In vitro evaluation of antifilarial effect of *Azadirachta indica* leaves extract in different solvents on the microfilariae of *Setaria cervi*

Sharba Kausar¹

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Abstract Polar solvent extracts of *Azadirachta indica* leaves were investigated to evaluate in vitro anthelmintic activity on the microfilariae of *Setaria cervi*. Four extracts of *A. indica* leaves were prepared with different solvents in increasing order of polarity (diethyl ether < chloroform < ethanol < methanol). Stock solution of the extract of *A. indica* leaves was prepared in Ringer's solution at the concentrations of 50, 100 and 200 µg/ml and used against microfilariae in three replicates. Ringer's solution was used as experimental control. Mortality rates of microfilariae were recorded for each concentration after 45, 90 and 135 min, respectively. All the solvent extracts show significant anthelmintic activity at the concentration of 200 µg/ml after 135 min of incubation. Highest mortality rate of microfilariae was observed in methanol and ethanol extracts throughout the incubation period compared to chloroform and diethyl ether extracts which showed low mortality rates. Dose dependent effect of ethanol and chloroform showed highest and lowest ranking when analyzed by regression values and correlation of regression. Solvents extracts of *A. indica* leaves showed anthelmintic effect and therefore may be used as phytopharmacological agents in future.

Keywords *Azadirachta indica* · Extracts · Microfilariae · Polar solvents · *Setaria cervi*

Introduction

Filariasis is a chronic infection of considerable morbidity in tropical and subtropical countries (Katiyar and Singh 2011; Murthy et al. 2011). *Setaria cervi* is an important filarid inhabiting the peritoneal cavity of buffaloes, causing peritonitis, intestinal occlusion and cerebro-setariasis in rare instances (Sarwar 1945; Innes and Shoho 1953; Pachauri 1972; Tung et al. 2003) and neurological disorders in human by *Setaria* sp. (Kadenatsii et al. 1974). Usually studies are conducted on the bovine filarid *S. cervi* in laboratory models as this worm resembles the human species in having microfilarial periodicity and chemotherapeutic response to known antifilarial agents (Singhal et al. 1973; Ahmad and Srivastava 2007).

Azadirachta indica (neem) is a large evergreen tree, common throughout the Indian subcontinent and is perhaps the most resourceful medicinal plant for more than two millennia. In Ayurveda, extract preparations of leaves, barks and fruits of *A. indica* had been used against different ailments (Biswas et al. 2002). Preparations from the leaves of this plant possess several effects that had been well documented like antipyretic, hypoglycemic, antiulcer, antimalarial, antibacterial, antifungal, antiviral, antifertility (Biswas et al. 2002), coccidiocidal (Dkhil et al. 2013) as well as anticarcinogenic effects (Balasenthil et al. 1999). The identified compounds in different crude extracts from *A. indica* are hydrocarbons, terpenoids, phenolic, alkaloids and their derivatives and are biologically active molecules (Kar et al. 2007; Cock et al. 2009; Lucantoni et al. 2010; Hossain et al. 2013). Aqueous extract of *A. indica* leaves and flowers showed anthelmintic activity by inhibiting mitochondrial enzymes (malate dehydrogenase and malic enzyme) of *S. digitata* (Banu et al. 1992). Alcoholic and aqueous extracts of flowers of *A. indica* was tested in vitro

✉ Sharba Kausar
sharbakauser@gmail.com

¹ Section of Parasitology, Department of Zoology, Faculty of Life Sciences, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

for their antifilarial activity against whole worm, nerve muscle preparation and microfilariae of *S. cervi* (Mishra et al. 2005c). Recently reactive oxygen species mediated apoptosis of *S. cervi* has also been demonstrated by polyphenol rich ethanolic leaves extract of *A. indica* (Mukherjee et al. 2014).

In present study the antifilarial activity of *A. indica* leaves in different solvents (methanol, ethanol, diethyl ether and chloroform) extracts has been observed against the microfilariae of *S. cervi*. Despite being one of the most debilitating diseases filariasis has been neglected because it does not cause fatality and mainly affects poor people (WHO 2005). Drug resistance has opened an opportunity for research in phytopharmacology. Plant extracts could be a cheap and non toxic alternative to routine antifilarial drugs and may offer recipe for some of the notable health challenges of today for the control of filarial nematodes (Nisha et al. 2007; Misra et al. 2011; Ode et al. 2011).

Materials and methods

Collection of plant material

Fresh leaf samples of neem plant were obtained and identified in the Department of Botany, Aligarh Muslim University, Aligarh.

Collection of microfilariae of *S. cervi*

Adult *S. cervi* worms were collected from freshly slaughtered cattle and brought to the laboratory. The worms were washed in normal saline and adult females were dissected to recover microfilariae from the gravid segment of the uterus for in vitro study.

Preparation of plant extract

The leaves of *A. indica* were dried in the shade, ground to fine powder by mortar pestle and stored in cellophane bags at 4 °C until used. Extraction of the powder was done in different solvents like methanol (m), ethanol (e), chloroform (c) and diethyl ether (d). The extract was prepared by soaking 1 g of plant leaf powder in 50 ml of solvent by cold maceration at room temperature for 3 days. Then solvents were evaporated at a constant temperature of 60 °C until no detectable solvent remained in the extracts. The plant extracts prepared in different solvents were stored at −4 °C until used. Extracts were dissolved in Ringer's solution on the day of the experiments to prepare a stock solution and its different dilutions were made to evaluate pharmacological activity of these extracts on the microfilariae in vitro.

In vitro anthelmintic activity against the microfilariae of *S. cervi*

The anthelmintic activity of different solvent extracts of *A. indica* was determined by keeping 10 microfilariae in 100 µl of Ringer's solution in 96-well titer plate. Three replicates for each solvent extract were run at the concentrations of 50, 100, 200 µg/ml. The inhibition of motility/mortality of the microfilariae in the above treatments was used as the criterion for anthelmintic activity. The motility/mortality was observed after 45, 90 and 135 min.

Statistical analysis

Data of the present findings were statistically analyzed using SPSS version 17.0 software. The statistical significance of the data was assessed by ANOVA for comparing means. Pearson correlation was done to describe the relationship between the percentage of larval motility/mortality with extract concentrations. Regression and correlation of regression were also done to indicate rank and dose dependent effect of four solvent extracts of *A. indica* leaves on the mortality of *S. cervi* microfilariae.

Results

Interpretation of results of the present study was based on the time taken for motility/mortality of microfilariae of *S. cervi* worm and dose dependent response of microfilariae to different solvent extract of *A. indica* leaves. All the extracts used in this study exhibited anthelmintic activity against the microfilariae of *S. cervi*.

Microfilariae incubated for 45 min in methanol extract showed 60, 67 and 87 % of mortality at 50, 100 and 200 µg/ml concentrations, respectively. After 90 min of incubation mortality rate was increased to 77, 80 and 97 % which further increased and reached to the higher levels of 87, 90 and 100 % after 135 min (Table 1).

Ethanol extract showed 23, 43 and 60 % mortality when microfilariae were incubated for 45 min in 50, 100 and 200 µg/ml concentrations, respectively. The rate of mortality increased after 90 min of incubation and was recorded as 37, 47 and 73 %, which further increased to 40, 57 and 90 % when incubation period was increased to 135 min (Table 1).

Chloroform extract of *A. indica* leaves showed 3, 13 and 23 % mortality when microfilariae was incubated for 45 min at the concentration of 50, 100 and 200 µg/ml. Mortality rate increased when incubation period was increased to 90 min and mortality recorded was 37, 43 and 57 %. After 135 min of incubation in above concentration

Table 1 Effect of exposure of *A. indica* on the mortality of microfilariae of *S. cervi* during 45, 90, 135 min of treatment

Extract concentrations (µg/ml)	Mortality of microfilariae of <i>S. cervi</i> (Mean ± SD)		
	45 min	90 min	135 min
Control	0.00a (0 %)	0.00a (0 %)	0.00a (0 %)
Methanol extract			
50	6.00 ± 1.00b (60 %)	7.66 ± 0.57b (77 %)	8.66 ± 0.57b (87 %)
100	6.66 ± 0.57b (67 %)	8.00 ± 0.10ab (80 %)	9.00 ± 0.00b (90 %)
200	8.66 ± 0.57a (87 %)	9.66 ± 0.57a (97 %)	10.0 ± 0.00a (100 %)
Ethanol extract			
50	2.33 ± 1.15b (23 %)	3.66 ± 0.57b (37 %)	4.00 ± 1.73b (40 %)
100	4.33 ± 0.57b (43 %)	4.66 ± 0.57ab (47 %)	5.66 ± 1.52ab (57 %)
200	6.00 ± 1.00a (60 %)	7.33 ± 0.57a (73 %)	9.00 ± 1.00a (90 %)
Chloroform extract			
50	0.33 ± 0.57a (3 %)	3.66 ± 0.57a (37 %)	4.00 ± 1.73b (40 %)
100	1.33 ± 0.57ab (13 %)	4.33 ± 0.57a (43 %)	6.66 ± 1.52ab (67 %)
200	2.33 ± 0.57a (23 %)	5.66 ± 1.15a (57 %)	8.00 ± 1.00a (80 %)
Diethyl ether extract			
50	0.00 ± 0.00a (0 %)	2.33 ± 0.57a (23 %)	3.66 ± 0.57a (37 %)
100	1.33 ± 0.57a (13 %)	4.00 ± 1.00ab (40 %)	6.00 ± 2.00a (60 %)
200	2.33 ± 2.08a (23 %)	5.00 ± 1.00a (50 %)	7.33 ± 1.52a (73 %)

Means followed by a common letter do not differ significantly (Tukey's test, $p < 0.05$)

the mortality rate was 40, 67 and 80 %, respectively (Table 1).

Anthelmintic activity of diethyl ether extract showed 0, 13 and 23 % mortality at 50, 100 and 200 µg/ml concentration after 45 min of incubation. The rate of mortality increased to 23, 40 and 50 % when microfilariae incubated for 90 min. When incubation period was increased to 135 min mortality increased to 37, 60 and 73 % (Table 1).

In Ringer's solution (control) experiment, all the microfilariae of *S. cervi* were active and motile as shown in Fig. 1. There was a significant relationship between the different solvent extract concentrations of *A. indica* leaves and percentage of mortality of microfilariae of *S. cervi* worm ($p < 0.05$) (Table 2). Variations, however recorded in the anthelmintic activity of different polar solvent extracts of *A.*

indica used against the microfilariae of *S. cervi* worm. The data obtained from regression and correlation of regression (Table 3) revealed the best dose dependent effect on the mortality rate of microfilariae of *S. cervi* with ethanolic extract ($R^2 = 0.871$) followed by methanol ($R^2 = 0.637$), diethyl ether ($R^2 = 0.523$) and chloroform ($R^2 = 0.484$).

Discussion

From the findings obtained during present study it is evident that time and concentration both, play significant roles in all the four extracts of *A. indica* leaves. Highest mortality rate of 100 and 90 % was caused by methanol and ethanol extract at 200 µg/ml concentration after 135 min,

Fig. 1 Showing (Mean $\pm$ SE) mortality of microfilariae of *S. cervi* at different concentration of *A. indica* leaves extract in solvents at different time intervals

indicating that the mortality rate of methanol and ethanol are highest and highly effective against the microfilariae of *S. cervi*. In contrast in earlier study on *Butea monosperma* inhibitory effect on the microfilariae of *S. cervi* was observed at the higher concentration of 1.25 mg/ml methanol and 3.6 mg/ml hexane-ethanol extracts when incubated for 24 h (Deshmukh et al. 2014). Anthelmintic effect of pure *A. indica* leaves extract on L3 larvae of *Haemonchus contortus* was maximum at 1000 µg concentration (Radhakrishnan et al. 2007). In vitro anthelmintic activity of methanol extract of *A. indica* against L3 larva of *H. contortus* from goats showed 40 % mortality at 4 mg/ml after 24 h treatment (Rahman et al. 2011). In contrast methanolic extract of *Aegle marmelos* Corr. leaves and *Vitex negundo* against adult *S. cervi* worm showed significant reduction of IC₅₀ at 0.168 and 0.049 mg/ml (Sahare and Singh 2013a, 2014). In vitro effect of ethanol extract of *A. indica* has also been observed in fowl against gastrointestinal nematodiasis which showed 100 % efficacy at a concentration of 50 mg/ml (Alam et al. 2014). In another study ethanolic extract of *A. indica* showed significant worm reduction at 25 µg/ml and highest mortality at 100 µg/ml after 24 h of incubation when applied against the microfilariae of *S. cervi* (Mukherjee et al. 2014).

Chloroform and diethyl ether also show the anthelmintic effect at 200 µg/ml causes 80 and 73 % mortality after 135 min of incubation. Therefore, it is clear that all the solvent extracts of *A. indica* show a significant mortality rate at 200 µg/ml concentration. In earlier study

chloroform extract of *V. negundo* leaves showed IC₅₀ at 0.084 mg/ml concentration after 24 h indicating significant antifilarial effect (Sahare and Singh 2013b). Studies conducted on alcoholic and aqueous extracts of *A. indica* on microfilariae of *S. cervi* showed LC₅₀ at 15 and 18 ng/ml (Mishra et al. 2005c) which is comparatively low concentration compared to my study. However earlier many studies were conducted on in vitro anthelmintic activity of plants/plant parts both aqueous and alcoholic extracts like *Sencio nudicaulis* (leaves), *Asparagus adscendens* (roots), *Mallotus philippensis* (leaves), *Ficus racemosa* (fruits) and *Pluechea lanceolata* (leaves and stem) on the microfilariae of *S. cervi*. In these studies alcoholic extract LC₅₀ ranges between 3 and 21 ng/ml after 6 h of incubation and LC₉₀ ranges between 12 and 35 ng/ml and in aqueous extract LC₅₀ ranges between 8 and 25 ng/ml and LC₉₀ between 16 and 42 ng/ml against the microfilariae of *S. cervi* (Singh et al. 1996, 1997a, b; Mishra et al. 2005a, b). The range of LC₅₀ and LC₉₀ was higher in *Leucas cephalotes* alcoholic alcohol extract of flower and stem against microfilariae of *S. cervi* i.e. LC₅₀ was 30 ng/ml and LC₉₀ was 50 ng/ml for flower and LC₅₀ and LC₉₀ for stem was 45 and 75 ng/ml respectively (Qamaruddin et al. 2002). Alcoholic extract of *A. indica* seeds was also recommended as anthelmintic against *Hymenolepis diminuta*, *Enterobius vermicularis* and hookworm as it is quite efficacious in naturally infected rats (Lawson et al. 2003). In vitro and in vivo study on gemmotherapeutic extract of *A. indica* leaves against gastrointestinal helminthes of sheep

Table 2 Pearson product correlation coefficient describing the relationship between 2 continuous variables

Relationship between methanol extract concentration and % of motility/mortality of microfilariae of <i>S. cervi</i>		
	Methanol extract concentration (µg/ml)	% of motility/mortality of microfilariae
Methanol extract concentration (µg/ml)		
Pearson correlation	1	.798 ^a
Sig. (1-tailed)	–	.001
N	12	12
% of motility/mortality of microfilariae		
Pearson correlation	.798 ^a	1
Sig. (1-tailed)	.001	–
N	12	12
Relationship ethanol extract concentration and % of motility/mortality of microfilariae of <i>S. cervi</i>		
	Ethanol extract concentration (µg/ml)	% of motility/mortality of microfilariae
Ethanol extract concentration (µg/ml)		
Pearson correlation	1	.933 ^a
Sig. (1-tailed)	–	.000
N	12	12
% of motility/mortality of microfilariae		
Pearson correlation	.933 ^a	1
Sig. (1-tailed)	.000	–
N	12	12
Relationship between chloroform extract concentration and % of motility/mortality of microfilariae of <i>S. cervi</i>		
	Chloroform extract concentration (µg/ml)	% of motility/mortality of microfilariae
Chloroform extract concentration (µg/ml)		
Pearson correlation	1	.696 ^a
Sig. (1-tailed)	–	.006
N	12	12
% of motility/mortality of microfilariae		
Pearson correlation	.696 ^a	1
Sig. (1-tailed)	.006	–
N	12	12
Relationship between diethyl ether extract concentration and % of motility/mortality of microfilariae of <i>S. cervi</i>		
	Diethyl ether extract concentration (µg/ml)	% of motility/mortality of microfilariae
Diethyl ether extract concentration (µg/ml)		
Pearson correlation	1	.723 ^a
Sig. (1-tailed)	–	.004
N	12	12
% of motility/mortality of microfilariae		
Pearson correlation	.723 ^a	1
Sig. (1-tailed)	.004	–
N	12	12

^a Correlation is significant at the 0.01 level (1-tailed)

detected 100 % inhibitory effect at 2.0 mg/ml concentration (Dogar et al. 2012). In vitro and in vivo observation on antimicrofilaricidal effects of *Trachispermum ammi* seed

extract in methanol, chloroform and diethyl ether solvent extract at 37 °C for 45 min showed significant activity against microfilariae of *S. cervi* (Rizvi et al. 2012).

Table 3 Regression values and correlation of regression of the effect of different solvent extract of *A. indica* leaves on mortality of microfilariae of *S. cervi*

<i>A. indica</i> extract	Regression values and correlation of regression	Ranking
Methanol extract	$Y = 0.403x + 2.665$ ($R^2 = 0.637$)	2
Ethanol extract	$Y = 0.354x + 0.820$ ($R^2 = 0.871$)	1
Chloroform extract	$Y = 0.252x + 0.821$ ($R^2 = 0.484$)	4
Diethyl ether extract	$Y = 0.239x + 0.577$ ($R^2 = 0.523$)	3

Present work reported the anthelmintic effect of diethyl ether and chloroform extract of *A. indica* on the microfilariae of *S. cervi* for the first time and also demonstrated that all the solvent extract of *A. indica* leaves possesses antifilarial activity against the microfilariae of *S. cervi* therefore screening experiments should be conducted on the refined phytochemical agents of *A. indica* to see their efficacy on the microfilariae and adult *S. cervi* worm and their mode of action must be worked out.

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