Vol. 9/1 (2009) 56 - 61

JOURNAL OF NATURAL REMEDIES

Screening of fruit of *Diospyros montana* for anti-filarial activity

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Abstract

Objective: To screen the potential antifilarial activity of petroleum and alcoholic extracts prepared from fruit of *Diospyros montana*. Materials and Methods: *In vitro* study was carried out to see the effect of extracts on both the whole worm(w.w). preparation and nerve muscle (n.m) preparation of *Setaria cervi*, on the survival of microfilariae. Results: Petroleum extract produced initial stimulation followed by reversible paralysis in whole worm The initial stimulation is not seen with petroleum extract on nerve muscle preparation. The alcoholic extract caused reversible paralysis in whole worm whereas irreversible in nerve muscle preparation. Conclusion: Fruit of *D. montana* was found to possess potential antifilarial activity.

Key words: Antifilarial, Screening, *Diospyros montana*, Setaria.

1. Introduction

Forty one species of Diospyros are found in India mostly in evergreen forests of Daccan, Assam and Bengal and in North-India [1, 2] *Diospyros montana* is an erect plant with a height of 15 - 20 ft. According to Indian traditionalsystem of medicine, the bark is used to prevent delirium in high fever, fruits for cracks in sole of feet and roots as abortifacient. [3]. Chemical constituents like α-amyrin, β-sitosterol, ursolic acid are isolated from fruit pulp [4], diospyrin from stem bark [5] and lupeol, iododiospyrin from wood [6] of *Diospyros montana*. *Setaria cervi*, a nematode and parasite of cattle water buffalo (*Bubalis bubalis* Lin), can be used for screening the antifilarial agents since its response to drugs is almost similar to human filarial worm [7, 8]. Both *Setaria cervi* and its nerve muscle preparation [9] exhibit vigorous rhythmical movements, which can be recorded on a slow moving drum by suspending the worm in an isolated organ bath. In the present study we have observed the effect of petroleum and alcoholic extracts of the fruit of *D. montana* on the spontaneous movements of whole worm, nerve muscle preparation and also on the survival of microfilariae of *S.cervi in vitro*.

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2. Materials and Methods

2.1 Plant material

The fruits of *D. montana* were procured from village Rampur, Bulandshahr, (UP) and the plant was identified by taxonomist Prof. Wazahat Hussain, Department of Botany, AMU., Aligarh (India) its voucher specimen was deposited in the same department.

2.2 Preparation of extract

The shade dried and powdered fruits of *D. montana* was taken in a round bottom flask and was steeped in desired solvent. The ethyl alcohol was used as a solvent for alcoholic extract, and petrol for petroleum extract. The flask contents were refluxed over steam bath for 18-24 hours. The solvents (Petrol and ethyl alcohol) was removed by distillation under reduced pressure. After the complete removal of the solvent, the residual material obtained was diluted with distilled water to make a stock solution of 1mg/ml.

2.3 Collection of filarial worm Setaria Cervi

Motile adult *S.cervi* (*Nematoda: Filarioidea*) of average length 6.0 ± 1.0 cm were collected from the peritoneal cavity of freshly slaughtered cattle and brought to the laboratory in a vacuum flask containing modified Ringer's solution (NaCl 9g, KCl 0.42g, $CaCl_2$ 0.24g $NaHCO_3$ 0.5g, glucose 0.25 per liter) at 37°C [8].

2.4 Whole worm (w.w.) preparation

Adult *S. cervi* were suspended in an ideal isolated organ bath of 20 ml capacity, in modified Ringer's solution at 37°C. Spontaneous movements of the worm were recorded on a slow moving kymograph drum [10]. Air or Oxygen was not bubbled through the solution, as it did not improve the movements of the worm. Approximately 15 min were allowed for the movements of worm to stabilize before eliciting the response of the extract. The extract was added in increasing concentration to the bath fluid and allowed to remain in contact for 15min, the effect was observed for 6h. If there was no response within 15 min it was considered inactive.

2.5 Nerve-muscle (n.m) complex

A worm was placed in a petri-dish containing modified Ringer's solution at 37°C. Two dissecting needles were inserted into the worm at one end, and the cuticle was split longitudinally. The intestine and uterus were cut at both ends and removed. The anterior 1 cm of the worm was removed to eliminate the influence of the nerve ring and cephalic ganglia. The remaining part was tied at either end and suspended in an isolated organ bath, containing modified Ringer's solution at 37°C. The preparation served to expose the n.m. complex directly to the action of the drugs, and also could exhibit spontaneous rhythmical movements similar to those of the whole worm. The drug concentrations were tested for their response as with whole worm preparation. The concentration of extract, which modified the movements, was tested in at least six preparations and the duration of observation in each case was 6 hr.

2.6 Collection of microfilariae (m.f.)

The uterus of a female *S. cervi* was cut at its junction with the vagina just below the bifurcation, and removed from the worm. It was teased with a fine needle in the Ringer solution and microfilariae (mf) were freed. The microfiliariae were suspended in a human serum and ringer mixture and the mf count was adjusted to 100/ml. 0.5 ml aliquots of the microfilariae suspension were placed in sterilized screw capped bottles containing extract of *Diospyros montana* in equal serum and ringer mixture (v/v). Extract was added in doubling concentration from 5 ng/ml. The

bottles were kept in an incubator at 37°C and examined under a microscope every 30 min till 6 hours to observe the survival / mortality of microfilariae. The lethal concentrations (LC_{50} and LC_{90}) were calculated from a concentration vs death graph.

In the preliminary set of experiment it was ascertained that the concentration of alcohol/ petrol in the suspending medium did not influence the survival / mortality of the m.f. and also the petroleum and alcoholic extracts of *Diospyros montana* were added to m.f. in concentration of 5, 10, 15, 20, 25 ng/ml to determine the limits of activity within 6 hours at 37°C, within these limits six concentrations were selected to observe the survival of m.f. The effect of each dose was observed 10 times. The mean of the values were plotted on a graph.

3. Results

3.1 Effect of petroleum extract of the fruits of D. montana on whole worm (w.w.) preparation of Setaria cervi

The spontaneous movements of w.w. were modified on addition of 200 μg/ml of petroleum extract characterized by increase in amplitude and rate of contractions with no effect on tone (upper panel, fig.1). The effect was evident immediately after the addition of extract. The stimulation lasted for 60 min. and was followed by decrease in rate leading to paralysis which could be restored on repeated washing of bath fluid i.e. the effect was reversible in nature (lower panel, fig. 1).

3.2 Effect of petroleum extract of the fruits of D. montana on nerve-muscle(n.m) preparation of S. cervi.

Depressant effect could be elicited with 100 μg/ ml of petroleum extract on the nerve-muscle preparation of *S. cervi* characterized by a decrease in tone, rate and amplitude of contractions (upper panel, fig. 2) leading to complete cessations of movements at 90 min. Repeated changes of bath fluid restored the movements to pre drug level (lower panel, fig.2).

3.3 Effect of alcoholic extract of fruits of D. montana on whole worm preparation of S. cervi.

The depressant effect of alcoholic extract was evident immediately at a concentration of 200 μg/ml characterized by decrease in the rate, amplitude and tone of contractions (upper panel, fig.3). This effect continued for 90 min, after which the amplitude started to declined leading to complete cessation of the contractions i.e. paralysis of the worm (lower panel, fig.3). The paralysis continued for 6 hrs. (total time for which the preparation was observed). Repeated changes of the bath fluid restored the movements, suggesting reversible paralysis.

3.4 Effect of alcoholic extract of fruits of D. montana on nerve-muscle preparation of S. cervi.

The effect produced by the alcoholic extract on nerve-muscle preparation at 200 μg/ml was depressant in nature, characterized by decrease in the tone, amplitude and rate of contractions (upper panel, fig,4), leading to irreversible paralysis in 60 min (lower panel, fig.4).

3.5 Effect of alcoholic extract on the survival of microfilariae

Alcoholic extract caused concentration related effect on the survival of microfilariae of *S. cervi*. The LC_{50} and LC_{90} as observed after 6 hrs is presented in table no.1.

Concentration related lethal effect of alcoholic as well as petroleum extract at a concentration of 25ng/ml is shown in fig.5.

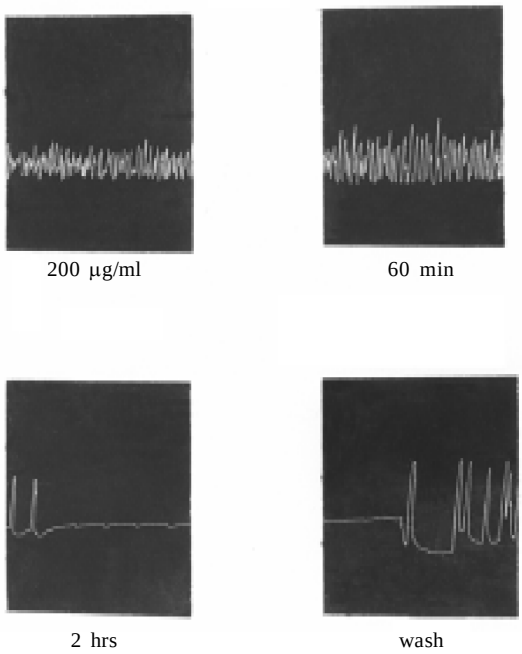

Fig. 1: Effect of Petroleum extract of fruit of D. montana on w.w, showing initial stimulation followed by reversible paralysis.

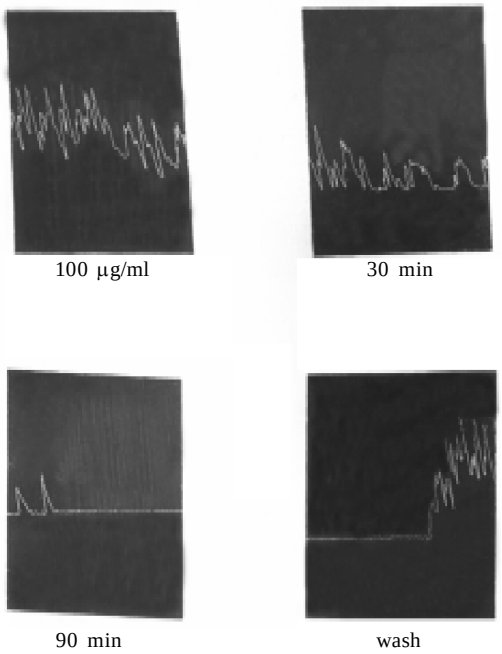

Fig. 2: Depressant effect followed by reversible paralysis of petroleum extract on n.m complex of S. cervi.

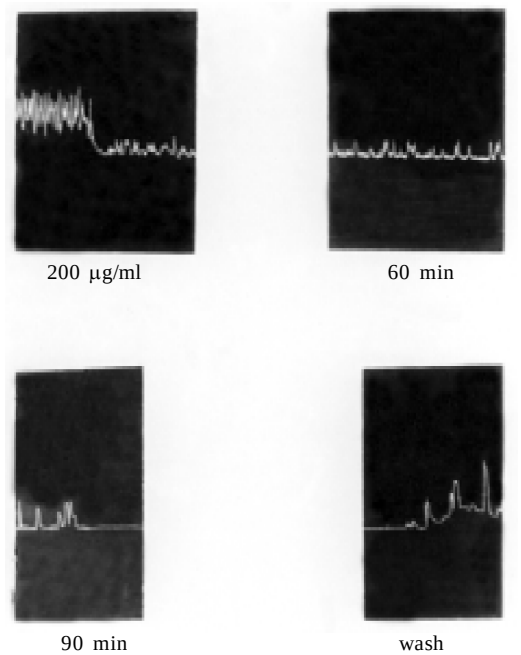

Fig. 3: Depressant effect of alcoholic extract of fruit of D. montana on w.w, followed by reversible paralysis.

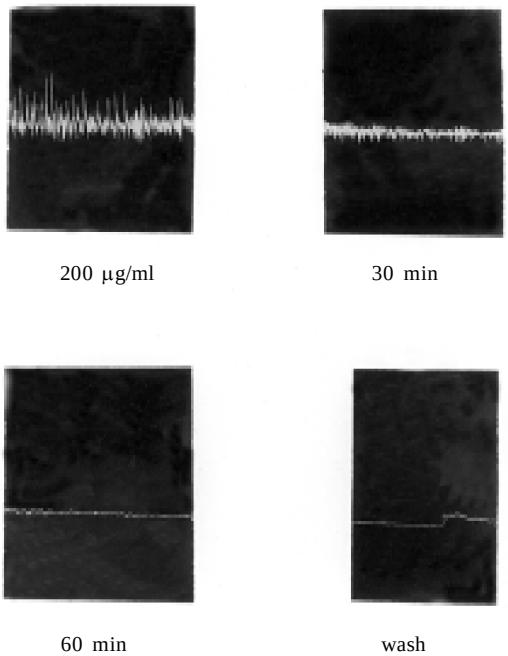

Fig. 4: Effect of alcoholic extract of fruit of D. montana on n.m complex, showing irreversible paralysis.

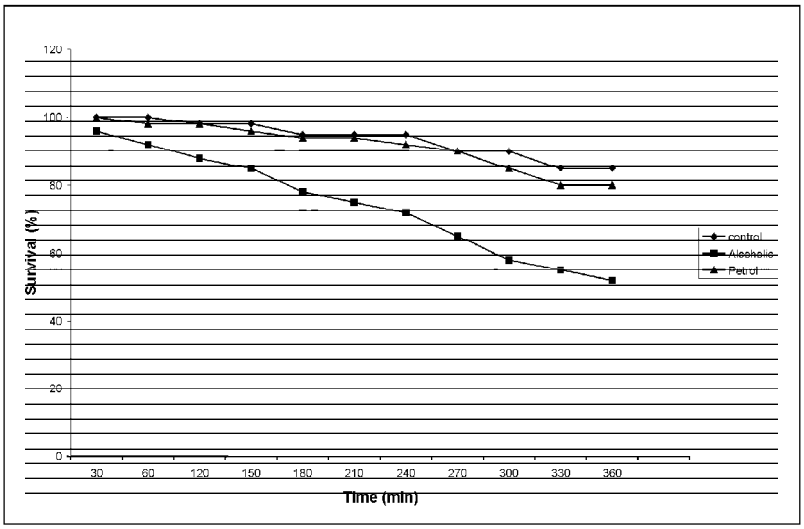

Fig. 5: Shows effect of alcoholic and petroleum extracts on survival of microfilariae at a concentration of 25 ng/ml.

Table 1. The effect of alcoholic extracts of fruit of *D. montana* on the survival of microfilariae of *S. cervi*.

Extract	Concentration (ng/ml)*
Alcoholic	
LC_{50}	40
LC_{90}	75

*Mean of 10 readings

4. Discussion

It is interesting to note that the effect produced by the two extracts is different in nature. Petroleum extract produced initial stimulation in whole worm leading to reversible paralysis, whereas on the nerve muscle, the effect was depressant in nature. The initial stimulation produced in case of whole worm could possibly be due to the cuticular irritation as seen with other substances including neurotransmitters like acetylcholine [12]. The initial stimulation did not manifest on removal of cuticle in case of nerve muscle preparation. The alcoholic extract produced inhibitory effect on both the whole worm and nerve muscle preparation followed by reversible paralysis of whole worm, whereas irreversible paralysis of the nerve muscle preparation. It could be due to the presence of more than one active ingredient, or it may be possible that the active ingredient is not able to cross the cuticle in sufficient amount due to low lipid solubility [9]. This is evident once the cuticle is stripped off to prepare nerve muscle preparation and now the extract is in direct contact with the preparation.

It has been reported that addition of acetylcholine (which is a stimulatory neurotransmitter in *S. cervi*) to the bath fluid during the phase of paralysis did not modify the effect indicating that the effect could be due to the blockade of the cholinergic receptors [13].

The survival time of the microfilariae was reduced in a concentration dependent manner with alcoholic extract (fig.5). If such concentration could be presented *In Vivo*, the fruit could be a useful tool in the treatment of filariasis. Further studies with the isolation of pure compounds is in progress to find out the active principle.

5. Acknowledgements

Grateful acknowledgement is made to ICMR, New Delhi for providing financial assistance to one of the authors Dr. Rakesh Kumar (Research Associate). A part of the findings were presented at the 39th annual conference of Indian Pharmacological Society, held at Jaipur from 21st to 23rd Dec, 2006.

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