vehicle and compounds were administered for 10 days in *Leishmania-donovani*-infected hamsters)

Groups	Dose	Spleen weight (mg)	Reduction in spleen weight (%)	Spleen parasitic burden ($\times 10^9$)	Reduction in spleen parasitic burden (%)	Animals died/ animals tested
Vehicle (DMSO)	2.0 ml/kg, i.p.	728 ± 62	–	1.41 ± 0.11	–	0/8
Sodium stibogluconate	50.0 mg/kg, i.p.	446 ± 66*	37	0.28 ± 0.04*	80	0/5
Miltefosine	12.5 mg/kg, i.p.	288 ± 23*	58	0.07 ± 0.00*	95	0/5
Cubebin (**2**)	100.0 mg/kg, i.p.	873 ± 85	–	1.33 ± 0.14	5.6	0/4
Piplartine (**5**)	30.0 mg/kg, i.p.	367 ± 87*	50	0.90 ± 0.19*	36	0/5

Values represented as mean ± standard error of mean (SEM). Significantly different from vehicle-treated group, * $P < 0.05$

DMSO dimethylsulfoxide

ature comparison of spectral data showed compound **5** was a pyridone amide piplartine [16].

All of the isolated compounds were tested for in vitro antileishmanial activity against promastigotes of *L. donovani* at 100 μM concentration. Lignan cubebin (**2**) showed 79.4 ± 5.5% inhibition of promastigotes; (–)-sesamin (**3**) and pellitorine (**4**) were inactive, whereas piplartine (**5**) showed 89.1 ± 2.9% inhibition at 100 μM. Further experiments were conducted to determine the IC_{50} values, and results are shown in Table 1. Piplartine and cubebin showed IC_{50} values at 7.5 and 28.0 μM, respectively (Table 1).

Cubebin (**2**) and piplartine (**5**), which showed significant in vitro activity, were evaluated in vivo in golden hamsters against amastigotes of *L. donovani*. Ten days' treatment with piplartine (**5**) at 30 mg/kg/10 ml i.p. significantly reduced parasitic burden and spleen weight (Table 2). A 36% reduction in parasitic burden and 50% reduction in spleen weight were observed on piplartine (**5**) treatment. It was toxic at a dose of 300 mg/kg per 10 ml i.p. Standard drugs such as sodium stibogluconate and miltefosine produced 80% and 95% reduction in parasitic burden at 50 and 12.5 mg/kg doses, respectively.

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