ANTIFILARIAL POTENTIAL OF VITEX NEGUNDO L. LEAVES AND DIETHYLCARBAMAZINE CITRATE AGAINST SETARIA CERVI

IN VITRO

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ABSTRACT

Herbal medicine is quite well known in Ayurveda and Siddha system of medicine. Hence the present study was designed to study the antifilarial effect of Vitex negundo L. leaves Methanolic extract and DEC against Setaria cervi in vitro. Activity was explored after 24 hrs incubation with concentrations ranging of 0.006 to 1.5 mg/ml for possible antifilarial effect, in terms of motility inhibition assay and MTT reduction assay. Vitex negundo L. Leaves showed significant antifilarial activity as compared to DEC was comparatively less significant in dose dependent manner. Inhibitory concentrations (50%) for with significant antifilarial activity in vitro system have been found to be 0.049 mg/ml for Methanol extract and for DEC it was 0.30 mg/ml calculated. The present research study shown significant antifilarial activity of Vitex negundo L. leaves Methanolic extracts highlights the further, isolation of active molecule from this plant for future drug designing.

Keywords: Anti-filarial, In-vitro, Drug designing, medicinal plant, DEC.

INTRODUCTION

WHO has launched research and development programme for filariasis. The World Health organization (WHO) has precisely recognized human lymphatic filariasis as one of the ten diseases in its tropical disease research (TDR) scheme highlighting the enormous disease burden. Filariasis is mainly caused by vector - borne nematode parasite Wuchereria bancrofti, Brugia malayi, and Brugia timori. Approximately 120 million population affected all over the world. In India, around 45% of its 1 – billion population lives in endemic areas and 48 million are infected[1], accounting 40% of the worldwide filariasis burden[2]. Socioeconomic
studies shown that the yearly loss caused by this debilitating disease is near to a billion U.S. dollars\textsuperscript{3}.

Diethylcarbamazine (DEC) is the drug of choice for treatment of patients suffered from filariasis. Major limitation of DEC is, it kills only blood circulating microfilariae, but less effective against the adult worms. Adult worms live for several years in the lymphatic system of infected individuals producing microfilariae and thereby facilitate transmission of the disease through the vector mosquitoes to more individuals. Therefore elimination of the parasite by means of microfilaricide alone is extremely difficult\textsuperscript{4}. India has traditionally well known knowledge of herbal medicine\textsuperscript{5}. WHO has already outlined the nature of traditional medicine including herbal therapeutics\textsuperscript{6}. So for, there is an urgent need to evaluate and validate the therapeutic impact of herbal drugs as per WHO guideline\textsuperscript{5}. Because of these reasons, it is very important to find out potent antifilarial drug candidate against the adult filarial worms.

With the perspective of these encouraging advancements, in the present study plant \textit{Vitex negundo} L. leaves methanolic extract and DEC was screened \textit{in vitro} for their antifilarial activity against \textit{Setaria cervi} adult filarial parasite.

**MATERIALS AND METHODS**

(i) Plant Materials / DEC drug

Leaves of \textit{Vitex negundo} L. plant leaves were collected from the local areas of Bhopal. The botanical identity was confirmed by a Botanist Prof. Zia-Ul-Hasan Department of Botany, Safia Science College, Bhopal and reference No. 410/Bot./Safia/2012 allotted. Diethylcarbamazine citrate (DEC) (Wyeth, Limited) drug was also used for antifilarial study.

(ii) Extraction

Leaves (1.5 kg) of \textit{Vitex negundo} L. was extracted successively with petroleum ether (60\textdegreeC - 80\textdegreeC) (Qualigens Fine Chem, Mumbai, SQ - grade), CHCl\textsubscript{3} (Ranchem, Mumbai, LR - grade), ethyl acetate (Merk India, Syntheisis Grade) and methanol (Ranchem, Mumbai, AR – Grade) by percolation method\textsuperscript{7,8}.

(iii) Parasite

Adult \textit{Setaria cervi} were obtained from the peritoneal cavity of freshly slaughtered cattle. The worms were washed repeatedly with normal saline (0.85\%) to free them of any extraneous material and used for assay.
(iv) In-vitro Motility Inhibition Assay
The worms were transferred 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% heat-inactivated fetal bovine serum (Hi - Media, Mumbai, India). Dilutions of the plant extract / DEC 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 3mL medium would give the required test concentration. Screening was done at concentrations ranging from 0.3 to 1 mg/mL. A simultaneous control was kept without the test solution but with 100µl DMSO in 3mL 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 37ºC for 24 hrs in 5% CO₂ incubator and motility observed after 2 to 24 hrs. 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.

(v) MTT – Formazan Colorimetric Assay
Effect of the plant extract / DEC on adult female Setaria worms was studied by MTT (3-[4,5dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) (Hi-media, Mumbai, India) - formazan reduction assay following the method described by Comely. 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 colour 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 (56ºC 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 by following the formula:
% inhibition (parameter) = 100 - [(T - H) / (C – H)] ×100
where T, C, and H are absorbance values obtained for the formazan produced in treated, control and heat killed worms respectively.

(v) Statistical Analysis
The results were expressed as mean ± s.e.m for the triplicate observations made in each observation. *P < 0.05 was considered as significant.

RESULTS
(i) Preparation of Plant Extract
The solvent removed from the plant extract under reduced pressure from *Vitex negundo* L. leaves resulted in a semisolid residue.

(ii) In-vitro Motility Inhibition Assay
Methanol extract and DEC was used for anti filarial screening against adult parasite *Setaria cervi*. Concentrations for Methanol extract 0.006 to 0.3 mg/ml caused complete immobilization of the worms and for DEC it was 0.5 to 1.5 mg/ml at 2 to 24 h incubation at 37ºC, whereas in untreated control, all the worms were active (Table 1). Post exposure incubation in fresh medium (without test solution) for 2 h, the worms was not revive; confirm their death due to the effect of drug treatment at different time duration and concentrations. The results revealed that, for plant extract at lower concentrations antifilarial activity was found significant but for DEC it was found at higher concentration of drug in dose and concentration dependent manner.

(ii) MTT - Reduction Assay
The antifilarial effect was further confirmed by comparison of the treated worms to untreated control and heat-killed worms, in terms of MTT- colorimetric assay. MTT is light yellow colour solution, when incubated with living parasite, is reduced by live mitochondria to yield dark blue formazan within the cells, the formazan formed is extracted with DMSO and quantitated colorimetrically during the assay. The very low absorbance values (<0.323) observed for the heat-killed worms was due to the least production of formazan in dead parasite. The percentage inhibition (>50%) was considered significant for plant extracts, it was achieved at very low concentrations 0.06, 0.1 and 0.3 mg/ml indicating the significant antifilarial effect and for DEC it was achieved at high concentrations 0.37, 0.75 and 1.5 mg/ml (Table 2). Thus, inhibitory concentration of drugs at which 50% of the motility inhibition achieved (IC50), was calculated by plotting the graph of percentage reduction in
MTT – reduction assay against different concentrations of drugs and the obtained value for extract 0.049 mg/ml and for DEC 0.30 mg/ml was calculated respectively. Both worm motility assay and MTT - reduction assay indicates the significant macrofilaricidal activity of plant *Vitex negundo* L. leaves methanolic extracts but DEC failed to show such similar activity against *Setaria cervi* parasite.

Table 1: *In vitro* antifilarial activity of Methanol extract of *Vitex negundo* L. leaves / DEC against adult filarial parasite in terms of motility inhibition.

<table>
<thead>
<tr>
<th>Methanol extract (mg/mL)</th>
<th>DEC (mg/mL)</th>
<th>Incubation time (end point) in hrs</th>
<th>Worm motility (Test)</th>
<th>Worm motility (Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.006</td>
<td>0.5</td>
<td>24.0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>0.01</td>
<td>0.9</td>
<td>20.0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>0.03</td>
<td>0.18</td>
<td>14.0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>0.06</td>
<td>0.37</td>
<td>10.0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>0.75</td>
<td>6.0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>0.3</td>
<td>1.5</td>
<td>2.0</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

100% Complete motility inhibition.
0% Completely motile.

Table 2: *In vitro* antifilarial activity of *Vitex negundo* L. leaves Methanol extract / DEC against adult filarial parasite in terms of MTT reduction assay.

<table>
<thead>
<tr>
<th>Sample / drug</th>
<th>Treatment</th>
<th>Test concentration (mg/ml)</th>
<th>Incubation time (In hours)</th>
<th>Absorbance at 492 nm (mean±sem)</th>
<th>% reduction relative to solvent control^C^, heat killed^H^ &amp; treated worms^T^</th>
<th>IC50 (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plant extract</strong></td>
<td>^C^Control</td>
<td>-</td>
<td>24</td>
<td>1.009±0.03</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>^H^Heat killed</td>
<td>-</td>
<td>0.5</td>
<td>0.323±0.028</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>^T^Plant extract</td>
<td>0.006</td>
<td>24</td>
<td>0.925±0.036*</td>
<td>12.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.01</td>
<td>20</td>
<td>0.875±0.002*</td>
<td>19.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.03</td>
<td>14</td>
<td>0.786±0.001*</td>
<td>32.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.06</td>
<td>10</td>
<td>0.599±0.003*</td>
<td>59.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1</td>
<td>6</td>
<td>0.443±0.008*</td>
<td>82.1</td>
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<tr>
<td></td>
<td></td>
<td>0.3</td>
<td>2</td>
<td>0.328±0.002*</td>
<td>98.7</td>
<td></td>
</tr>
<tr>
<td><strong>DEC</strong></td>
<td>^C^Control</td>
<td>-</td>
<td>24</td>
<td>1.028±0.08</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>^H^Heat killed</td>
<td>-</td>
<td>0.5</td>
<td>0.321±0.02</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>^T^DEC</td>
<td>0.5</td>
<td>24</td>
<td>0.953±0.004*</td>
<td>10.7</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>0.9</td>
<td>20</td>
<td>0.869±0.005*</td>
<td>22.2</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>0.18</td>
<td>14</td>
<td>0.763±0.011</td>
<td>37.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.37</td>
<td>10</td>
<td>0.624±0.002*</td>
<td>57.2</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.75</td>
<td>6</td>
<td>0.502±0.001*</td>
<td>74.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>2</td>
<td>0.329±0.004*</td>
<td>98.9</td>
<td></td>
</tr>
</tbody>
</table>

^C^ Positive control, ^H^Negative control, ^T^Treated worm with plant extract / DEC

*P value represents the level of significance *P* < 0.05 when comparing the mean value of absorbance observed for the formazan formed between treated and control worms.
DISCUSSION

Filariasis causes permanent and long term disability in developing countries. Looking high socio-economic burden of filarial disease, development of potent antifilarial drug candidate is essential. Traditional medicines are reasonably popular and being largely used by world population mostly in the developing countries. These are safe, efficient and suitable for population. The chemical components of these medicines are believed to have better suitability with the human body and have fewer side effects\(^5\). So, WHO has referred the traditional medicine as holistic approach for health\(^12\).

In present work an effort was made to contribute to this database by screening of Vitex negundo L. leaves crude methanolic extract for anti-filarial activity against Setaria cervi. This plant is traditionally used medicinal plant in many Ayurvedic drug preparations in India, revealed promising adulticidal activity. Plant extract shown significant antifilarial activity in in vitro experiment in terms of Motility inhibition assay and MTT - reduction assay. Another study was carried out for same plant against Setaria cervi filarial nematode\(^13,14\). Other study was also carried out by various workers with other plant extracts against Setaria cervi.

Aqueous and alcoholic extracts of the leaves of Mallotus philippensis (Lam.) was reported antifilarial activity\(^15\). Antifilarial activity of Alcoholic extract of Plumbago indigo also identified\(^4\), alcoholic and aqueous extract of Azadirachta indica flowers\(^16\) and Excoecaria agallocha L. leaves extracts\(^17\) shown antifilarial activity. Antifilarial activity of Asparagus adscendens Roxb\(^18\), alcoholic and aqueous extracts of the fruits of the Ficus racemosa Linn.\(^19\), and DEC\(^20\) inhibited the spontaneous movements of the whole worm and in another study Methanolic extract of leaves of Hibiscus mutabilis exhibited activity against Setaria cervi in vitro\(^21\). In present experiment DEC was also assessed for antifilarial activity. At lower concentration it was failed to show significant antifilarial activity than leaves extract. In similar study some synthetic compounds also shown antifilarial activity against filarial parasite\(^22,23\).

CONCLUSION

In present investigation Vitex negundo L. leaves methanolic extract shown significant adulticidal activity as compared to DEC. Some active molecules may be present in this extract may be responsible for the actual effect. Therefore, it would be interesting to find out the phytochemical / structural analysis of extract and study of pharmacological approach to development new antifilarial therapeutic drug molecule.
ACKNOWLEDGEMENT
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REFERENCES