Phytomedicine 20 (2013) 124–132

Contents lists available at SciVerse ScienceDirect

Phytomedicine

journal homepage: www.elsevier.de/phymed

Antifilarial diarylheptanoids from *Alnus nepalensis* leaves growing in high altitude areas of Uttarakhand, India

Deepti Yadav[a], Subhash Chandra Singh[b], Ram Kishore Verma[a], Kirti Saxena[c], Richa Verma[c], Puvvada Kalpana Murthy[c,**], Madan Mohan Gupta[a,*]

[a] *Analytical Chemistry Department, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow, India*
[b] *Botany and Pharmacognosy Department, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow, India*
[c] *Division of Parasitology, CSIR-Central Drug Research Institute, Lucknow, India*

ARTICLE INFO

Keywords:
Alnus nepalensis
Diarylheptanoids
Antifilarial
HPTLC

ABSTRACT

Lymphatic filariasis continues to be a major health problem in tropical and subtropical countries. A macrofilaricidal agent capable of eliminating adult filarial parasites is urgently needed. Platyphyllenone (**A**), alusenone (**B**), hirustenone (**C**) and hirsutanonol (**D**) are important biologically active diarylheptanoids present in *Alnus nepalensis*. In the present study, we report the antifilarial activity in diarylheptanoids isolated from the leaves of *A. nepalensis*. Out of four compounds (**A**–**D**) tested *in vitro* one has shown promising anti-filarial activity both *in vitro* and *in vivo* studies. This is the first ever report on antifilarial efficacy of a compound of the plant and warrants further studies around this scaffold.

In addition, a sensitive, selective and robust densitometric high-performance thin-layer chromatographic method was developed and validated for the above four biomarker compounds. The separation was performed on silica gel 60F_{254} high-performance thin layer chromatography plates using chloroform:methanol (9:1, v/v) as mobile phase. The quantitation of marker compounds was carried out using densitometric reflection/absorption mode at 600 nm after post-chromatographic derivatization using vanillin–sulfuric acid reagent. The method was validated for peak purity, precision, robustness, limit of detection (LOD) and quantitation (LOQ) etc., as per the International Conference on Harmonization (ICH) guidelines.

Introduction

Alnus nepalensis (D. Don), an alder species, family Betulaceae, is an actinorhizal tree found in the hilly regions of Eastern and Northeastern India (Chauhan and Misra 2002). The leaf, roots and bark of *A. nepalensis* are used in dysentery, stomachache, and diarrhea in Indian system of medicine, ayurveda (Pande et al., 2006). A decoction of the root of *A. nepalensis* is prescribed to treat diarrhea and paste from the leaves is applied on cuts and wounds as a hemostatic (Changkija 1999). The diarylheptanoids, which are characteristic components of *Alnus* species, have been reported to have several biological activities. Platyphyllenone (**A**), alusenone (**B**), hirustenone (**C**), and hirsutanonol (**D**) are important biologically active diarylheptanoids. Hirustenone and hirsutanonol show anti-influenzal (Tung et al., 2010a,b), anti oxidant (Kuroyanagi et al., 2005), cytotoxic (Choi et al., 2008), hepatoprotective effects on t-BHP-induced damage to HepG2 cell (Park et al., 2010), NO and COX-2 inhibitory activities (Kim et al., 2005) and HIF-1 inhibitory activities (Jin et al., 2007). Furthermore, hirustenone has been reported to prevent cytokine and chemokine-mediated immune cell function and inflammatory reaction and was found to be an attractive starting point for the development of a topical drug for T cell-based anti-atopic dermatitis due to its calcineurin inhibitory effects (Lee et al., 2009; Joo et al., 2009). Alusenone and platyphyllenone showed significant antioxidant and hepatoprotective effects (Tung et al., 2010a,b). They need to search for some newer activities.

Filariasis causes major public health and socioeconomic problems in tropical areas. Recently, WHO (1995) estimated that 120 million people in at least 73 countries suffer from lymphatic filariasis; and 1.1 billion people living in endemic areas are at risk of infection. Existing antifilarial drugs diethylcarbamazine (DEC), ivermectin and albendazole are being used for the control for human filariasis, of which diethylcarbamazine and ivermectin,

Abbreviation: HPTLC, High-performance thin layer chromatography.

* Corresponding author at: Analytical Chemistry Department, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow 226015, India.
Tel.: +91 941 5927850; fax: +91 522 2342666.
** Corresponding author. Fax: +91 522 2623405.
E-mail addresses: drpkmurthy@yahoo.com (P.K. Murthy), guptammg@rediffmail.com (M.M. Gupta).

Communication No. CIMAP:2012-112J; CDRI: 8353.

0944-7113/$ – see front matter © 2012 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.phymed.2012.10.017

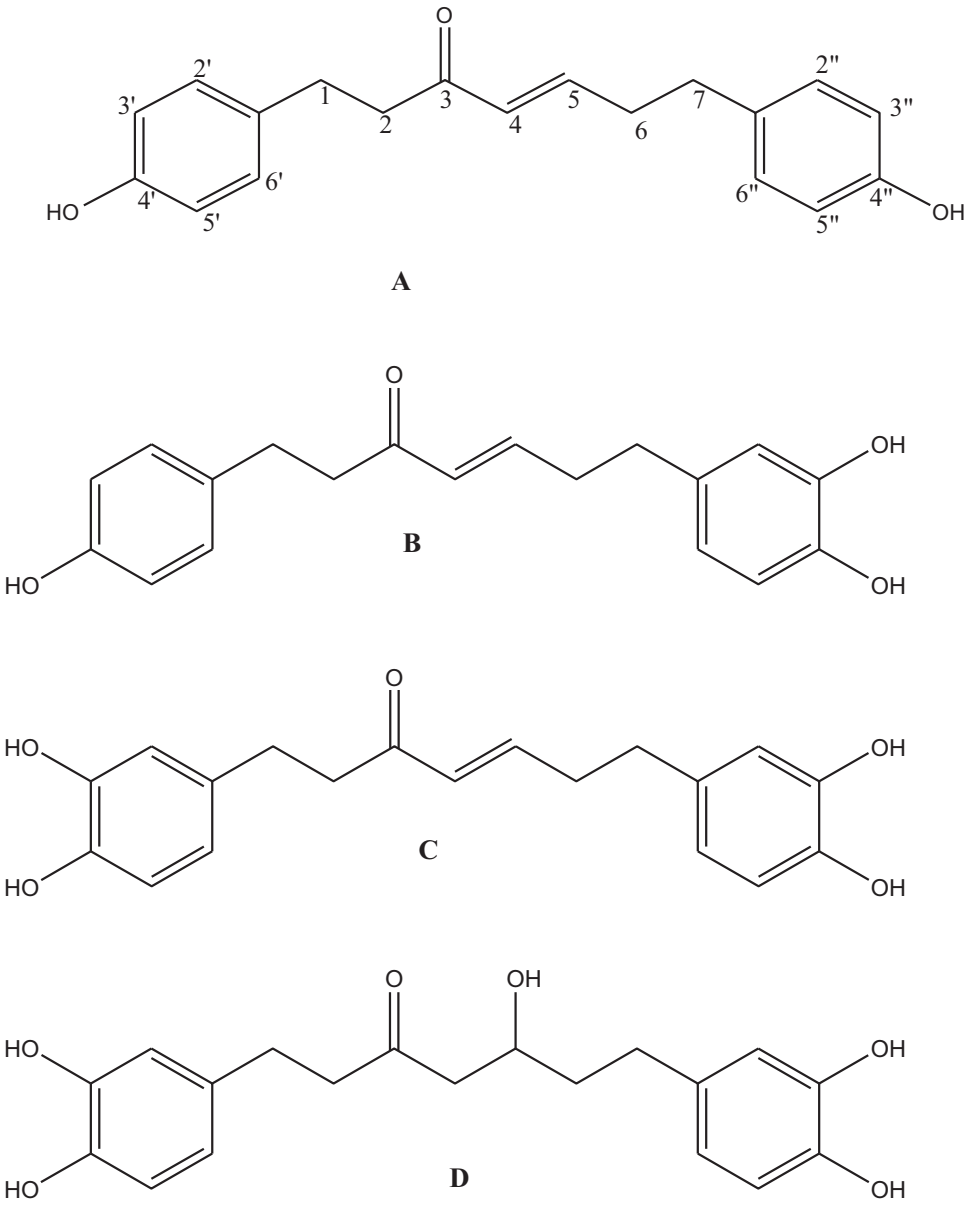

Fig. 1. Chemical structures of compounds **A**–**D**.

are principally microfilaricidal with limited or no action on adult parasites (Sashidhara et al., 2012) and are not devoid of adverse reactions (Sharma 1990; Fan, 1992). While ivermectin mainly affects the late stages of microfilarial development, albendazole has a transient effect on early embryogenesis. Besides, drug resistance to ivermectin appears to be another issue of concern (Singh et al., 2008). Moxidectin though looks promising in animal studies are still under development. In current therapy DEC and ivermectin are given either alone or in combination with albendazole. Advent of Mass Drug Administration (MDA) strategy raised hope for elimination of this disease; however, unfortunately this disorder is continuing due to the technical limitations of MDA strategy (Burkot et al., 2006). This discouraging scenario demands a pressing need for development of new agent(s) that can kill the adult worms and or sterilize the adult worms (Srivastava et al., 2000; Dhananjeyan et al., 2005) since adult parasites are the major fabricator of debilitating pathological lesions apart from producing millions of mf that are picked up by mosquito vector and transmitted. Therefore, we need macrofilaricidal agents, which not only adversely affect the target but also should have very low or no side effect. Several anti-filarial agents have been discovered through research on medicinal plants used by local healers. Therefore, efforts have been made to screen medicinal plants for antifilarial activities. In the present study, we report the isolation, characterization and antifilarial activity of diarylheptanoids from leaves of *A. nepalensis*. This plant is not reported for its antifilarial activity.

In addition, *A. nepalensis* lacks quality control methods for bioactive diarylheptanoids. HPTLC is the method commonly applied for the identification assay and stability study of herbal raw materials and formulations. Currently HPTLC emerge as an alternative to HPLC because of far less solvent consumption, reduction of the analysis time, cost of analysis, besides being eco-friendly. HPTLC method also facilitates repeated detection (scanning) of the chromatograms with same or different parameters. Very few HPTLC work is reported on diarylheptanoids mainly on curcuminoids (Gupta et al., 1999; Paramasivam et al., 2008). To the best of our knowledge there is no report on the simultaneous separation and quantitation of diarylheptanoids viz. hirsutanonol, hirustenone,

alusenone and platyphyllenone which prompted us to develop a validated HPTLC method for the quantification of these important biologically active markers **A**–**D** (Fig. 1).

Materials and methods

Collection of plant material

Leaves of *A. nepalensis* were collected from Kausani, Uttrakhand, India. A voucher specimen (no. 13644) has been deposited in the herbarium of CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow, India.

Extraction and isolation of markers from A. nepalensis

The air-dried powdered leaves of *A. nepalensis* (600 g) were macerated with MeOH (2 L) overnight and evaporated in vacuo to yield crude extract (200 g). This crude extract was suspended in H_2O (600 ml) and extracted successively with hexane (3×500 ml), $CHCl_3$ (3×500 ml) and butanol (3×500 ml). Vacuum concentration yielded hexane extract (32 g), $CHCl_3$ extract (20 g) and butanol extract (90 g). Vacuum liquid chromatography of $CHCl_3$ extract over silica gel eluted with a gradient of hexane–ethyl acetate (each 4.5 L, *n*-hexane–EtOAc 1:0, 19:1, 23:2, 9:1, 17:3, 4:1, 39:11, 3:1, 7:3, 13:7 3:2,) afforded eleven corresponding fractions (C1–11). Fr.6 was subjected to reverse phase preparative HPLC (RP-C18, MeOH:H_2O, 65:35, 15 ml/min) to give compound **A** (t_R 7.6). Fr.8 was subjected to reverse phase preparative HPLC (RP-C18, MeOH:H_2O, 50:50, 15 ml/min) to give compound **B** (t_R 15.6). Fr.9 was also subjected to reverse phase preparative HPLC (RP-C18, MeOH:H_2O, 50:50, 15 ml/min) to give compounds **C** and **D** (t_R 7.5 and 4.7), respectively. HPLC purity of compounds **A**–**D** were found to be 98%, 99%, 99% and 98%, respectively on an analytical HPLC. (LC-10, Shimadzu make; A – MeOH:H_2O, 65:35; B, C, and D – MeOH:H_2O, 50:50)

A–**D** (Fig. 1) were characterized as platyphyllenone, alusenone, hirsutenone and hirsutanonol. All sprectroscopic data are in agreement with these reported elsewhere (Tung et al., 2010a,b; Ohta et al., 1984; Chen et al., 1998; Lee et al., 2000).

Reagents and chemicals

Markers **A**–**D** were isolated in the lab from *A. nepalensis*. TLC plates (Silica gel 60 F_{254}) and silica gel (60–120 mesh) for column chromatography was purchased from m/s Merck (Mumbai, India).

Apparatus

Preparative HPLC (Shimadzu, Japan) consisting column (Supelcosil LC-18, 21.2 mm × 250 mm, 12 μm), pumps LC-8A and PDA detector was used for isolation and purification of reference compounds. Precoated TLC silica gel $60F_{254}$, (10 cm × 10 cm, 20 cm × 10 cm, E. Merck, Darmastadt, Germany) were used for optimization of analytical protocol. Vario System, TLC Scanner winCATS-III, Reprostar 3, twin trough chamber, immersion device III, TLC plate heater (Camag, Muttenz, Switzerland) was used for digital image scanning, method development and validation. NMR spectra were recorded in MeOD with TMS as internal standard using 300 MHz spectrometer (Avance, Bruker, Switzerland). ESI-MS spectra were obtained on LCMS-2010EV (Shimadzu, Japan) hyphenated to LC system (LC-20AD, CTO-20A, SIL-10AF, SPDM20A, and ABM-20A) for authenticity and purity of the reference compounds.

Antifilarial activity

Parasites

Parasites. Sub-periodic strain of *Brugia malayi* was maintained in *Mastomys coucha* and jirds (*Meriones unguiculatus*) through *Aedes aegypti* mosquitoes (Murthy et al., 1983, 1997). Microfilariae (mf) and adult worms were harvested from the peritoneal cavity of the animals, which were exposed to infective larvae (L_3) approximately 5–6 months back. Freshly isolated live parasite stages were washed thoroughly and used for *in vitro* assays (Murthy and Chatterjee, 1999). The Adult worms (10 females and 5 males) were transplanted into peritoneal cavity of the jirds (Gaur et al., 2007).

In vitro assay

Test samples and reference drug diethylcarbamazine (DEC) were dissolved in DMSO or sterile distilled water, respectively. The final concentration of DMSO in the incubation medium was kept below 0.1%. DMSO was used in place of test compounds for controls. Incubation medium used was Hanks Balanced Salt Solution (HBSS; pH 7.2) containing mixture of antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; Sigma, USA).

Initially, efficacy of the test samples was assessed *in vitro* on mf of *B. malayi* using motility assay (Murthy and Chatterjee, 1999; Lakshmi et al., 2010). In the 1st set of experiment, the concentration of the test samples and DEC were used at 1000/500/250 μg/ml (test extracts/fractions/subfractions) or 800/1000 μM (DEC) respectively, in the assay systems. After exposure for 24 h at 37 °C in 5% CO_2 atmosphere, the effect on motility of mf was assessed. The agents causing ≥50% inhibition in motility over untreated control mf were considered active and subjected for secondary *in vitro* screening for assessing IC_{50} value. In secondary screening both mf and adult worms were used using motility and or 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) reduction assays (Murthy and Chatterjee, 1999; Lakshmi et al., 2010). For assessing IC_{50}, adult worms and mf stage of the parasites were incubated with two fold serial dilutions of the test agents and DEC starting from 3.91 to 1000 μg/ml or 1.56–800 μM, respectively using triplicates of 40–50 mf/100 μl/well in 96 well-plate and one adult female worm/ml/well in 48-well-plate (Nunc, Denmark). After assessing motility status the same worms were processed and assessed percent inhibition in MTT reduction potential of the treated worms over control parasites (Murthy and Chatterjee, 1999). For assessing CC_{50}, cells of VERO Cell line C1008 (African green monkey kidney cells) were incubated with a three fold serial dilutions of the test samples and DEC (starting from >20 times greater LC100 of the test sample).

Assessment of in vitro efficacy

The viability of the treated worms was assessed by calculating percent inhibition in motility and MTT reduction over control worms (DMSO treated) as per method described by Murthy and Chatterjee (1999). 100% inhibition in motility of the worms or mf and or ≥50 inhibition in MTT reduction of treated adult parasites were considered acceptable anti-filarial activity of the test samples.

Briefly, parasite motility was assessed under a microscope after 24 h exposure to the test substance and scored as: 0 = dead; 1–4 = loss of motility (1 = 75%; 2 = 50%; 3 = 25% and 4 = no loss of motility) i.e. the worms failed to gain pretreatment level of motility even after transferring the treated worms in fresh medium without any test agent at 37 °C for 25–30 min and expressed as percent inhibition over control. 100% inhibition in motility of the worms or

mf and or ≥50 inhibition in MTT reduction ability of adult parasites was considered acceptable antifilarial activity.

Determination of inhibitory conc.50 (IC_{50}) and cell cytotoxicity50 (CC_{50})

IC_{50} (the concentration of the test agent at which the motility of the parasites was inhibited by 50%) and CC_{50} were determined as per methods described by Lakshmi et al. (2010), Page et al. (1993) and Mosmann (1983). Data were transferred into a graphic program (Excel); IC_{50} and CC_{50} were calculated by linear interpolation between the two concentrations above and below 50% inhibition (Huber and Koella, 1993).

Selectivity Index (SI) of the agents were computed by the formula $SI = CC_{50}/IC_{50}$.

In vivo efficacy

Host–parasite models

All the experiments in animals were conducted in compliance with the Institutional Animal Ethics Committee guidelines for use and handling of animals. Throughout the study, the animals were housed in climate (23 ± 2 °C; RH: 60%) and photoperiod (12 h light–dark cycles) controlled animal quarters. Both the species of animals were fed standard rodent chow. In addition, dried shrimps were also given to *M. coucha* only. The animals had free access to drinking water.

Jirds infected with sub-periodic strain of *B. malayi* was used for present study.

The animals were autopsied under deep anesthesia (as per guide lines of Animal Ethics Committee).

Administration of the test agents and reference drug

The agents were pulverized to fine powder and suspended in 0.1% Tween-80 prepared in distilled water whereas DEC was prepared in distilled water. In jirds the test agent(s) were administered at 100 mg/kg body weight through s.c. route for 5 consecutive days while DEC was given to them at 25 mg/kg body weight. The suspensions/solutions of the agents were prepared daily before administration to the animals.

B. malayi–M. unguiculatus model

Male jirds of 8–10 weeks old were transplanted intraperitoneally with freshly isolated adult worms from peritoneal cavity of the jirds harboring 5–6 months old *B. malayi*. (Gaur et al., 2007). Each animal received 10 female and 5 male adult worms. On day 2 or 3 post-adult worm transplantation (p.a.t.), the peritoneal fluid was aspirated and checked for the presence of mf. The treatment was started on day 7 or 8-p.a.t. The animals were killed on day 42 post initiation of treatment (p.i.t.) and parasites recovered were collected and counted.

Assessment of microfilaricidal efficacy

Microfilaricidal efficacy of each test agent was evaluated on days 7/8 and 14 post intiation of the treatment (p.i.t.) and expressed as percent reduction in mf count over the pretreatment level (Chatterjee et al., 1992; Gaur et al., 2007; Lämmler and Wolf, 1977).

Assessment of macrofilaricidal and worm sterilization efficacy

Macrofilaricidal/adulticidal efficacy of the agents was assessed and expressed as percent reduction in adult worm recovery in treated group over untreated animals. Treated and untreated animals were killed on day 42 since start of the treatment to recover adult worms from the peritoneal cavity washings. Parasites were examined for their motility, cell adherence on their surface, death, or encapsulation under microscope and all the surviving females were teased individually in a drop of saline to examine the condition of intrauterine stages (Chatterjee et al., 1992; Gaur et al., 2007).

Percent mortality or female worm sterilization were assessed on the basis of the results obtained from untreated control animals.

Table 1
Extraction efficiency of different solvent for chemical markers.[a]

Solvent	Amount of compounds quantified[b] (%, w/w)			
	A	**B**	**C**	**D**
Hexane	ND	ND	ND	ND
Chloroform	0.10	0.14	ND	ND
Ethylacetate	0.17	0.17	0.94	0.51
Acetone	0.23	0.21	1.13	0.64
Methanol	0.27	0.32	1.18	0.75

[a] Microwave extraction at 50 °C and 350 W for 10 min.
[b] Plant dry weight basis.

Table 2
Extraction efficiency of different techniques for chemical markers.[a]

Techniques[c]	Amount of compounds quantified[b] (%, w/w)			
	A	**B**	**C**	**D**
Cold	0.27	0.32	1.18	0.75
Hot	0.28	0.35	1.36	0.87
Sonication	0.27	0.31	1.49	0.88
Microwave	0.32	0.36	1.59	0.92

[a] Plant dry weight basis.
[b] Methanol was used as solvent.
[c] Cold, kept for 24 h at 30 °C; sonication, for 30 min at 50 °C; hot, boiling water bath for 30 min; microwave, 50 °C and 350 W for 10 min.

Statistical analysis

Statistical analyses were carried out using GraphPad Prism 3.0 version software. Results were presented as mean ± S.D. of data obtained from 4 to 6 animals in two experiments. Student's '*t*' test was used to analyze the statistical significance of the data. Significance was established at $P < 0.05$ level.

Standard solution and sample preparation

Powdered leaves (100 mg) were extracted with 10 ml different solvents by microwave extraction method at 50 °C and 350 W for 10 min to choose most suitable solvent for further analysis. Methanol was preferred due to high extractive value (Table 1). Different techniques like cold percolation (kept for 24 h at 30 °C), microwave extraction (at 50 °C and 350 W for 10 min), sonication (for 30 min at 50 °C) and hot extraction (at boiling water bath for 30 min) methods were applied in order to improve the quantity of the extracted compounds. Microwave extraction was found to be most appropriate (Table 2). The extracts were concentrated on a rotavapour to afford different extracts. The standards solution of markers (**A**–**D**) were prepared by dissolving accurately weighed amount in methanol as a stock solution (1 mg/ml) and stored at 4 °C. These standards were further diluted and mixed as per the requirements to obtain a desired concentration for quantification. The concentrations of reference standards used for calibration were in the range of 250–2000 ng/band for **A**–**D.** The known volumes of mixed standards were spotted on the TLC plates to prepare 6 points calibration curves.

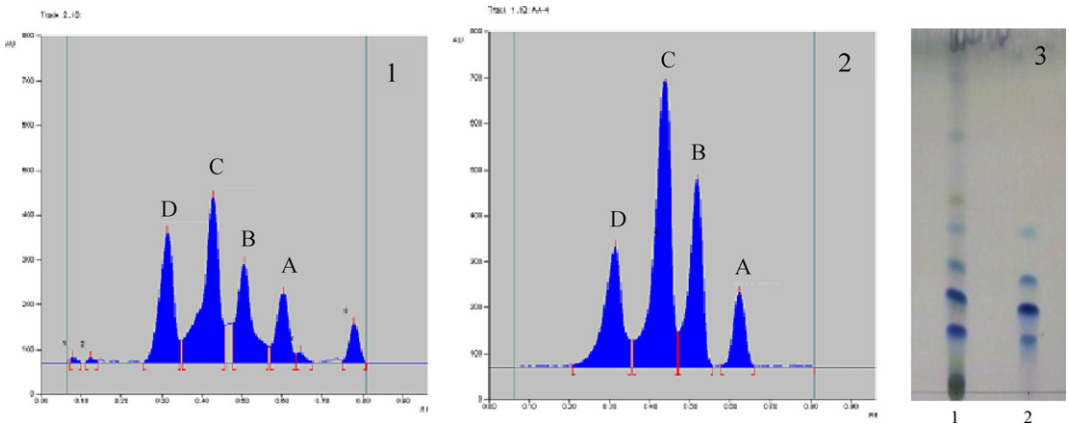

Fig. 2. The representative HPTLC chromatograms (**1** and **2**) and photo-documentation (**3**) after derivatization of *Alnus nepalensis* leaf extract (**1**) and mixture of standards (**2**).

Chromatographic experiments

Precoated silica gel $60F_{254}$ TLC plates were prewashed using methanol and activated at 60 °C for 5 min, prior to chromatography. Standard and sample solutions were spotted in the form of spots 10 mm from both the lower and left edge and 10 mm space between two spots, with a microlitre syringe using an automatic TLC Sampler, under continuous nitrogen current. A constant application rate of 5 μl/min was employed. Linear ascending development was carried out in twin trough glass chambers saturated with the mobile phase. The mobile phase selection was carried out using the Vario System wherein different compositions consisting of different ratios of solvents of varying polarity were tried. Finally, a mobile phase consisting of chloroform:methanol (9:1, v/v) was optimized for quantitative chromatography. The saturation time of the TLC chamber in the mobile phase was optimized to 5 min for a better resolution of the tested markers. After development, the plates were dried and dipped into vanillin–sulphuric acid reagent (vanillin:EtOH:H_2SO_4, 5.0 g:475 ml:25 ml) using the Immersion device (dipping time 2 s, dipping speed 5 cm s^{-1}) followed by air drying for 5 min. The plates were then heated for 5 min at 110 °C using TLC plate heater-III (Camag) and quantified densitometrically at 600 nm. TLC Scanner-III controlled by win CATS 1.4.2.8121 software was used for quantitative evaluation. The densitometry scanning was performed in the reflectance/absorbance mode: slit width 6.00 mm × 0.45 mm, scanning speed 20 mm s^{-1} and data resolution 10 m $step^{-1}$. Savitsky-Golay-7 was used for data filtering and the lowest slope for baseline correction in order to integrate the area. For recording of characteristic derivatized spot of compounds and sample tracks in the range 400–700 nm, tungsten lamp was used. Reprostar 3 with cabinet cover and mounted digital camera (Power Shot G5 with Neck Strap NS-DC2, Canon, Japan) was used for imaging and archiving the thin layer chromatograms. Quantitation was performed using area under peak with linear regression of amount μg/band. Peak profiling was performed in visible region after derivatization (Fig. 2).

Method validation

Specificity

The specificity, expressed as the ability of the method to assess the analyte clearly in the presence of other compounds from analyzing sample, was confirmed by comparing the R_f of analytes and standards. Good correlation was also obtained between standards and sample overlay spectra (>0.999). Peak resolutions and quantitation of the fortified peaks (see recovery) of individual **A**–**D** were taken in additional indications of the specificity.

Linearity

For providing linearity six calibration points were analyzed over the range of 250–2000 ng/band for **A**–**D**. Standard solution was applied in triplicates and analyzed using the TLC method described in the experimental part.

LOD and LOQ

The calibration graphs were constructed by plotting the peak area vs concentration of **A**–**D,** and the regression equations were calculated using the least square method in Graph PAD Prism 4 (Table 3). The calibration graphs were plotted over the concentration range 250–2000 ng for **A**–**D**. Aliquots of the standard working solutions of markers were applied in triplicate to the plates**.**

Precision

Repeatability of the method was validated as instrumental, intra-assay and inter-assay precisions. Instrumental precision was measured for 5 spots for each of the reference compounds at three different concentrations. Intra-assay precision was studied by analyzing five individual spots for each compound applied onto different plates on the same day in the same lab. Inter-assay precision included analysis of the same three concentrations of each solution, analyzed five times on the same day for three different days. The intra and inter-day variation study in the analysis of marker compounds was determined at the LOQ level. The results depicted in Table 3 showed no significant inter and intra-day variation in the analysis of the compounds **A**–**D**.

Accuracy

The accuracy of the method was tested by performing recovery of the compounds in the sample at three levels. Pre quantified methanolic extract of *A. nepalensis* was spiked with known amount of each compound and analyzed. The recovery percentage was calculated by using the formula: recovery (%) = (amount found − original amount)/amount spiked × 100% (Table 4).

Table 3
Statistical analysis of calibration curves in the HPTLC determination of diarylheptanoids.[a,b,c]

Parameters	Compounds			
	A	**B**	**C**	**D**
R_f value	0.58 ± 0.02	0.47 ± 0.04	0.38 ± 0.03	0.26 ± 0.02
Slope	2102 ± 24.36	1149 ± 16.20	2159 ± 35.04	3125 ± 35.56
95% confidence limits of slope	2025–2180	1098–1201	2047–2270	3011–3238
Intercept	5223 ± 80.78	7223 ± 53.71	4161 ± 116.2	2788 ± 117.9
95% confidence limits of intercept	4966–5480	7052–7394	3791–4531	2412–3163
F (value of Fisher test)	7450	5035	3794	7721
Goodness of fit ($S_{y,x}$)	77.02	51.21	110.80	112.40
Coefficient of determination (r^2)	0.9996	0.9994	0.9992	0.9996
LOD (ng)	110	134	154	108
LOQ (ng)	336	446	513	360
Repeatability (% RSD)	1.05	1.15	1.07	1.03
Reproducibility (% RSD)	0.98	1.06	1.21	1.19

[a] Concentration range: 250–2000 ng.
[b] Number of concentration levels – 6.
[c] For all equations the significance levels $P < 0.0001$.

Table 4
Recovery study to evaluate accuracy of the method.

Compounds	Amount in sample (ng)	Spiked amount (ng)	Recovery%	Average recovery	RSD%
A	320	250	93.41	95.94	2.43
		500	96.43		
		750	97.99		
B	360	250	101.2	99.67	1.45
		500	98.35		
		750	99.47		
C	1600	250	95.22	95.91	2.56
		500	93.87		
		750	98.65		
D	920	250	96.22	98.59	2.21
		500	98.87		
		750	100.70		

Robustness

Robustness is a measure of the capacity of a method to remain unaffected by small but deliberate variations in the method conditions, and is an indication of the reliability of the method. The amount of mobile phase, composition of mobile phase, temperature, derivatising time, heating time and the chamber saturation time were varied in the range ± 5% and the effects on the analytical values were studied. The effect of variation in chamber dimension (20 × 20 and 10 × 10) was also studied by varying one parameter at a time. The S.D. of peak areas was calculated for each parameter and the% R.S.D. was found to be less than 2% in each case. The low values of % R.S.D. show the robustness of the method (Table 5).

Results and discussion

Tested diarylheptanoids revealed promising *in vitro* activity on both adult parasite and mf. Compounds **A** and **C** showed better efficacies as indicated by complete inhibition in motility of adult worms (LC100: 15.63 μg/ml) and >50% inhibition in reduction potential of MTT by treated adult parasites *in vitro* (Table 6); their IC_{50} values were found to be 11.05 and 4.55 μg/ml for adult worms and 11.05 and 44.19 μg/ml for mf, respectively. Compound **B** and **D** showed low *in vitro* antifilarial activity as pointed out by comparatively higher IC_{50} values. The standard drug DEC killed adult worms (LC100: 800; IC_{50}: 289 μg/ml) and mf (LC100: 500; IC_{50}: 354 μg/ml) at very high concentrations (Table 6).

Compound **C** exhibited promising *in vitro* adulticidal activity as evidenced by low LC100 and IC_{50} values, therefore followed up for *in vivo* testing in jird model harboring transplanted adult worms of *B. malayi*. The test agent was administered in animals at 100 mg/kg, s.c. for 5 days. Compound **C** killed around 57% ($P < 0.001$) of the transplanted worms showing either totally inactive or calcified at one or other end; these worms were considered dead. An insignificant embryostatic activity was noticed in female worms. DEC administered at 25 mg/kg for 5 days by the same route exerted 23% adulticidal activity (statistically not significant as compared to untreated controls) with 11% sterilizing effect on the female worms. None of the worms recovered from DEC treated animals was found to show calcification (Table 7).

The developed TLC method was validated according to the guidelines of the International Conference on Harmonization (ICH)

Table 5
Robustness testing of the HPTLC method.

Parameters	RSD of compounds (peak area %)			
	A	**B**	**C**	**D**
Mobile phase composition	0.42	0.49	0.69	0.58
Time gap between spotting and plate development	0.25	0.32	0.24	0.38
Scanning wavelength ± 5 nm	1.23	1.56	1.95	0.98
Chamber saturation time	0.45	0.42	0.38	0.56

Table 6
In vitro activity of *Alnus nepalensis* on adult worms and microfilariae of *Brugia malayi*.

Antifilarial agent	Effect on female adult worm			Effect on microfilariae (Mf)		CC_{50}[c] (mg/ml)	SI w.r.t. adults motility	SI w.r.t. Mf motility
	LC100[a] (μg/ml) in motility assay	IC_{50} (μg/ml) in motility assay[b]	Mean % inhibition in MTT	LC100 (μg/ml) in motility assay	IC_{50} (μg/ml) in motility assay[b]			
A	15.63	11.05	61	31.25	11.05	ND	ND	ND
B	31.25	18.58	46	62.50	44.19	ND	ND	ND
C	15.63	4.55	59.22	125	44.19	6.40	1406.59	144.83
D	>250	ND	32.95	125	44.11	ND	ND	ND
DEC-citrate[d]	800	289	62	500	354	9103	31.50	25.70

SI, selectivity index (CC_{50}/IC_{50}); w.r.t., with respect to; DEC, diethylcarbamazine.
[a] 100% reduction in motility indicates death of parasite.
[b] 50% concentration of the agent at which 50% inhibition in motility is achieved.
[c] Concentration at which 50% of cells are killed.
[d] In μM.

Table 7
Antifilarial activity of *Alnus nepalensis* and DEC against *Brugia malayi* in jirds (*Meriones unguiculatus*). Values are mean ± SD.

Compound no.	Dose in mg/kg s.c. × 5 days (*n*)	Status of microfilaraemia in peritoneal cavity on day 7 post initiation of treatment	No. of worms recovered (% mortality over untreated control)	% Ster. female worms
C	100 (5)	Active	5.80 ± 1.30 (56.50[a])	17
DEC-citrate	25 mg/kg (6)	Active	10.3 ± 2.90 (22.73[NS])	11
Untreated control	Vehicle treated (4)	Active	11 ± 1.53	0

n, number of animals. DEC, diethylcarbamazine; NS, not significant over untreated control (Student's '*t*' test).
[a] $P < 0.001$ (vs untreated control; Student's '*t*' test).

(ICH, 2005) and the IUPAC (IUPAC, 2002) for the following parameters: specificity, linearity, range, LOD, LOQ, precision, robustness and accuracy. The R_f values of **A**–**D** were 0.58, 0.47, 0.38 and 0.26. A linear relationship was found for the 250–2000 ng/spot concentration range for **A**–**D**. The coefficients of correlation (r^2) 0.9996, 0.9994, 0.9992 and 0.9994 revealed a good linearity response for developed method. The LOD values were found to be 110, 134, 154 and 108 ng/band for compounds **A**–**D,** respectively, whereas LOQ values were 336, 446, 513 and 360 ng/band, respectively (Table 3). The results show a high sensitivity of the method for the investigated test compounds (A–D). The average recoveries for compound **A**–**D** were found to be 95.9%, 99.7%, 95.9% and 98.6% respectively within the acceptable RSD%. Good recoveries are in support of accuracy of method. (Table 4). The results depicted in Table 3 showed no significant inter and intra-day variation in the analysis of the test compounds**.** The low values of %R.S.D. show the robustness of the method (Table 5). Taking into account that the validation parameters were found within the recommended limits, the proposed method is suitable for the quantification of test markers from real samples.

Conclusion

It may be summarized that compound **C** possessed promising antifilarial activity against *B. malayi*. This is the first ever report on the adulticidal activity of a test compound isolated from *A. nepalensis*. The present findings appear important in view of the observed efficacy against a human lymphatic filarial species. Our products are diarylheptanoids, which are entirely different from currently available three synthetic drugs diethylcarbamazine (DEC), which is a piperazine derivative, ivermectin, which is a macrocyclic lactone and albendazole, which is a benzimidazole.

Most importantly, our diarylheptanoids showed macrofilaricidal (adult worm killing) activity. This activity is absent in the available synthetic drugs mentioned above which are mostly microfilaricidal agents. Killing adult worms is considered as a superior filarial control strategy (WHO recommends new drug discovery directed to this strategy) since killing one adult worm will prevent production of thousands of mf that circulate in blood and are responsible for transmission of infection (mosquitoes that transmit infection take-up mf only during their blood meal).

Thin layer chromatography is globally accepted practical solution to characterize the raw herbs, active constituent enriched extracts and their formulation. The method reported here is very simple, rapid and suitable for the rapid screening of *A. nepalensis* for chemical profile. The assay can be applied without any special pre-treatment of the sample. All four compounds can be assayed on a single plate with a single solvent system and at a single scanning wavelength (600 nm). A large number of samples can be analyzed at the same time without compromising accuracy. Moreover, the chromatographic patterns evidenced that the extraction techniques influence only the quantitative results, not the qualitative ones. The developed method demonstrates once again that using appropriate resources, TLC combined with photo documentation and image processing can be a powerful analytical tool.

Conflict of interest

The authors have no conflict of interest to report.

Acknowledgements

The authors thank the Directors, CSIR-CIMAP and CSIR-CDRI, Lucknow, for their keen interest and encouragement during the course of work. This work was supported by a Network Project (NWP0037) of Council of Scientific and Industrial Research (CSIR), New Delhi. Ms. Deepti Yadav and Richa Verma are thankful to CSIR, New Delhi for the award of Senior Research Fellowship and Ms. Kirti Saxena to the Network Project NWP0037 for financial assistance as a project assistant.

References

Burkot, T.R., Durrheim, D.N., Melrose, W.D., Speare, R., Ichimori, K., 2006. The argument for integrating vector control with multiple drug administration campaigns to ensure elimination of lymphatic filariasis. Filaria 5, 10.

Changkija, S., 1999. Folk medicinal plants of the Nagas in India. Asian Folklore Studies, 205–230.

Chatterjee, R.K., Fatma, N., Murthy, P.K., Sinha, P., Kulshreshtha, D.K., Dhawan, B.N., 1992. Macrofilaricidal activity of the stem bark of *Streblus asper* and its major active constituents. Drug Development Research 26, 67–78.

Chauhan, V.S., Misra, A.K., 2002. Development of molecular markers for screening of *Alnus nepalensis* (D. Don) genotypes for the nitrogenase activity of actinorhizal root nodules. Molecular Genetics and Genomics 267, 303–312.

Chen, J., Karchesy, J.J., Gonzalez-Laredo, R.F., 1998. Phenolic Diarylheptenones from *Alnus rubra* Bark. Planta Medica 64, 74–75.

Choi, S.E., Kim, K.H., Kwon, J.H., Kim, S.B., Kim, H.W., Lee, W.M., 2008. Cytotoxic activities of diarylheptanoids from *Alnus japonica*. Archives of Pharmacal Research 31, 1287–1289.

Dhananjeyan, M.R., Milev, Y.P., Kron, M.A., Nair, M.G., 2005. Synthesis and activity of substituted anthraquinones against a human filarial parasite, *Brugia malayi*. Journal of Medicinal Chemistry 48, 2822–2830.

Fan, P.C., 1992. Diethylcarbamazine treatment of bancroftian and malayan filariasis with emphasis on side effects. Annals of Tropical Medicine and Parasitology 86, 399–405.

Gaur, R.L., Dixit, S., Sahoo, M.K., Khanna, M., Singh, S., Murthy, P.K., 2007. Anti-filarial activity of novel formulations of albendazole against experimental brugian filariasis. Parasitology 134, 537–544.

Gupta, A.P., Gupta, M.M., Kumar, S., 1999. Simultaneous determination of curcuminoids in *Curcuma* samples using high performance thin layer chromatography. Journal of Liquid Chromatography and Related Technologies 22, 1561–1569.

Huber, W., Koella, J.C., 1993. A comparison of three methods of estimating EC50 in studies of drug resistance of malaria parasites. Acta Tropica 55, 257–261.

ICH, 2005. Q2 Proceeding of the International Conference on Harmonization , November, Step 4 Version: Geneva.

IUPAC, 2002. Harmonized guidelines for single-laboratory validation of methods of analysis (IUPAC technical report). Pure and Applied Chemistry 74, 835–855.

Jin, W.Y., Cai, X.F., Na, M.K., Lee, J.J., Bae, K.H., 2007. Triterpenoids and Diarylheptanoids from *Alnus hirsuta* Inhibit HIF-1 in AGS Cells. Archives of Pharmacal Research 30, 412–418.

Joo, S.S., Kim, S.G., Choi, S.E., Kim, Y.B., Park, H.Y., Seo, S.J., Choi, Y.W., Lee, M.W., Lee, D.I., 2009. Suppression of T cell activation by hirsutenone, isolated from the bark of *Alnus japonica*, and its therapeutic advantages for atopic dermatitis. European Journal of Pharmacology 614, 98–105.

Kim, H.J., Yeom, S.H., Kim, M.K., Shim, J.G., Paek, I.N., Lee, M.W., 2005. Nitric oxide and prostaglandin E2 synthesis inhibitory activities of diarylheptanoids from the barks of *Alnus japonica* Steudel. Archives of Pharmacal Research 28, 177–179.

Kuroyanagi, M., Shimomae, M., Nagashima, Y., Muto, N., Okuda, T., Kawahara, N., Nakane, T., Sano, T., 2005. New diarylheptanoids from *Alnus japonica* and their antioxidative activity. Chemical and Pharmaceutical Bulletin 53, 1519–1523.

Lakshmi, V., Joseph, S.K., Srivastava, S., Verma, S.K., Sahoo, M.K., Dube, V., Mishra, S.K., Murthy, P.K., 2010. Antifilarial activity *in vitro* and *in vivo* of some flavonoids tested against *Brugia malayi*. Acta Tropica 116, 127–133.

Lämmler, G., Wolf, E., 1977. Chemoprophylactic activity of filaricidal compounds on *Litomosoides carinii* infection of *Mastomys natalensis* (author's transl). Tropenmed Parasitol 28, 205–225.

Lee, C.S., Ko, H.H., Seo, S.J., Choi, Y.W., Lee, M.W., Myung, S.C., Bang, H., 2009. Diarylheptanoid hirsutenone prevents tumor necrosis factor-α-stimulated production of inflammatory mediators in human keratinocytes through NF-κB inhibition. International Immunopharmacology 9, 1097–1104.

Lee, M.W., Park, M.S., Jeong, D.W., Kim, K.H., Kim, H.H., Toh, S.H., 2000. Diarylheptanoids from the leaves of *Alnus hirsuta* Turcz. Archives of Pharmacal Research 23, 50–53.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65, 55–63.

Murthy, P.K., Chatterjee, R.K., 1999. Evaluation of two in vitro test systems employing *Brugia malayi* parasite for screening of potential antifilarials. Current Science 77, 1084–1089.

Murthy, P.K., Murthy, P.S.R., Tyagi, K., Chatterjee, R.K., 1997. Fate of infective larvae of *Brugia malayi* in the peritoneal cavity of *Mastomys natalensis* and *Meriones unguiculatus*. Folia Parasitologica (Praha) 44, 302–304.

Murthy, P.K., Tyagi, K., Roy Chowdhury, T.K., Sen, A.B., 1983. Susceptibility of *Mastomys natalensis* (GRA strain) to a subperiodic strain of human *Brugia malayi*. Indian Journal of Medical Research 77, 623–630.

Ohta, S., Aoki, T., Hirata, T., Suga, T., 1984. The structures of four diarylheptanoid glycosides from the female flowers of *Alnus serrulatoides*. Journal of the Chemical Society, Perkin Transactions 1, 1635–1642.

Page, C., Page, M., Noel, C., 1993. A new fluorimetric assay for cytotoxicity measurements *in vitro*. International Journal of Oncology 3, 473–476.

Pande, P.C., Tiwari, L., Pande, H.C., 2006. Folk-Medicine and Aromatic Plants of Uttaranchal. Bishen Singh, Mahendra Pal Singh Publishers, New Connaught Place, Dehradun, India.

Paramasivam, M., Wasim Aktar, Md., Poi, R., Banerjee, H., Bandyopadhyay, A., 2008. Occurrence of curcuminoids in Curcuma longa: quality standardization by HPTLC. Bangladesh Journal of Pharmacology 3, 55–58.

Park, D., Kim, H.J., Jung, S.Y., Yook, C.S., Jin, C., Lee, Y.S., 2010. A new diarylheptanoid glycoside from the stem bark of *Alnus hirsuta* and protective effects of diarylheptanoid derivatives in human HepG2 Cells. Chemical and Pharmaceutical Bulletin 58, 238–241.

Sashidhara, Koneni, V., Kumar, A., Bhaskara Rao, K., Kushwaha, V., Saxena, K., Murthy, P.K., 2012. In vitro and in vivo antifilarial activity evaluation of 3,6-epoxy [1,5]dioxocines: A new class of antifilarial agents. Bioorganic and Medicinal Chemistry Letters 22, 127–132.

Sharma, S., 1990. Vector borne diseases. Progress in Drug Research 35, 365.

Singh, B.K., Mishra, M., Saxena, N., Yadav, G.P., Maulik, P.R., Sahoo, M.K., Gaur, R.L., Murthy, P.K., Tripathi, R.P., 2008. Synthesis of 2-sulfanyl-6-methyl-1,4-dihydropyrimidines as a new class of antifilarial agents. European Journal of Medicinal Chemistry 43, 2717–2723.

Srivastava, S.K., Chauhan, P.M.S., Bhaduri, A.P., Fatima, N., Chatterjee, R.K., 2000. Quinolones: novel probes in antifilarial chemotherapy. Journal of Medicinal Chemistry 43, 2275–2279.

Tung, N.H., Kim, S.K., Ra, J.C., Zhao, Y.Z., Sohn, D.H., Kim, Y.H., 2010a. Antioxidative and hepatoprotective diarylheptanoids from the bark of *Alnus japonica*. Planta Medica 76, 626–629.

Tung, N.H., Kwon, H.J., Kim, J.H., Ra, J.C., Ding, Y., Kim, J.A., Kim, Y.H., 2010b. Anti-influenza diarylheptanoids from the bark of *Alnus japonica*. Bioorganic and Medicinal Chemistry Letters 20, 1000–1003.