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Antileishmanial phenylpropanoids from *Alpinia galanga* (Linn.) Willd.

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Hexane, chloroform and ethyl acetate extracts (100 µg/ml) of *Alpinia galanga* rhizomes exhibited significant activity *in vitro* against promastigotes of *L. donovani*. Twelve compounds namely, methyleugenol (**1**), *p*-coumaryl diacetate (**2**), 1'-acetoxychavicol acetate (**3**), 1'-acetoxyeugenol acetate (**4**), *trans*-*p*-acetoxycinnamyl alcohol (**5**), *trans*-3,4-dimethoxycinnamyl alcohol (**6**), *p*-hydroxybenzaldehyde (**7**), *p*-hydroxycinnamaldehyde (**8**), *trans*-*p*-coumaryl alcohol (**9**), galangin (**10**), *trans*-*p*-coumaric acid (**11**) and galanganol B (**12**) were isolated from these extracts. Of these, compounds **2**, **3**, **4** and **5** were found most active *in vitro* against promastigotes of *L. donovani* with IC_{50} values of 39.3, 32.9, 18.9 and 79.9 µ*M* respectively. This is the first report of antileishmanial activity of the extracts and isolated constituents of *A. galanga*.

Keywords: *Alpinia galanga*, *Leishmania donovani*, Leishmaniasis, Phenylpropanoids, Promastigotes

Leishmaniasis is a wide spread life-threatening disease caused by protozoa of genus *Leishmania*. According to available WHO estimates, the disease is spread across 88 countries causing serious health problems especially in developing countries[1-3]. The three main manifestations of disease are visceral, cutaneous and muco-cutaneous leishmaniasis. Visceral leishmaniasis (VL), also known as kala-azar is caused by *L. donovani*. More than 90% of world's cases of VL are reported in India, Bangladesh, Nepal, Sudan, Brazil and Ethiopia[3]. In India, most of the leishmaniasis cases are reported in Bihar, Orissa and Uttar Pradesh states[2-3]. Cutaneous and muco-cutaneous leishmaniases are more prevalent in Afganistan, Saudi Arabia and some Latin American countries[1]. The first line drugs used for the treatment of leishmaniasis suffer from serious side effects such as toxicity, high cost and rapid emergence of resistance. More recently, emergence of co-infection of leishmaniasis with HIV has made the treatment even more challenging[4]. As a part of research program to find antileishmanial leads from Indian medicinal plants, antileishmanial activity of extracts and compounds of *Piper cubeba* and *Piper retrofractum* have been reported[5]. In the present communication, isolation of chemical constituents from *Alpinia galanga* (Linn.) Willd. and evaluation of their antileishmanial activity *in vitro* against promastigotes of *L. donovani* are discussed.

Alpinia galanga (Linn.) Willd. is a commonly used spice in south and south-east Asian countries. The rhizomes of the plant are extensively used as a spice or ginger substitute for flavoring foods and in traditional medicine for several purposes, such as stomachic, carminative, antiflatulent, antifungal and anti-itching agents. Rhizomes of *A. galanga* have been tested for wide range of biological activities. These include antifungal[6], antibacterial[7], antimycobacterial[8], antiviral[9], anticancer[10], antitrypanosomal[11] etc. *A. galanga* rhizomes mainly contain phenylpropanoids most abundant of which are 1'*S*-1'-acetoxychavicol acetate (**3**), 1'*S*-1'-acetoxyeugenol acetate (**4**), *p*-coumaryl diacetate (**2**) etc. Most of the biological activities reported for the plant are attributed to the presence of phenylpropanoids[6, 8-11].

Materials and Methods

Chemicals and instruments—Plant material was extracted using soxhlet extractor (Perfit, India Ltd., India). All solvents used were of analytical grade (CDH Laboratory Reagents, India). Extracts were

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concentrated using vacuum rotary evaporator (Buchi R-200, Switzerland). Precoated TLC plates of silica gel GF_{254} and RP-18 (Merck, Germany) were used for TLC analysis. Silica gel 60-120 mesh (CDH Laboratory Reagents, India) was used for column chromatography. TLC grade silica, silica gel G was used for vacuum liquid chromatography (VLC) of extracts. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on 400 and 100 MHz spectrometer, respectively (Bruker, Germany). Deuterated chloroform ($CDCl_3$) and methanol (CD_3OD) (Aldrich, USA) were used for recording NMR and tetramethylsilane (TMS) was used as an internal standard. Shimadzu HPLC system was used for analytical and semi-preparative HPLC of the samples. Reverse phase analytical column C_{18} (250×4.6 mm), Kromasil (Phenomenex, USA) and semi-preparative column C_{18} (250×10 mm), SPHER (Princeton, USA) were used for analytical and preparative separation of compounds.

Plant materials and preparation of extracts—Dried rhizomes of *A. galanga* were purchased locally. These were identified in the Department of Natural Products, NIPER, S.A.S. Nagar and voucher specimen of plant material is maintained in the laboratory. Rhizomes (2 kg) were powdered and sequentially extracted by soxhlet extraction with hexane, chloroform and ethyl acetate for 48 h. The residue was extracted with methanol at room temperature for one week. Extracts were filtered and concentrated on a rotary evaporator under reduced pressure at 40°C. The yields of hexane, chloroform, ethylacetate and methanol extracts were 2.7, 0.8, 0.6 and 1.2% respectively.

Antileishmanial evaluation—In vitro promastigote cell toxicity assay using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] cell proliferation assay was used to test the antileishmanial activity *in vitro*. *L. donovani* (0.125×10^6) promastigotes from logarithmic phase culture were 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 post-treatment viable cells was calculated relative to control and results were expressed as concentration inhibiting the parasite growth. The standard pentamidine was used at reported IC_{50} value, while the IC_{50} value of standard miltefosine was taken from our previous experiments[5].

Isolation of compounds—Hexane extract (AGH, 55 g) was obtained as light brown oil. 50 g of this extract was subjected to vacuum liquid chromatography (VLC) over silica gel G using hexane-ethylacetate (0-100%) as mobile phase. Six major fractions were obtained – AGH1 (7.4 g), AGH2 (9.5 g), AGH3 (5 g), AGH4 (3 g), AGH5 (1.9 g) and AGH6 (6 g). Fraction AGH2 on repeated silica gel column chromatography using hexane-ethyl acetate (0-40% gradient elution) yielded **1** (50 mg). Fraction AGH4 was separated by semi-preparative reverse-phase HPLC using acetonitrile-water (40:60) to give **2** (15 mg) and **3** (40 mg). Fraction AGH5 was separated by VLC over silica gel G using hexane-ethylacetate (0-100%) as mobile phase in 30 subfractions (AGH5-1 to AGH5-30). Fractions AGH5-14 to AGH5-18 were pooled based on their similar TLC profiles. This pooled fraction was then separated by semi-preparative reverse-phase HPLC using acetonitrile-water (30:70) to give **2** (5 mg) and **4** (10 mg). Fraction AGH5-30 was also separated by semi-preparative reverse-phase HPLC using acetonitrile-water (30:70) to give **5** (5 mg).

Chloroform extract (AGC, 15 g) was subjected to VLC on silica gel G using hexane-ethylacetate-methanol (gradient elution). Five major fractions were collected – AGC1 (3 g), AGC2 (1.4 g), AGC3 (1 g), AGC4 (960 mg) and AGC5 (4 g). Fraction AGC2 was separated by column chromatography on silica gel using chloroform-methanol (0-10%) to give **6** (15 mg), **7** (5 mg) and **8** (8 mg).

Ethyl acetate extract (AGE, 10 g) was subjected to VLC on silica gel G using chloroform-methanol gradient elution. Five major fractions were collected – AGE1 (2 g), AGE2 (1 g), AGE3 (2 g), AGE4 (600 mg) and AGE5 (2.5 g). Fraction AGE2 was separated by column chromatography on silica gel in 50 fractions. Fraction AGE2-23 and AGE2-24 were pooled and separated by semi-preparative reverse-phase HPLC [methanol-water (10-100% in 50 min)] to give 9 (4 mg). Fraction AGE3 (2 g) on silica gel column chromatography using chloroform-methanol (0-100%) yielded **10** (17 mg). Fraction AGE4 (600 mg) was separated by semi-preparative reverse-phase HPLC [methanol-water (10-100% in 50 min)] to give **11** (7 mg) and **12** (15 mg).

Results and Discussion

Hexane, chloroform, ethyl acetate and methanol extracts showed 87.2, 76.6, 53.6 and 31.8% inhibition respectively of promastigotes of *L. donovani in vitro* at 100 µg/ml concentration. Chromatographic separation of hexane extract led to isolation of five compounds. These were characterized as methyleugenol[12] (**1**), *p*-coumaryl diacetate[13] (**2**), 1'-acetoxychavicol acetate[6,13] (**3**), 1'-acetoxyeugenol acetate[6,13] (**4**) and *trans-p*-acetoxycinnamyl alcohol[14] (**5**). Chromatographic separation of chloroform extract resulted in purification of three compounds. These were characterized as *trans*-3,4-dimethoxycinnamyl alcohol[10] (**6**), *p*-hydroxybenzaldehyde[12,13] (**7**) and *p*-hydroxycinnamaldehyde[15] (**8**). Five compounds were isolated from ethyl acetate extract. These were characterized as *trans-p*-coumaryl alcohol[12] (**9**), galangin[14,16] (**10**), *trans-p*-coumaric acid (**11**) and galanganol B[12] (**12**). All the compounds were characterized by comparison of their spectroscopic data with literature values. The structures of isolated compounds are shown in Fig. 1.

All the isolated compounds were tested in *in vitro* promastigote assay at 100 µ*M* concentration. The results of antileishmanial evaluation of extracts and isolated compounds are shown in Table 1. Of these compounds, *p*-coumaryl diacetate (**2**), 1'-acetoxychavicol acetate (**3**), 1'-acetoxyeugenol acetate (**4**) and *trans-p*-acetoxycinnamyl alcohol (**5**) showed more than 50% inhibition of the parasite. These four compounds were tested at lower concentrations and the IC_{50} values determined from percent inhibition at three different concentrations were found to be 18.9, 32.9, 39.3 and 79.9 µ*M* for **4**, **3**, **2** and **5** respectively (Fig. 2). The activity of compound **4** was comparable to the standard drug miltefosine. These results suggest that phenylpropanoid compounds containing one or two acetyl groups have better antileishmanial activity. Compounds **8** and **11**, lacking acetyl group showed no or less activity. Compounds **10** and **12** also showed diminished activity.

It can be concluded that acetylated phenylpropanoids present in *A. galanga* have antileishmanial principles of the plant. Not many phenylpropanoids have been reported for antileishmanial activity. The present study indicated a considerable potential for further evaluation of this

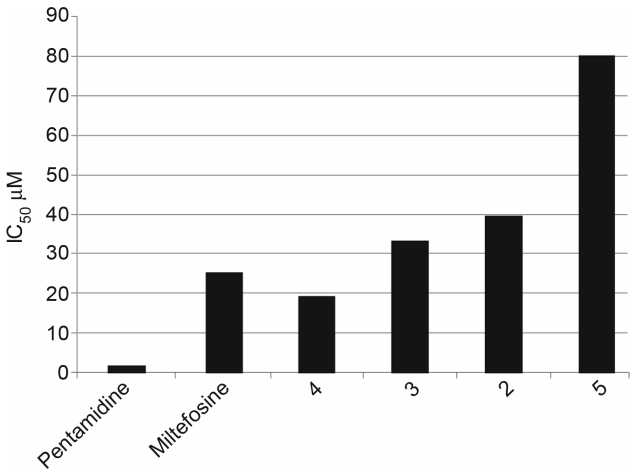

Fig. 2—IC_{50} values of standards and active phenylpropanoids of *A. galanga*

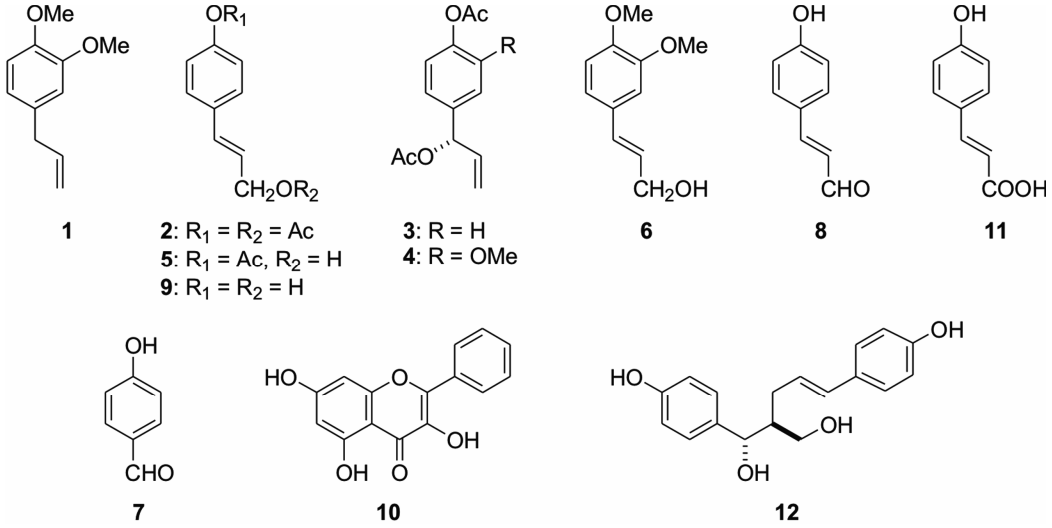

Fig. 1—Compounds isolated from *A. galanga*

Table 1—*In vitro* antileishmanial activity of extracts and compounds of *A. galanga*

Extracts/Compounds	Concentration (μg/ml or μ*M*)[a]	Inhibition of promastigotes % (mean ± SEM; n = 3)	IC_{50} (μ*M*)
Hexane	100	87.2 ± 1.1	-
Chloroform	100	76.6 ± 4.6	-
Ethyl acetate	100	53.7 ± 19.1	-
Methanol	100	31.8 ± 1.4	-
Methyleugenol (**1**)	100	Nil	-
p-Coumaryl diacetate (**2**)	100	81.5 ± 2.0	39.3
	50	67.0 ± 8.3	
	25	34.5 ± 7.3	
1'-Acetoxychavicol acetate (**3**)	50	71.7 ± 7.3	32.9
	25	51.7 ± 9.4	
	12.5	9.4 ± 6.5	
1'-Acetoxyeugenol acetate (**4**)	25	74.6 ± 0.2	18.9
	12.5	17.6 ± 9.0	
	5	11.0 ± 6.6	
trans-p-Acetoxy-cinnamyl alcohol (**5**)	100	69.0 ± 7.8	79.9
	50	21.1 ± 6.3	
	25	0.0 ± 0.0	
trans-3,4-Dimethoxycinnamyl alcohol (**6**)	100	Nil	-
p-Hydroxyben-zaldehyde (**7**)	100	43.0 ± 7.2	> 100
p-Hydroxy-cinnamaldehyde (**8**)	100	Nil	-
p-Coumaryl alcohol (**9**)	100	40.3 ± 9.6	> 100
Galangin (**10**)	100	53.4[b]	~ 100
trans-p-Coumaric acid (**11**)	100	41.4 ± 7.8	> 100
Galanganol B (**12**)	100	29.0 ± 6.3	> 100
Pentamidine			ca 1.75
Miltefosine			ca 25
Control	NA	0.0 ± 0.0	-

[a]μg/ml for extracts and μ*M* for pure compounds; [b]Results are expressed as mean [*n* = 2]

class of compounds for antileishmanial activity. It has been reported that ethanolic extract of the plant is non-toxic up to acute dose of 3 g/kg body weight in mice[17]. Rhizomes are being used as a spice since ages, therefore there lies a scope of developing antileishmanial herbal formulation with *A. galanga* as a major ingredient.

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