Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading doi:

Antifilarial activity of ethyl acetate extract of *Vitex negundo* leaves *in vitro*

KN Sahare, V Singh*

Department of Microbiology, Barkatullah University, Bhopal-462 026, M.P., India

ARTICLE INFO

Article history:
Received 10 June 2013
Received in revised form 15 July 2013
Accepted 15 August 2013
Available online September 2013

Keywords:
Antifilarial activity
In vitro assay
Medicinal plant
Setaria cervi
Vitex negundo

ABSTRACT

Objective: To evaluate the possible antifilarial effect of ethyl acetate extract of *Vitex negundo* (Verbenaceae) leaves against *Setaria cervi* filarial parasite *in vitro*. **Methods:** *In vitro* screening was done by the method of motility inhibition and MTT reduction assay with concentrations of 0.03 to 1.00 mg/mL for 2 to 24 h incubation periods respectively, for possible antifilarial effect by comparing with control. **Results:** In motility assay, complete inhibition of motility was observed and in MTT reduction assay which gave >50% reduction for concentrations 0.20, 0.50 and 1.00 mg/mL at 10, 6 and 2 h incubation periods respectively in a dose dependent manner (P<0.05). An antifilarial effect imparted by plant extract was found to be a function of their relative concentrations. Inhibitory concentration (IC_{50}) for the plant extract was found to be 0.16 mg/mL. **Conclusions:** The present study recorded significant antifilarial effect of *Vitex negundo* plant extract and contributed toward the development of database for novel drug candidates for lymphatic filariasis.

1. Introduction

Filariasis is a vector-borne parasitic disease affecting millions of people and is the second leading cause of long-term and permanent disability in the world. In India, around 45% of its 1-billion plus population lives in known endemic areas[1] and 48 million are infected[2], accounting for 40% of the global filariasis burden[3]. Socioeconomic studies showed that the annual loss caused by this disease is near to a billion U.S. dollars[4]. However, considering the filariasis as a public health problem, relatively few drugs are available for its treatment. The most widely employed drug in the treatment of lymphatic filariasis for decades is diethylcarbamazine in India and ivermectin is recommended in areas of Africa for onchocerciasis. Though both drugs have activities against microfilariae in interrupting transmission of the disease, they are less effective against the adult worm[5,6]. Adult worms may live for several years in the infected individual[7], producing microfilariae and thereby facilitating transmission of the disease through the vector mosquitoes to healthy person. Hence, elimination of the parasite by means of microfilariae alone is extremely difficult. This warrants an effective and safe drug targeted against the adult filarial worm.

Herbal medicines from plant sources have extensive past and present use in treatment of various diseases. The importance of natural products in modern medicine has been well acknowledged. Variety of herbal drugs originate from natural products[8]. Plant *Vitex negundo* (*V. negundo*; Verbenaceae) is an important source of such natural drugs. It is a reputed medicinal herb and its parts have been employed as a traditional cure in Asian systems of medicine (Indian, Chinese, and Malaysian) for a variety of disease conditions. A number of pharmacological activities have been attributed to *V. negundo*, such as analgesic and anti-inflammatory activity[9], enzymes inhibition[10], nitric oxide scavenging activity[11], snake venom neutralization activity[12], anti-feeding activity[13], anti-radical and anti-lipoperoxidative[14], CNS activity[15], hepatoprotective activity[16], anti-bacterial activity[17], anti-fungal[18], larvicidal activity[19], anti-androgenic effects[20], mosquito repellent activity[21], anti-microfilarial[22-25], anti-amnesic activity[26], anti-inflammatory activity[27] and mast cell stabilizing activity[28]. Here we report *in vitro* macrofilaricidal activity of ethyl acetate extract of *V. negundo* leaves against adult *Setaria cervi* (*S. cervi*) filarial parasite.

*Corresponding author: V Singh, Associate Professor & Head, Department of Microbiology, Hoshangabad Road, Barkatullah University, Bhopal-462 026 (M.P.), India.
Tel: +91755 4242581
E-mail: vinodsingh63@yahoo.co.in

2. Materials and methods

2.1. Procuring plant material

Leaves of plant *V. negundo* were collected from the local areas of Bhopal (M.P.) and identified taxonomically by expert Botanist Dr Zia-Ul Hasan, Department of Botany, Safia Science College, Bhopal (M.P.). Voucher specimen (no. 410/Bot/Safia/2012) was deposited in department. Plant materials were washed in tap water, dried in shade and powdered.

2.2. Extraction

Leaves (1.5 kg) of *V. negundo* was extracted successively with petroleum ether (60-80 ℃) (Qualigens Fine Chem, Mumbai, SQ-grade), chloroform (Ranchem, Mumbai, LR-grade), ethyl acetate (Merk India, Syntheisis Grade) and methanol (Ranchem, Mumbai, AR-Grade) by percolation method[29,30].

2.3. Parasite

Adult *S. cervi* were obtained from the peritoneal cavity of freshly slaughtered cattle. The worms were washed repeatedly with normal saline (8.5 g/L) to free them from any extraneous material and used for assay.

2.4. In-vitro motility inhibition assay

The worms were transferred immediately to DMEM (Dulbecco's modified eagle's medium) (Hi-Media, Mumbai, India) with 0.01% strepto-penicillin (Hi-Media, Mumbai, India) and supplemented with 10% (v/v) heat-inactivated fetal calf serum (Hi-Media, Mumbai, India). Dilutions of the extract of *V. negundo* were made in DMSO (Dimethyl sulphoxide) (Merck India, drug use grade) in such a way that 100 μL of which, when distributed to sterile disposable Petri dishes (35-mm diameter and 5-mL capacity) containing 3 mL medium, would give the required test concentration. Screening was done at concentrations ranging from 0.03 to 1.00 mg/mL. A simultaneous control was kept without the test solution but with 100 μL DMSO in 3 mL of the medium. Two worms (one male and one female) were introduced into each Petri dish. Three replicates each were set up for both test and control. The worms were incubated at 37 ℃ for 24 h in 5% (v/v) CO_2 incubator and motility observed after 2 to 24 h. After exposure, the worms were washed twice with fresh medium and transferred to another set of fresh Petri dish containing fresh medium without the test solution to find out whether any of the immotile worms regained motility in the 2 h post treatment period in drug free medium. If the worms did not revive, the condition was considered as irreversible and the concentration lethal. Each experiment was repeated three times[31].

2.5. MTT-reduction assay

Effect of the plant extract on adult female *Setaria* worms was studied by MTT [3-(4,5dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Hi-media, Mumbai, India)-farmazan reduction assay following the method described by Comely *et al*[32]. Because of the scarcity of male worms, only female worms were used for these tests. The parasites were further incubated for 30 min individually in 0.5 mL phosphate buffered saline (pH 7.4) containing 0.25 mg/mL MTT. At the end of the incubation, worms were carefully transferred to a microtiter plate containing 400 μL of DMSO (Hi-media, Mumbai, India, Spectroscopic grade) and allowed to be at room temperature for 1 h, with occasional gentle shaking to extract the color developed. The absorbance of the resulting formazan solution was then determined at 492 nm in an enzyme-linked immunosorbent assay reader (LISA plus, Microtitre plate Reader) relative to DMSO blank. High values of absorption correlate with high viability of the worms. Positive control was set up with adult females not treated with the test solution but exposed to DMSO as described in the above experiment. Adult worms that had previously been heat killed (56 ℃ for 30 min) and incubated with MTT served as the negative control. Viability of the worms was estimated as percentage inhibition in formazan formation relative to solvent controls and heat killed worms[33] by following the formula:

$$\text{Percentage inhibition} = 100-[(T-H)/(C-H)]\times 100. \quad (1)$$

Where, T, C, and H are absorbance values obtained for the formazan produced in treated, control, and heat killed worms, respectively. The percentage inhibition >50% was considered significant.

2.6. Statistical analysis

For comparison of results between extract and respective controls, Student's t test was used. $P<0.05$ was considered as significant.

3. Results

3.1. Obtainment of plant extract

The solvent removed from the plant extract under reduced pressure from *V. negundo* leaves resulted in a semisolid residue.

3.2. In vitro antifilarial activity in terms of inhibition assay

Crude ethyl acetate extract was used for antifilarial screening against adults of the cattle filarial worm *S. cervi*. The plant extract at concentrations of 0.03, 0.06, 0.10, 0.20, 0.50 and 1.00 mg/mL caused complete immobilization of the worms after 24, 14, 10, 6 and 2 h exposure at 37 ℃, respectively, whereas in untreated control, all the worms were active. Exposure incubation in fresh medium (without test solution) for 2 h did not revive the worms, confirming their death due to the treatment. Thus, the results indicated that the inhibition in motility was faster at higher concentrations, while it was relatively slow at lower concentrations.

3.3. In vitro antifilarial activity in terms of MTT-reduction assay

The macrofilaricidal effect of the plant extract was further confirmed by comparing the treated worms to untreated control and heat-killed worms, in terms of MTT-formazan colorimetric assay (Table 1). MTT is pale yellow in solution, but it is reduced by active mitochondria to yield dark blue formazan within the cells when incubated with living cells. During the assay, the formazan formed is extracted with DMSO and quantitated colorimetrically. The very low absorbance value (0.319) observed for the heat-killed worms was due to the least production of formazan in dead worms. The percentage inhibition considered significant was found to be 58.7%, 74.5% and 98.5% at concentrations 0.20, 0.50 and 1.0 mg/mL at 10.0, 6.0 and 2.0 h incubation periods (Table 1), indicating the significant effect of the plant extract at lower concentration. Inhibitory concentration at which 50% of the motility inhibition achieved (IC_{50}) was calculated by plotting the graph of percentage reduction in MTT-assay against different concentrations of herbal drug and the obtained values was 0.16 mg/mL. Both worm motility assay and MTT-reduction assay confirmed the macrofilaricidal activity of the leaves extracts of *V. negundo*. The effects of this plant extracts were shown in dose dependent manner.

Table 1
In vitro antifilarial activity of *Vitex negundo* L. against adult filarial parasite in terms of MTT reduction assay.

Treatment	Incubation time (h)	Test concentrations (mg/mL)	Percentage reduction (%)
Positive control	24.0	–	–
Negative control[a]	0.5	–	–
Plant extract	24.0	0.03	14.2
	20.0	0.06	20.5
	14.0	0.10	41.3
	10.0	0.20	58.7
	6.0	0.50	74.5
	2.0	1.00	98.7

[a]Adult worms that had previously been heat killed and incubated with MTT served as the negative control.

4. Discussion

In view of the enormous socio-economic burden of filarial disease on the developing countries, where this disease is much more preponderant in accordance to WHO/TDR mandate, detection and establishment of novel antifilarial therapeutic candidates emerged up as a necessity. Herbal medicines are quite popular and being used by about 80% of the world population mostly in the developing countries. These are time tested for their safety, efficacy, and cultural suitability. The chemical ingredients of these plants are believed to have better compatibility with the human body with most likely lesser side effects[34]. Hence, very appropriately, the WHO has referred this system of medicine as holistic approach towards health[35]. A growing body of evidence assembled from previous studies identified antifilarial activities of various herbal medicines[36]. The present work is an attempt to contribute to this database by screening crude plant extracts for antifilarial activity on *S. cervi*. The plant *V. negundo*, a traditionally used medicinal plant in many Ayurvedic drug preparations in India, revealed promising adulticidal activity, against adult filarial worm *S. cervi* in the *in vitro* system. The treated worms were completely immobilized due to the lethal effect of the plant extract at lower concentrations in a dose dependent manner. MTT reduction assay of the worms treated with the drug confirmed its effect on the viability of the worms by acting at the cellular level, as indicated by the reduced level of mitochondrial enzyme that reduces the MTT to formazan. Antifilarial activity at lower concentration recorded in terms of loss of motility in comparison to the suitable controls indicates that these can be considered as potential drugs, though this should be further confirmed by studying corresponding actual loss of viability of the parasites. Consequently, IC_{50} was also calculated. Ethyl acetate extracts of *V. negundo* leaves showed promising result at lower concentration. These results indicate that certain active compounds present in this plant extracts apply the actual therapeutic impact depending on their permeability properties. Thus, it would be interesting to find out the rationale behind the pharmaceutical efficacy of this potential drug candidate in the light of phytochemical analysis of this extract. A study with aqueous and alcoholic extracts of the leaves of *Mallotus philippensis* (Lam.) against *S. cervi* reported antifilarial effect and also highlighted the importance of permeability factor[37]. Other studies were carried out to test the antifilarial efficacy of *Plumbago indigo/rosea*[38], flowers extracts of *Azadirachta indica*[39], isolated molecules from fruits of *Trachyspermum ammi*[40] and leaves extracts of *Excoecaria agallocha* L.[41] against related filarial worm *Setaria degitata*.

Our findings indicate the importance of in depth study of this herbal drug for fortification of the antifilarial therapeutic range; this traditional therapeutic alternative may actually prove better in terms of cost effectiveness and patient fulfillment in combating the filariasis.

Conflict of interest statement

The authors declare that they have no conflict of interest.

References

[1] World Health Organization. Eliminate filariasis: attack poverty. The global alliance for lymphatic filariasis. Proceedings of the First Meeting at Santiago de Compostela, Spain, WHO/CDS/CPE/CEE/2000.5; 2002 May 4-5; Geneva: WHO; 2000.
[2] Michael E, Bundy DA, Grenfell BT. Reassessing the global prevalence and distribution of lymphatic filariasis. *Parasitology* 1996; **112**: 409-428.
[3] Das PK, Ramaiah KD, Augustin DJ, Ashok Kumar. Towards elimination of lymphatic filariasis in India. *Trends Parasitol* 2001; **17**: 457-460.
[4] Ramaiah KD, Kumar KN, Ramu K, Pani SP, Das PK. Functional

impairment caused by lymphatic filariasis in rural areas of south India. *Trop Med Int Health* 1997; **2**: 832-838.

[5] Ottesen EA, Duke BOL, Daram M, Behbehani K. Strategies and tools for the control/elimination of lymphatic filariasis. *Bull World Health Organ* 1997; **75**: 491-503.

[6] Noroes J, Dreyer G, Santos A, Mendes VG, Medeiros Z, Addis D. Assessment of the efficacy of diethylcarbamazine on adult *Wuchereria bancrofti in vivo*. *Trans R Soc Trop Med Hyg* 1997; **91**: 78-81.

[7] Vanamail P, Subramanian S, Das PK, Pani SP, Rajagopalan PK. Estimation of fecundic life span of *Wuchereria bancrofti* from longitudinal study of human infection in an endemic area of Pondicherry (South India). *Indian J Med Res* 1990; **91**: 293-297.

[8] Chin YW, Balunas MJ, Chai HB, Kinghorn AD. Drug discovery from natural sources. *AAPSJ* 2006; **8**: e239-253.

[9] Dharmasiri MG, Jayakody JR, Galhena G, Liyanage SS, Ratnasooriya WD. Anti-inflammatory and analgesic activities of mature fresh leaves of *Vitex negundo*. *J Ethnopharm* 2003; **87**: 199-206.

[10]Azhar-Ul-Haq, Malik A, Khan MT, Anwar-Ul-Haq, Khan SB, Ahmad A, et al. Tyrosinase inhibitory lignans from the methanol extract of the roots of *Vitex negundo* Linn. and their structure-activity relationship. *Phytomedicine* 2006; **13**: 255-260.

[11]Jagetia GC, Baliga MS. The evaluation of nitric oxide scavenging activity of certain Indian medicinal plants *in vitro*: a preliminary study. *J Med Food* 2004; **7**: 343-348.

[12]Alam MI, Gomes A. Snake venom neutralization by Indian medicinal plants (*Vitex negundo* and *Emblica officinalis*) root extracts. *J Ethnopha* 2003; **86**: 75-80.

[13]Chandramu C, Manohar RD, Krupadanam DG, Dashavantha RV. Isolation, characterization and biological activity of betulinic acid and ursolic acid from *Vitex negundo*. *Phytother Res* 2003; **17**: 129-134.

[14]Munasinghe TC, Seneviratne CK, Thabrew MI, Abeysekera AM. Antiradical and antilipoperoxidative effects of some plant extracts used by Sri Lankan traditional medical practitioners for cardioprotection. *Phytother Res* 2001; **15**: 519-523.

[15]Gupta M, Mazumder UK, Bhawal SR. CNS activity of *Vitex negundo* Linn. in mice. *Indian J Exp Biol* **1999; 37**: 143-146.

[16]Avadhoot Y, Rana AC. Hepatoprotective effect of *Vitex negundo* against carbon tetrachloride-induced liver damage. *Arch Pharm Res* 1991; **14**: 96-98.

[17]Perumal Samy R, Ignacimuthu S, Sen A. Screening of 34 Indian medicinal plants for antibacterial properties. *J Ethnopharm* 1998; **62**: 173-182.

18]Damayanti M, Susheela K, Sharma GJ. Effect of plant extracts and systemic fungicide on the pineapple fruit-rotting fungus, *Ceratocystis paradoxa*. *Cytobios* 1996; **86**: 155-165.

[19]Pushpalatha E, Muthukrishnan J. Larvicidal activity of a few plant extracts against *Culex quinquefasciatus* and *Anopheles stephensi*. *Indian J Malariol* 1995; **32**: 14-23.

[20]Bhargava SK. Antiandrogenic effects of a flavonoid-rich fraction of *Vitex negundo* seeds: a histological and biochemical study in dogs. *J Ethnophar* 1989; **27**: 327-339.

[21]Hebbalkar DS, Hebbalkar GD, Sharma RN, Joshi VS, Bhat VS. Mosquito repellent activity of oils from *Vitex negundo* Linn. leaves. *Indian J Med Res* 1992; **95**: 200-203.

[22]Sahare KN, Anandhraman V, Meshram VG, Meshram SU, Gajalakshmi D, Goswami K. *In vitro* effect of four herbal plants on the motility of *Brugia malayi*. *Indian J Medi Res* 2008; **127**: 467-471.

[23]Sahare KN, Anandhraman V, Meshram VG, Meshram SU, Singh V, Reddy MVR, et al. Antifilarial potential of *Butea monosperma* L. against microfilaria *in vitro*. *Int J Pharm Tech Res* 2012; **4**(3): 1181-1184.

[24]Sharma RD, Veerpathran AR, Dakshinamoorthy G, Sahare KN, Goswami K, Reddy MVR. Possible implication of oxidative stress in anti filarial effect of certain traditionally used medicinal plants *in vitro* against *B. malayi* microfilariae. *Pharmacogn Res* 2010; **2**(6): 350-354.

[25]Sahare KN, Anandhraman V, Meshram VG, Meshram SU, Reddy MVR, Tumane PM, et al. Anti-microfilarial activity of methanolic extracts of *Vitex negundo* and *Aegle marmelos* and their phytochemical analysis. *Indian J Exp Bio* 2008; **46**: 128-131.

[26]Kanwal A, Mehla J, Kuncha M, Naidu V, Gupta Y, Sistla R. Anti-amnesic activity of *Vitex negundo* in scopolamine induced amnesia in rats. *Pharmacol Pharm* 2010; **1**(1): 1-8.

[27]Swapnadeep P, Jain DC, Joshi SB. Anti-inflammatory activity of *Vitex negundo* leaves extracts obtained through different extraction methods. *Drug Invention Today* 2009; **1**(2): 157-159.

[28]Patel JI, Deshpande SS. Mast cell stabilizing activity of various subfractions of leaves of *Vitex negundo*. *Int J Green Pharm* 2011; **5**(4): 273-275.

[29]Gautam LN, Shrestha SL, Wagle P, Tamrakar BM. Chemical constituents from *Vitex negundo* (Linn.) of Nepalese origin. *Scientific World* 2008; **6**(6): 27-32.

[30]Kokate CK. *Practical pharmacognosy*. 4th ed. Delhi: Vallabh Prakashan; 1993.

[31]Murthy PK. Evaluation of two *in vitro* systems employing *Brugia malayi* parasite for prescreening of potential antifilarials. *Curr Sci* 1999; **77**(8): 1084-1089.

[32]Comley JCW, Rees MJ, Turner CH, Jenkins DC. Colorimetric quantitation of filarial viability. *Int J Parasi* 1989; **19**: 77-83.

[33]Strote G, Bonow I, Kromer M, de Kromer TR, Attah S, Opoku N. Chemotherapy for onchocerciasis: results of *in vitro* experiments with promising new compounds. *Trop Med Int Health* 1998; **3**: 397-407.

[34] Kamboj VP. Herbal medicine. *Curr Sci* 2000; **78**: 35-39.

[35]World Health Organization. *The promotion and development of traditional medicine: Report of a WHO meeting, WHO Technical Report Series*. Geneva: WHO; 1978; p. 622.

[36]Temjenmongla T, Arun Kumar Y. Anticestodal efficacy of folklore medicinal plants of Naga Tribes in North-East India. *Afr J Trad CAM* 2005; **2**: 129-133.

[37]Singh R, Singhal KC, Khan NU. Antifilarial activity of *Mallotus philippensis* Lam. on *Setaria cervi* (Nematoda: Filarioidea) *in vitro*. *Indian J Phys Pharmacol* 1997; **41**: 397-403.

[38]Mathew N, Paily Abidha KP, Vanamail P, Kalyansundram M, Balraman K. Macrofilaricidal activity of plant *Plumbago Indica/ rosea in vitro*. *Drug Dev Res* 2002; **56**(1): 33-39.

[39]Mishra V, Parveen N, Singhal KC, Khan NU. Antifilarial activity of *Azadirachta indica* on cattle filarial parasite *Setaria cervi*. *Fitoterapia* 2000; **76**: 54-61.

[40]Mathew N, Bhattacharya SM, Perumal V, Muthuswamy K. Antifilarial lead molecules isolated from *Trachyspermum ammi*. *Molecules* 2008; **13**: 2156-2168.

[41]Patra JK, Mahapatra AD, Rath S, Dhal NK, Thatoi H. Screening of antioxidant and antifilarial activity of leaves extracts of *Excoecaria agallocha* L. *Int J Integr Bio* 2009; **7**(1): 9-15.