European Journal of Medicinal Chemistry 50 (2012) 230–235

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Galactolipids from *Bauhinia racemosa* as a new class of antifilarial agents against human lymphatic filarial parasite, *Brugia malayi*☆

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ARTICLE INFO

Article history:
Received 22 November 2011
Received in revised form
20 January 2012
Accepted 29 January 2012
Available online 3 February 2012

Keywords:
Bauhinia racemosa
Galactolipids
Antifilarial
Brugia malayi
Jird

ABSTRACT

Bioassay guided fractionation of ethanolic extract of the leaves of *Bauhinia racemosa* led to the isolation of galactolipid and catechin class of the compounds (**1**–**7**) from the most active *n*-butanol fraction (F4). Among the active galactolipids, **1** emerged as the lead molecule which was active on both forms of lymphatic filarial parasite, *Brugia malayi*. It was found to be better than the standard drug ivermectin and diethylcarbamazine (DEC) in terms of dose and efficacy.

1. Introduction

Lymphatic filariasis (LF) is one of the neglected mosquito-borne tropical diseases. It is endemic in 81 countries putting 1.2 billion people at risk globally with an estimated 120 million infected [1]. The filarial nematode *Wuchereria bancrofti*, accounts for 91% of LF infections while *Brugia malayi* and *Brugia timori* are responsible for the remaining 9% in South and Southeast Asia. These lymphatic dwelling parasites cause damage to the lymphatic system leading to the clinical symptoms such as lymphedema, elephantiasis and hydrocele. LF is the second largest cause of permanent and long term disability [2,3].

The Global Programme to Eliminate Lymphatic Filariasis (GPELF) was established in 1999 with the objective of interrupting transmission of the parasites in all endemic countries by 2020 through annual mass drug administration (MDA) with diethylcarbamazine or ivermectin monotherapy, or either drug in combination with albendazole [4]. Current drugs used for MDA implementation by national programmes are mainly microfilaricidal and are effective in reducing microfilariae (mf) counts but not effective in killing adult worms. Besides this, adverse reactions may occur following treatment, thus their use is crippled and needs to be replaced with some more effective treatment regime which could be effective on both forms (adult and mf) of parasite.

Medicinal plants have been used as therapeutic aid for alleviating human ailments from vedic age [5] and still provide ingredients for formulations of new medicines in pharmaceutical industry. In fact, WHO has listed over 21,000 plant species to be of medicinal use around the world. More than 60% of the world's human population relies on plant medicine for primary health-care needs [6]. The previous work on medicinal plants like *Butea monosperma* and *Aegle marmelos* showed significant antifilarial activity against *B. malayi* microfilariae [7], *Caesalpinia bonducella* showed microfilaricidal and female-sterilizing efficacy against *B. malayi* in animal models [8], extracts prepared from *Carapa procera*, *Polyalthia suaveolens* and *Pachypodanthium staudtii* exhibited microfilaricidal activity on *Onchocerca volvulus* [9] and recently fruit extract of *Xylocarpus granatum* exhibited promising *in vitro* and *in vivo* antifilarial activity against human lymphatic filarial parasite, *B. malayi* which could be attributed to the presence of two pure compounds gedunin and photogedunin [10].

Bauhinia racemosa Lam. belongs to family Caesalpinaeceae popularly known as 'Kachnal' (Hindi). It is a small deciduous tree widely distributed throughout the tropics including India, Ceylon, China and Timor. The stem bark of the plant has been used traditionally for diarrhea, dysentery and as an astringent while the

☆ Part 10 in the series "Search for new natural leads".

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doi:10.1016/j.ejmech.2012.01.057

decoction of the leaves was utilized as the remedy for malaria and headache [11]. The alcoholic extract of the leaves showed different degrees of anti-inflammatory, analgesic, antipyretic as well as antispasmodic properties [12]. In another study, the methanolic extract of the stem bark exhibited strong free radical scavenging, antimicrobial and antitumor activity [13,14]. Fresh flower buds of this plant had shown inhibition of ulcer formation [15]. Cytotoxicity against CA-9 KB in cell culture, hypotensive and hypothermic activities were also reported from the hydroalcoholic extract of this plant [16]. As a part of our continuing efforts directed toward the unearthing of the structurally interesting and biologically promising molecules from the Indian medicinal plants, the ethanolic extract of the leaves of *B. racemosa* was taken up to assess its antifilarial activity against *B. malayi* followed by bioassay guided fractionation, isolation of the phytoconstituents and identification of the lead molecule/s from the active fraction. This is the first report on the antifilarial effects of this plant.

2. Results and discussion

Previous phytochemical investigation showed the presence of polycyclic phenolics from heartwood [17,18], flavonoids from leaves [19], triterpenoids and sterols from stem bark of *B. racemosa* [20], but none of the constituents have ever been identified to establish their various bioactivities. In the present study, we have established the antifilarial activity in the leaves of the plant and also identified the lead molecule among the various phytoconstituents isolated from the active sub-fraction. To the best of our knowledge the isolated phytoconstituents **1**–**8** from the leaves of *B. racemosa* have never been tested against filarial parasite.

2.1. Antifilarial activity of the plant

In the preliminary study, the crude ethanolic extract (F1) showed 80% reduction in the motility of the adult filarial parasite, *B. malayi in vitro*. Though the worm did not show 100% reduction in the motility, however, it was paralyzed and only a small part of it sometimes showed slight and sluggish movement. This prompted us to fractionate ethanolic extract (F1) into *n*-hexane (F2), chloroform (F3) and *n*-butanol fraction (F4) to find out the locus of activity. They were subjected to *in vitro* antifilarial assays and it was found that only *n*-butanol fraction (F4) showed promising adulticidal (IC_{50} 5.46 μg/mL) and microfilaricidal (IC_{50} 4.89 μg/mL) activity with Minimum Inhibitory Concentration (MIC) of 15.6 μg/mL while others were found inactive. The results thus revealed that the active constituents were mainly concentrated in the *n*-butanol fraction.

2.2. Isolation of the active constituents from n-butanol fraction (F4)

With the objective to identify the constituents responsible for the activity, sub-fraction F4 was purified using successive column chromatographic separations on normal phase silica gel and reverse phase silica gel; and gel filtration over sephadex LH20 was also utilized to obtain seven constituents **1**–**7** in their pure forms (Fig. 1). Two classes of compounds (galactolipid and catechin) were identified from the active fraction. Galactolipids were characterized as (2*S*)-1, 2-di-*O*-linolenoyl-3-*O*-α-galactopyranosyl-(1→6)-*O*-β-galactopyranosyl glycerol (**1**), (2*S*)-1-*O*-linolenoyl-2-*O*-palmitoyl-3-*O*-α-galactopyranosyl-(1→6)-*O*-β-galactopyranosyl glycerol (**2**) and (2*S*)-1-*O*-oleoyl-2-*O*-palmitoyl-3-*O*-α-galactopyranosyl-(1→6)-*O*-β-galactopyranosyl glycerol (**3**), whereas catechins were characterized as (−)-epiafzelechin (**4**), (−)-epicatechin (**6**) and (−)-catechin (**7**) together with protocatechuic acid (**5**). The identity of the compounds and their structure elucidations were accomplished with the help of extensive 1D and 2D NMR and other spectroscopic tools. The absorption bands observed at 3383 and 1733 cm^{-1} in the IR spectrum indicated the presence of hydroxyl and ester functions, while ^1H and ^{13}C NMR spectra of **1** showed signals at δ_H 4.44 (1H, dd, 12.0, 2.8), 5.26 (1H, m), 4.25 (1H, d, 6.8), 4.88(1H, d, 3.4) and δ_C at 64.0, 71.7, 68.7, 105.2, 100.6, 174.9, 174.6 which were characteristically attributable to a digalactosyl diacylglycerol. ESI-MS showed molecular ion at *m/z* 959 $[M + Na]^+$ and a fragment ion peak at *m/z* 613 $[M - 323]^+$ confirmed the molecular formula. The identification of fatty acid residue and the sugar moiety was achieved by characterization of the fatty acid obtained by alkaline hydrolysis with NaOMe–MeOH and D-galactose obtained by acidic hydrolysis. The configuration at C-2 was assigned to be 'S' on the basis of specific rotation studies. Further the detailed study of ^1H–^1H COSY, HSQC and HMBC spectra coupled with mass spectral analysis and their comparison with the reported literature values established the structure of active galactolipid **1** [21,22]. Spectral analysis of **2** and **3** showed structural similarities with **1** but displayed characteristic differences in their fatty acid residues, thus were characterized accordingly (Please refer to Supplementary data). Although **3** has been synthesized and isolated previously but its complete peak assignment could not be found in the literature thus it has been presented in Table 1.

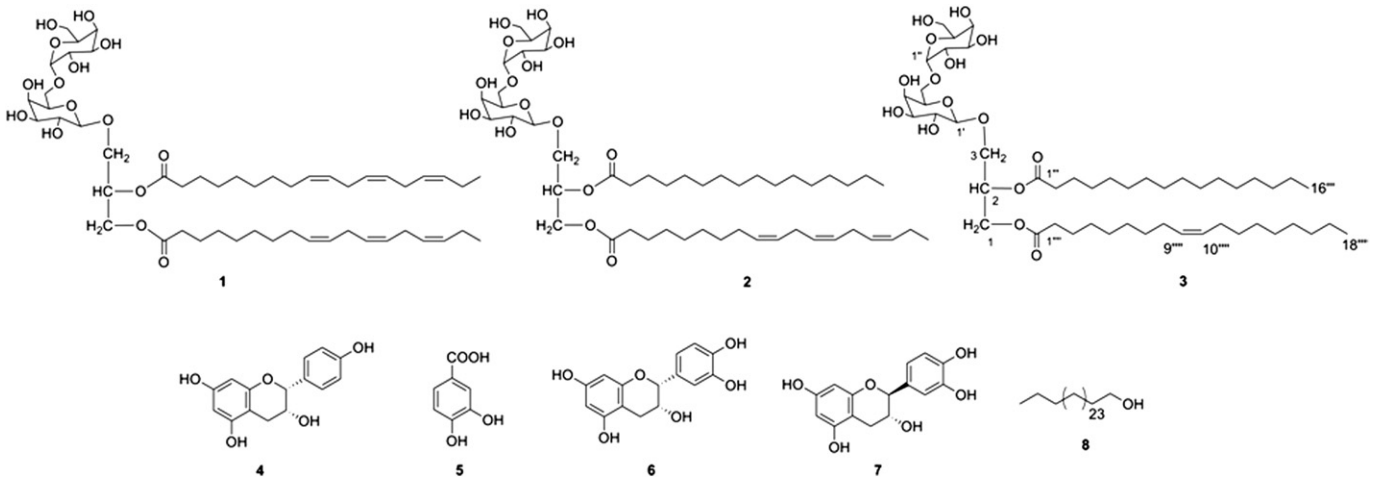

Fig. 1. Chemical structure of the isolated compounds from the leaves of *B. racemosa*.

Table 1
^{1}H and ^{13}C NMR assignment of the galactolipid **3**.[a]

Position	Chemical shift δ_C	Multiplicity	Chemical shift δ_H (J in Hz)
1	64.0	CH_2	4.44, dd (12.0, 2.6) 4.21, fused
2	71.7	CH	5.26, m
3	68.7	CH_2	3.94, m 3.75, m
1′; 1″	105.3; 100.6	CH; CH	4.26, d (6.7); 4.88, merged
2′; 2″	72.5; 72.4	CH; CH	3.52, m; 3.85, m
3′; 3″	74.6; 71.4	CH; CH	3.52, m; 3.77, m
4′; 4″	70.0; 71.1	CH; CH	3.89, m; 3.91, m
5′; 5″	74.5; 70.2	CH; CH	3.76, m; 3.77, m
6′; 6″	67.7; 62.8	CH_2; CH_2	3.91, m; 3.74, m 3.69, m
1‴; 1⁗	175.0; 174.6	qC; qC	–
2‴; 2⁗	35.1; 35.0	CH_2; CH_2	2.34, td (7.3, 4.2) for both
3‴; 3⁗	26.0; 26.0	CH_2; CH_2	1.62, br s for both
4‴–13‴ 4⁗–7⁗ 12⁗–15⁗	30.2–30.8	CH_2	1.30, br s
14‴; 16⁗	33.1; 33.1	CH_2	1.30, br s for both
15‴; 17⁗	23.8; 23.8	CH_2	1.30, br s for both
16‴; 18⁗	14.5; 14.5	CH_2	0.92, t (6.3) for both
8⁗	28.2	CH_2	2.06, m
9⁗	130.9	CH	5.36, m
10⁗	130.8	CH	5.36, m
11⁗	28.2	CH_2	2.06, m

[a] The spectra were recorded in CD_3OD at 300 MHz (^{1}H NMR) and 75 MHz (^{13}C NMR) and multiplicity was determined by DEPT experiments.

Selected ^{1}H–^{1}H COSY and ^{1}H to ^{13}C correlations (HMBC) helped in confirming the bond connectivities and the overall structure of galactolipid **3** (Fig. 2). Similarly, compounds **4**–**7** were characterized; optical rotations, splitting pattern and coupling constant '*J*' values helped in assigning the configuration at stereo-centers [23,24]. It is noteworthy to mention that compounds **1**–**4** are first time reported from this genus '*Bauhinia*'.

2.3. In vitro screening of the isolated compounds

Galactolipids such as monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG) are major constituents of the chloroplast membrane in plant kingdom. Their occurrence and distribution is an area of intense interest and investigation due to their various biological functions such as antitumor promoting activity, DNA polymerase inhibition, hemolytic activity, antiviral, anti-inflammatory, antimicrobial and anti-hyperlipidemic activities [25–27]. The isolated compounds were subjected to the *in vitro* testing against both forms (adult and mf) of *B. malayi*. The comprehensive results have been summarized in Table 2. Galactolipid **1** was found to be the most active of all the tested compounds which showed significant inhibition in the motility of both adult and mf, and also in the viability of adult worm. It killed

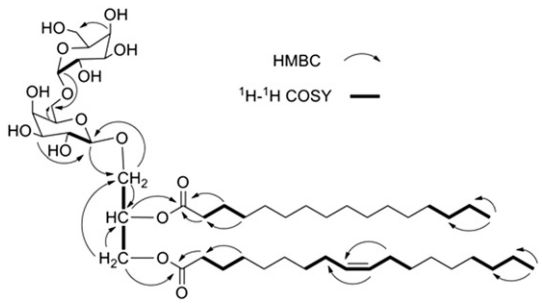

Fig. 2. Selected ^{1}H–^{1}H COSY and HMBC correlations of compound **3**.

Table 2
In vitro antifilarial activity of the fraction F4 and galactolipids **1**–**3** against adult and microfilariae of *B. malayi*.[a]

Test samples	Adult			Microfilariae			CC_{50}
	MIC	IC_{50}	SI	MIC	IC_{50}	SI	
Fraction F4	15.6	5.46	>18.31	15.6	4.89	>20.45	>100
1	3.9	1.25	>80	15.6	1.607	>62.23	>100
2	Inactive	–	–	31.25	2.7	>37.03	>100
3	Inactive	–	–	31.25	1.05	>95.24	>100
8	Inactive	–	–	31.25	3.26	>30.67	>100
Ivermectin	7.8	1.61	>62.11	125	3.62	>27.62	52.84
DEC	Inactive	–	–	Inactive	–	–	–

[a] MIC, IC_{50} and CC_{50} values are in μg/mL.

adult (MIC 3.9 μg/mL) as well as mf (MIC 15.6 μg/mL) with IC_{50} values 1.25 and 1.61 μg/mL respectively. Galactolipids **2** and **3** were not equally active as **1** on adults as they brought only 50% inhibition in motility at 31.25 μg/mL but they killed mf (MIC 31.25 μg/mL), revealing IC_{50} values of 2.70 and 1.05 μg/mL, respectively. Rest of the tested compounds appeared inactive as they showed only 50–60% inhibition in parasite motility at 31.25 μg/mL. The standard drug ivermectin killed adult worms (MIC 7.8 μg/mL) with IC_{50} of 1.61 μg/mL and mf (MIC 125 μg/mL) with an IC_{50} of 3.62 μg/mL. The other standard drug diethyl carbamazine (DEC) was found inactive against both. From these results it was clear that only the galactolipids were active against filarial parasite while catechins appeared inactive. Further, **1** was found to be the most active compound against adult worms and **3** was the most active against mf among all. It is noteworthy that galactolipids **1**–**3** were found to be effective and superior to the standard drug ivermectin in the *in vitro* assays. To check the role of long chain fatty acid in the activity, another compound **8**, a long chain fatty acid alcohol, which was isolated from chloroform fraction F3 (isolation not discussed here), was tested *in vitro* along with these compounds, it was inactive against adult worm but killed mf (MIC 31.25 μg/mL) with IC_{50} of 3.26 μg/mL . This suggested that the lipid part (side chain) of the galactolipids modulate the bioactivity. The detailed *in vitro* data has been presented in Table 3. All the active compounds were found safe in cytotoxicity assay on vero cell line (Selectivity Index (SI) for the tested compounds was found to be more than 20) and were further evaluated *in vivo* in primary screening model.

2.4. In vivo screening of the active compounds

The active sub-fraction F4 was evaluated *in vivo* in primary screen (adult *B. malayi* i.p. transplanted jird) model at 100 mg/kg subcutaneously for five days and exhibited $23.33 \pm 8.8\%$ adulticidal activity which was even less than DEC ($30.5 \pm 7.35\%$) at the same dose. Subsequently, active compounds from *n*-butanol fraction F4 were evaluated *in vivo* in the same primary screening model intraperitoneally (i.p.) for five consecutive days at the dose of 50 mg/kg. The galactolipid **1** brought about $58.3 \pm 8.33\%$ ($P < 0.01$) adult worm mortality, galactolipid **2** showed $45.8 \pm 4.2\%$ ($P < 0.05$) mortality, galactolipid **3** brought about $54.15 \pm 4.15\%$ ($P < 0.01$) mortality and long chain fatty acid alcohol **8** demonstrated $49.95 \pm 8.35\%$ ($P < 0.05$) adulticidal activity over control. The complete results have been summarized in Table 4. Although **2**, **3** and **8** were not effective on adult worms in *in vitro* assays but they brought significant adult worm mortality *in vivo*. Galactolipid **1** was found to be the most active compound with highest adult parasite mortality.

3. Conclusion

B. racemosa is a medicinal deciduous plant being traditionally used against various diseases and ailments, its stem and bark have

Table 3
In vitro motility scoring and MTT assay results of the fractions and constituents **1**–**8** against adult and microfilariae of *B. malayi*.

Test samples	Conc. (μg/mL)	% Inhibition[a]		
		Motility of adult worm	MTT reduction over control	Motility of microfilariae
Fraction F1	31.25	80 (1+)	42.00	70 (2+)
Fraction F2	31.25	30 (3+)	31.30	50 (2+)
Fraction F3	31.25	40 (3+)	10.40	40 (3+)
Fraction F4	31.25	100 (D)	75.00	100 (D)
	15.6	100 (D)	54.00	100 (D)
	7.8	60 (2+)	40.00	70 (2+)
	3.9	50 (2+)	35.02	55 (2+)
	1.9	50 (2+)	20.80	50 (2+)
1	31.25	100 (D)	82.9	100 (D)
	15.6	100 (D)	75.0	100 (D)
	7.8	100 (D)	61.8	99 (1+)
	3.9	100 (D)	52.1	95 (1+)
	1.9	90 (1+)	43.6	60 (2+)
2	31.25	50 (2+)	30.0	100 (D)
	15.6	inactive	0.0	95 (1+)
	7.8	inactive	0.0	90 (1+)
	3.9	inactive	0.0	70 (2+)
3	31.25	50 (2+)	24.6	100(D)
	15.6	inactive	0.0	99 (1+)
	7.8	inactive	0.0	90 (1+)
	3.9	inactive	0.0	80 (1+)
4	31.25	50 (2+)	14.8	59 (2+)
5	31.25	50 (2+)	20.6	60 (2+)
6	31.25	50 (2+)	34.8	50 (2+)
7	31.25	60 (2+)	43.2	60 (2+)
8	31.25	50 (2+)	32.6	100 (D)
	15.6	inactive	0.0	95 (1+)
	7.8	inactive	0.0	85 (1+)
Control	–	00 (4+)	–	4+

[a] Values in parenthesis are motility scores: 4+, 0%; 3+, 1–49%; 2+, 50–74%; 1+, 75–99%; D, 100%.

a great medical significance whereas leaves have also shown various pharmacological activities. Standard drugs ivermectin as well as DEC reduce the mf burden of the patients and are not so much effective against the adult *B. malayi* worms [28]. GPELF, an effort made by WHO against LF is also facing problem since the drugs administered in MDA programme have been found to exhibit adverse local as well as systemic reactions in many cases [29]. Unavailability of vaccines as well as the pressure of increased risk of development of drug resistant worms urge for an urgent need of excavating the drugs effective against the LF parasite. Thus, in the light of above facts we have used the *B. racemosa* leaves; extracted and fractionated them to get their purified forms and analyzed their antifilarial activities against LF parasites (adult female worm and mf). Bioassay guided fractionation of the ethanolic extract (F1) of the leaves of *B. racemosa* afforded *n*-hexane (F2), chloroform (F3) and *n*-butanol fraction (F4), among them only F4 was found to possess *in vitro* antifilarial activity which was further purified to obtain seven phytoconstituents (galactolipids and catechins, **1**–**7**). The *in vitro* and *in vivo* assays against LF parasite, *B. malayi* led to the discovery of antifilarial activity in the leaves of plant and galactolipid **1** was identified as the lead potent molecule active against adult worms as well as mf whereas galactolipids **2** and **3** were found effective against mf while other isolated compounds (catechins) were found ineffective against both the stages. With this study galactolipids emerged as the new class of antifilarial agent, which are being first time reported from this plant. Galactolipids are important secondary metabolites of the plants which could be the next chemotherapeutical cure to eliminate adamant lymphatic filaria.

4. Experimental

4.1. General experimental information

IR spectra were recorded on Perkin Elmer 399B spectrophotometer, optical rotation was measured on Rudolph Autopol III Polarimeter, NMR spectra were obtained from Bruker Avance DRX 300 MHz spectrometer (^{1}H at 300 MHz, ^{13}C at 75 MHz, respectively) in CD_3OD and ESI mass spectra were recorded on Thermo LCQ Advantage Max-IT. Purity of the isolated compounds was found to be greater than 95%. Organic solvents were distilled prior use. Column chromatography was carried out on Si gel (60–120 and 230–400 mesh, Merck) and reverse phase RP-18 silica gel (Sigma–Aldrich); sephadex LH 20 (Sigma–Aldrich) was used for gel filtration. Thin layer chromatography was performed on silica gel $60F_{254}$ aluminium and glass plates (Merck).

4.2. Plant material

The leaves of *B. racemosa* were collected from Bhimtal, Uttarakhand in 2007. The plant was authenticated and a voucher specimen (No. 8478) was deposited in the herbarium of the Botany Division, Central Drug Research Institute, Lucknow, India.

4.3. Extraction and fractionation

The shade dried and grounded leaves (4.5 kg) of the plant *B. racemosa* were extracted with ethanol (5% water, 25 L × 3) at room temperature. The combined alcoholic part was concentrated under high vacuum on rotary evaporator at bath temperature <50 °C, to give crude ethanolic extract, F1 (487 g). It was macerated with cold *n*-hexane (250 mL × 3) to give fraction F2 (29 g). Remaining of the extract was dissolved in methanol:water (1:9,

Table 4
In vivo antifilarial efficacy of the active fraction and galactolipids **1**–**3** against *B. malayi* i.p. transplanted jird.

Test samples	Dose (mg/kg)[a]	Mean number of adults recovered			% Adulticidal activity over control[b]	% Female worm sterilization
		Male	female	total		
Fraction F4	100, s.c. × 5 days	3.6	4.0	7.6	−23.33 ± 8.8	Nil
DEC	100, s.c. × 5 days	02	6.33	8.33	−30.5 ± 7.35	Nil
Control 1	–	2.67	7.33	10	–	–
1	50, i.p. × 5 days	02	03	05	−58.3 ± 8.33**	Nil
2	50, i.p. × 5 days	03	4.5	7.5	−45.8 ± 4.2*	Nil
3	50, i.p. × 5 days	02	3.5	5.5	−54.15 ± 4.15**	Nil
8	50, i.p. × 5 days	03	03	06	−49.95 ± 8.35*	Nil
Control 2	–	03	09	12	–	–

*$P < 0.05$ is low significant, **$P < 0.01$ is highly significant.
[a] Each group consisted of 5 animals.
[b] Mean ± SE.

1 L), the aqueous layer obtained was successively partitioned with chloroform (500 mL × 3) and *n*-butanol (500 mL × 3) to give fraction F3 (48 g) and F4 (300 g), respectively. All the organic solvents were concentrated under high vacuum to give the respective fractions. Bioassay guided fractionation and isolation was followed after the confirmation of the antifilarial activity in butanol fraction.

4.4. Phytochemical investigation of n-butanol fraction (F4)

Fraction F4 (300 g) was chromatographed over silica gel column packed in chloroform. It was subjected to gradient of methanol:-chloroform (0–100%) to obtain two hundred fractions (500 mL each), which were pooled according to their TLC profiles to furnish twenty-five major sub-fractions (I-XXV). Both, fraction VIII (4 g) and fraction IX (5 g) were separately subjected to gel filtration on sephadex LH 20 using methanol:chloroform gradient as mobile phase. Similar fractions were pooled to give six (A–F) and eight (G–N) sub-fractions, respectively. Sub-fraction A and sub-fraction G were combined together (1.13 g) and subjected to reverse phase RP-18 silica gel column chromatography utilizing methanol:water gradient (95–100%) to furnish five sub-fractions (O–S). Finally, sub-fraction P (240 mg) was purified over reverse phase RP-18 silica gel column chromatography using methanol:acetonitrile gradient (0–1%) to furnish pure compound **1** (70 mg), sub-fraction Q (140 mg) was purified to give **2** (60 mg) and sub-fraction R (90 mg) gave **3** (43 mg). Sub-fraction F (195 mg) was successively purified by sephadex LH 20 using methanol:water (4:6) as mobile phase and then over reverse phase PTLC on RP-18 silica gel coated glass plates to afford pure compounds **4** (66 mg) and **5** (10 mg). Similarly, sub-fraction N (170 mg) was purified by successive reverse phase column chromatography and PTLC on RP-18 coated glass plates in methanol:water (4:6) to obtain **6** (40 mg) and **7** (5 mg).

4.5. Antifilarial activity: in vitro assays

4.5.1. Infection maintenance

B. malayi (sub-periodic strain) was maintained in six-week old male rodent host, *Mastomys coucha* and *Meriones unguiculatus* (jird). Mastomys were subcutaneously inoculated with 100 infective larvae (L3) of *B. malayi* [30] while jirds received 150 L3 intraperitoneally [31]. L3 were obtained from gently crushed mosquitoes (laboratory bred *Aedes aegypti*) by Baerman's technique on day 9 ± 1 post infective feeding on donor mastomys. For *in vitro* studies the parasites were recovered by washing the peritoneal cavity of jird infected 4–6 months back while for *in vivo* primary screening, jirds intraperitoneally transplanted with adult *B. malayi* were used.

4.5.2. Sample preparation

Stock suspensions of the plant samples, standard drugs ivermectin and DEC (10 mg/mL) were prepared in DMSO for *in vitro* testing. The *in vitro* activity of the crude extract and fractions was assessed at serial two fold dilutions of the stock starting from 31.25 μg/mL to the lowest concentration of 1.9 μg/mL.

4.5.3. Parasite isolation

B. malayi adult worms and mf were isolated aseptically from the peritoneal cavity of infected jirds within 120–150 days of intraperitoneal inoculation of L3, mf was isolated by passing the peritoneal suspension through 5.0 μm membrane filter.

4.5.4. Activity evaluation criterion

Percentage reduction in the motility of adult female worm as well as mf and the percentage inhibition in MTT reduction in treated adult female worm parasite compared to their respective untreated controls were evaluated. The criterion for adulticidal activity was 100% irreversible immobility of adult worm with ≥50% inhibition in MTT reduction by treated parasite over untreated control. MIC of the tested extracts on adult *B. malayi* and mf was determined as the minimum concentration of test sample causing total irreversible immobility (death). Filaricide ivermectin (IC_{50} adult 1.61 μg/mL and IC_{50} mf 3.62 μg/mL) is used as a standard antifilarial drug in *in vitro* screen while DEC served as a standard filaricide for *in vivo* screen since latter was inactive on both adult and mf *in vitro*. For assessing 50% inhibitory concentration (IC_{50}), further two fold dilutions of each test material was tested starting from MIC (value up to 0.3 μg/mL). IC_{50} concentration is determined by Excel based line graphic template after plotting concentration values of each sample against percent inhibition in motility of parasite on *x*- and *y*-axis. Due to unproportionate correlation between the inhibitions in MTT and the motility, only the motility data was considered for evaluating IC_{50}.

4.5.5. In vitro screening

MIC of the samples was evaluated at various two fold dilutions (starting from 31.25 ug/mL) on fully motile female worms in 48-well culture plate in duplicate containing 1000 μL media with one female parasite/well (NUNC). RPMI 1640 medium containing antibiotics (penicillin 100 units/mL, streptomycin sulphate 100 μg/mL and neomycin mixture; Sigma, USA) and 10% fetal bovine serum was used. The worms exposed to the crude extract and fractions were incubated for 48 h at 37 °C in CO_2 incubator. Exposed adult worms were observed microscopically for their motility 1 h after transferring the parasite to fresh drug free medium. The adult female worms were then processed for the 3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) reduction assay as described earlier [32]. MIC of the extracts and fractions was also assessed against mf (20 live mf/well) using 96-well plate in duplicate containing 200 μL medium to which various two fold dilutions (starting from 31.25 μg/mL) of stock solution of compounds were added and incubated under same conditions as the adult female parasites.

4.5.6. IC_{50} evaluation

After evaluating the MIC values of the extracts, active samples were followed for their IC_{50} values. For assessing their 50% inhibitory concentrations (IC_{50}) the parasites (one female worm/mL/well and 20 mf/200 μL/well) were exposed to two fold dilutions (starting from their MIC values) of the active extracts and based on their % motility reduction IC_{50} values were evaluated.

4.5.7. CC_{50} evaluation

Assessment of cytotoxicity CC_{50} (50% cytotoxicity concentration) values of the active samples was done on the vero cells (monkey kidney cell lines) with the help of *in vitro* cytotoxicity assay following the method of O'Brien et al. with modifications [33]. Vero cells were trypsinized, counted in Neubauer chamber and plated as required (10,000 cells/100 μL/ well) in a 96-well plate. 100 μL/well of minimal essential medium (MEM) was added to each third column of the plate without vero cells to serve as negative control. 10,000 cells/100 μL/well were plated in to each well except the third, sixth, ninth and 12th column and incubated overnight at 37 °C in CO_2 incubator. Medium from all the wells was replaced by 100 μL of fresh medium. One hundred microliters of medium containing 300 μg/mL test sample (highest concentration) was added to row H in triplicates (i.e. compound **1** from columns 1–3, compound **2** from columns 4–6, compound **3** from columns 7–9 and compound **4** from columns 10–12). Serial dilutions (3:1) were prepared with a multichannel pipette by transferring 50 μL from row H to row G and mixing it and re-transferring 50 μL in the

same way to each consecutive row till row B. Row A was kept drug free to serve as positive control and the plate was incubated for 48 h at 37 °C in 5% CO_2 in air. After 72 h of drug exposure, 10 μL/well of viability marker dye Resazurin or Alamar blue (stock solution 12.5 mg/100 mL PBS) was added and the plate was incubated for another 2–4 h. Fluorimetric reaction was measured using an excitation wavelength of 536 nm and an emission of 588 nm in a fluorimetric plate reader. Data was transferred to Excel and plotted as per the template using fluorescent signal against corresponding drug concentration. CC_{50} values were determined directly.

4.5.8. Determination of selectivity index

Selectivity index (SI) values of the *in vitro* active samples were evaluated as the ratio of CC_{50} and IC_{50} ($SI = CC_{50}/IC_{50}$). Test samples with SI value of $\geq$10 were considered safe for further *in vivo* follow up.

4.6. Antifilarial activity: in vivo efficacy

4.6.1. Sample preparation

Crude extract was finely suspended in distilled water, whereas the compounds were solubilized in distilled water with the help of 0.1% Tween-80.

4.6.2. In vivo screening model

Naive six to eight-week old male jird was intraperitoneally transplanted with ten female and five male adult *B. malayi* worms recovered from infected jirds. Worms were taken out after washing the peritoneal cavity of infected jird. The given numbers of worms were implanted into the peritoneal cavity of these naive jirds by incising the abdomen under Ketamine anesthesia (50 mg/kg, i.p.). On day 6/7, peritoneal fluid (a drop) was aspirated with the help of 1 mL syringe and examined microscopically for the presence of live mf to ensure successful transplant.

4.6.3. Dosage

Fraction F4 was administered subcutaneously at the dose of 100 mg/kg for 5 consecutive days, while the pure compounds were given at 50 mg/kg by peritoneal route (i.p.).

4.6.4. In vivo activity

The treated and control i.p. transplanted jirds were euthanized on day 51 and worms were recovered from the peritoneal cavity after completing the observation period of 50 days. Recovered worms were analyzed microscopically for their motility, death or encapsulation. The condition of embryonic stages in the uteri was also examined microscopically after teasing the recovered live female worms in a drop of phosphate buffered saline (pH 7.2). Sterilization/embryostatic effect was considered to be any abnormality or death/distortion detected in the uterine contents, including oocytes, eggs and mf and the percentage of sterile females was assessed [34].

4.6.5. Statistical analysis

The analysis of data was carried out by PRISM 3.0 using one-way ANOVA (nonparametric) and Dunnett's multiple comparison test. $P < 0.05$ was considered as of low significance (*) while $P < 0.01$ was considered as highly significant (**).

Acknowledgments

Authors acknowledge the SAIF Division for providing the spectroscopic data and botany division for the plant material. We are also thankful to Dr T. K. Chakraborty (Director, CSIR-CDRI) for his constant support and encouragement. This is CDRI communication number 8190.

Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2012.01.057.

References

[1] WHO, Global programme to eliminate lymphatic filariasis, in: Wkly. Epidemiol. Rec. (2009), pp. 437–444.
[2] J.O. Gyapong, V. Kumaraswami, G. Biswas, E.A. Ottesen, Expert Opin. Pharmacother. 6 (2005) 179–200.
[3] M.J. Bockarie, M.J. Taylor, J.O. Gyapong, Expert Rev. Anti-infect. Ther. 7 (2009) 595–605.
[4] K. Ichimori, E.A. Ottesen, Bol. Med. Hosp. Infant Mex. 68 (2011) 134–137.
[5] K. Sullivan, C.N. Shealy, Complete Natural Home Remedies, Element Books Limited, Shafterburg, UK, 1997.
[6] M.M. Robinson, X. Zhang, Traditional Medicines: Global Situation, Issues and Challenges, WHO, Geneva, 2011.
[7] K.N. Sahare, V. Anandharaman, V.G. Meshram, S.U. Meshram, D. Gajalakshmi, K. Goswami, M.V. Reddy, Indian J. Med. Res. 127 (2008) 467–471.
[8] R.L. Gaur, M.K. Sahoo, S. Dixit, N. Fatma, S. Rastogi, D.K. Kulshreshtha, R.K. Chatterjee, P.K. Murthy, Indian J. Med. Res. 128 (2008) 65–70.
[9] V.P. Titanji, M.S. Evehe, J.F. Ayafor, S.F. Kimbu, Acta Leiden 59 (1990) 377–382.
[10] S. Misra, M. Verma, S.K. Mishra, S. Srivastava, V. Lakshmi, S. Misra-Bhattacharya, Parasitol. Res. 109 (2011) 1351–1360.
[11] K.R. Kirtikar, B.D. Basu, in: , second ed., Indian Medicinal Plants, vol. II. Lalit Mohan Basu, Allahabad, India, 1996, pp. 894–895.
[12] A.S. El-Khatib, A.E. Khaleel, Bull. Fac. Pharm. Cairo Univ. 33 (1995) 59–65.
[13] M. Gupta, U.K. Mazumder, R.S. Kumar, T.S. Kumar, Acta Pharmacol. Sin. 25 (2004) 1070–1076.
[14] R.S. Kumar, T. Sivakumar, R.S. Sunderam, M. Gupta, U.K. Mazumdar, P. Gomathi, Y. Rajeshwar, S. Saravanan, M.S. Kumar, K. Murugesh, K.A. Kumar, Braz. J. Med. Biol. Res. 38 (2005) 1015–1024.
[15] A.H. Akhtar, K.U. Ahmad, J. Ethnopharmacol. 46 (1995) 1–6.
[16] M.L. Dhar, M.M. Dhar, B.N. Dhawan, B.N. Mehrotra, C. Roy, Indian J. Exp. Biol. 6 (1968) 232–247.
[17] A.S.R. Anjaneyulu, A.V. Raghava Reddy, D.S.K. Reddy, R.S. Ward, D. Adhikesavalu, T. Stanley Cameron, Tetrahedron 40 (1984) 4245–4252.
[18] A.S.R. Anjaneyulu, A.V.R. Reddy, D.S.K. Reddy, T.S. Cameron, S.P. Roe, Tetrahedron 42 (1986) 2417–2420.
[19] G.A. El-Hossary, M.A. Selim, A.E. Sayed, A.E. Khaleel, Bull. Fac. Pharm. Cairo Univ. 38 (2000) 93–97.
[20] A. Prakash, R.L. Khosa, Curr. Sci. 45 (1976) 705.
[21] F. Cateni, G. Falsone, J. Zilic, P. Bonivento, M. Zacchigna, D. Zigon, S. Sosa, G. Altinier, Arkivoc 5 (2004) 54–65.
[22] D. Watanabe, R. Kerakawati, T. Morita, T. Nakamura, K. Ueno, T. Kumamoto, W. Nakanishi, T. Ishikawa, J. Uzawa, H. Seki, M. Tachi, K. Harada, Y. Higuchi, C. Chaichantipyuth, Heterocycles 78 (2009) 1497–1505.
[23] V.K. Sethi, S.C. Taneja, K.L. Dhar, C.K. Atal, Phytochemistry 23 (1984) 2402–2403.
[24] A.M. Balde, L.A. Pieters, A. Gergely, H. Kolodziej, M. Claeys, A.J. Vlietinck, Phytochemistry 30 (1991) 337–342.
[25] R. Tanaka, Y. Sakano, A. Nagatsu, M. Shibuya, Y. Ebizuka, Y. Goda, Bioorg. Med. Chem. Lett. 15 (2005) 159–162.
[26] F. Cateni, P. Bonivento, G. Procida, M. Zacchigna, G. Scialino, E. Banfi, Bioorg. Med. Chem. Lett. 17 (2007) 1971–1978.
[27] E. Larsen, A. Kharazmi, L.P. Christensen, S.B. Christensen, J. Nat. Prod. 66 (2003) 994–995.
[28] W.A. Stolk, G.J. Van Oortmarssen, S.P. Pani, S.J. De Vlas, S. Subramanian, P.K. Das, J.D. Habbema, Am. J. Trop. Med. Hyg. 73 (2005) 881–887.
[29] B.V. Babu, K. Rath, A.S. Kerketta, B.K. Swain, S. Mishra, S.K. Kar, Trans. R. Soc. Trop. Med. Hyg. 100 (2006) 464–469.
[30] G. Petranyi, H. Mieth, I. Leitner, Southeast Asian J. Trop. Med. Pub. Health 6 (1975) 328–337.
[31] J.W. McCall, J.B. Malone, A. Hyong-Sun, P.E. Thompson, J. Parasitol. 59 (1973) 436.
[32] M. Mukherjee, S. Misra, R.K. Chatterjee, Acta Trop. 70 (1998) 251–255.
[33] J. O'Brien, I. Wilson, T. Orton, F. Pognan, Eur. J. Biochem. 267 (2000) 5421–5426.
[34] S. Misra, R.K. Chatterjee, A.B. Sen, Indian J. Med. Res. 79 (1984) 749–754.