ANTIFILARIAL EFFECT OF *ARTEMISIA NILAGIRICA* EXTRACT AND ITS ULTRA HIGH DILUTIONS AGAINST CANINE DIROFILARIASIS

NIRMAL CHANDRA SUKUL, PAROMITA SARKAR, ANIRBAN SUKUL
AND SANTI PRASAD SINHABABU

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Abstract: An ethanolic extract of the flowering meristems of worm wood, *Artemisia nilagirica* was allowed to evaporate. The residue, thus obtained, was administered orally on 4 pariah dogs naturally infected with *Dirofilaria immitis* at 10 mg/kg/day for 15 days and then at 20 mg/kg/day for the next 15 days. Two homoeopathic potencies of the *A. nilagirica* extract, called Cina 200 and Cina 1000, were obtained commercially and administered orally at 0.1 ml/dog/day for 30 days on two separate batches, each consisting of 4 dogs. Blood was sampled from the dogs before treatment and on day 15, 30, 45 and 75 following the treatment. *A. nilagirica* extract (Cina θ) was diluted with 90% ethanol 1:100 and shaken by 10 manual strokes to prepare the 1st potency, called Cina 1. All subsequent potencies were prepared by mixing 1 part of the preceding potency with 99 parts of 90% ethanol and giving the mixture 10 manual strokes. Cina θ , Cina 200 and Cina 1000 reduced microfilarial densities in treated dogs by 78.38, 63.06 and 71.40%, respectively on day 30. There were 57.13, 42.44 and 64.20% reduction on day 75. No apparent toxic effect was observed in the treated dogs. Electronic spectra of Cina θ , Cina 200 and Cina 1000 showed comparable absorbance with the latter two giving a blue shift. Cina θ in CCl_4 showed a red shift suggesting molecular complexation and charge transfer (CT) interaction between aqueous ethanol and compounds of *A. nilagirica*. CT was further evidenced by the NMR spectra of the deuterium nuclei of Cina θ in 90% ethanol. NMR spectra of Cina θ , Cina 200, Cina 1000 and 90% ethanol indicated a change in the solution structure of Cina 200 and Cina 1000. This altered solution structure is thought to be responsible for inducing immune reaction of the hosts against the parasite.

Key words: *Artemisia nilagirica*, Antifilarial, *Dirofilaria immitis*, Homoeopathic potency, Ethanol solution structure, Electron transfer

INTRODUCTION

Species of *Artemisia* have long been used by rural people for expelling intestinal nematodes (Singh *et al.*, 1983). High dilutions of the ethanolic extract of the flowering meristems of *Artemisia nilagirica* (Clarke) Pamp have been used in the Homoeopathic system of medicine under the name Cina against intestinal worms (Kent, 1911; Boericke, 1927). The purpose of the present study is to see whether this plant extract, both in its crude form and also its homoeopathic dilutions, called potencies, is effective against canine dirofilariasis.

In Homoeopathy the ethanol extract of *A. nilagirica*, called Cina θ , is diluted with 90% ethanol 1:100 and the mixture is shaken with 10 powerful downward strokes to prepare the first centesimal potency called Cina 1. All subsequent potencies are prepared by adding

to one part of the preceding potency 99 parts of 90% ethanol and shaking the mixture in a similar way (Anonymous, 1920; Sukul and Klemm, 1988). Effective homoeopathic potencies could also be produced by sonication instead of mechanical agitation (Sukul *et al.*, 1996; Sukul, 1997). Two potencies of Cina, like Cina 200 and Cina 1000, purchased from King & Co., Calcutta, were used. Since these potencies are too dilute to have any drug molecules, electronic and NMR spectra of them were obtained to find out their difference from the solvent medium like 90% ethanol vis-a-vis the physical basis of their effectiveness. In order to find out the solvent effect on the solute, we prepared Cina θ in a neutral solvent like CCl_4 and obtained the electronic spectra of the solution.

MATERIALS AND METHODS

Treatment with Cina θ

Cina θ , purchased from King & Co., Calcutta was allowed to evaporate in an incubator at 40°C. The residue was dehydrated in a vacuum dessicator over anhydrous Calcium Chloride and stored at 4°C. Blood was sampled from 4 naturally infected dogs, 2 males and 2 females, every 15 days for a period of 2 months and microfilarial concentration per ml of blood was determined. Blood film was allowed to dry, dehaemoglobinised in distilled water and stained with Giemsa stain. The same dogs were then administered orally with the residue of Cina θ at 10 mg/kg body weight/day for 15 days. Blood was sampled from the dogs on day 15. The same dogs were treated again orally with the residue at 20 mg/kg/day for 15 days more. Blood was sampled on day 30, 45 and 75. Capsules were filled with the residue, kept inside a loaf of bread and then offered to the microfilaraemic dogs.

Treatment with Cina 200 and Cina 1000

Blood was sampled from a batch of 4 infected dogs, 2 males and 2 females, every 15 days for 2 months. Cina 200 was mixed with pure cow milk at 0.1 ml/4 ml of cow milk and 4 ml of the mixture was offered in a glass plate to a dog which immediately consumed the mixture. The schedule of treatment and blood sampling were the same as with Cina θ . However, the dosage of Cina 200 was same in the 2 phases of treatment. A batch of 4 naturally infected dogs (3 females and 1 male) was treated with Cina 1000 after determining the mf density for 2 months. The treatment schedule, dosage of the drug and blood sampling were same as with Cina 200.

Electronic spectra of drug

Using a UV-VIS spectrophotometer (Beckman DU 640) absorption spectra of Cina θ in 90% ethanol and Cina θ in carbon tetrachloride, Cina 200 and Cina 1000 against the corresponding solvent blanks were obtained in the wave length range of 190–750 nm at 29°C. The spectra were run in matched quartz cuvettes and were corrected for instrumental baseline errors. Test solutions were kept at the above temperature for at least 10 min to allow for the thermal equilibration.

NMR Spectra of drugs

The spin-lattice relaxation time (T_1 in msec) of the naturally abundant ^2H (0.015%) was measured in 90% ethanol, Cina θ in 90% ethanol, Cina 200 and Cina 1000 using a AMX-400 NMR spectrometer operating at 61.4

MHz at 22°C. The mechanism by which excess spin energy of a nucleus (here ^2H) is shared with the surroundings is referred to as the spin lattice relaxation. The time taken for a fraction $1/e=0.37$ of the excess energy to be dissipated is called the relaxation time. Such relaxation comes about by lattice motions like molecular tumbling in liquids (Banwell and McCash, 1994). Deuterium is a quadrupolar nucleus having a small quadrupole moment 1. Quadrupolar relaxation depends upon the interaction of the electric quadrupole moment with an electric field gradient. If the quadrupole moment is small, as it is for ^2H , the interaction is small and the relaxation will be slow. Like all quadrupolar nuclei its relaxation is sensitive to τ_c . τ_c is the average time taken to rotate through 1 radian or roughly the reciprocal of the rate of tumbling of the relevant piece of the molecule (Sanders and Hunter, 1993). T_1 values of ^2H of water, hydroxyl, methylene and methyl groups of ethanol were measured from the stacked spectra with the help of a computer.

RESULTS

Treatment effect on mf count

The mean microfilarial counts per ml of blood in 3 batches of dogs before treatment are shown in Table 1. The microfilarial concentration in each dog did not vary appreciably during the 2-month period of observation.

Table 1 Microfilarial concentration/ml blood of dogs naturally infected with *Dirofilaria immitis* before treatment

Microfilarial concentration/ml blood on different days					
Dogs	Day 0	Day 15	Day 30	Day 45	Day 60
Batch I					
Male	1,001	973	945	997	986
Male	437	423	480	505	440
Female	727	798	756	704	692
Female	813	852	808	826	874
Batch II					
Male	219	241	264	313	274
Male	304	298	253	221	234
Female	535	477	542	580	569
Female	330	374	385	423	450
Batch III					
Male	274	235	252	300	302
Female	236	287	259	247	272
Female	218	269	254	219	203
Female	251	275	244	235	279

Batch I: Treated later with Cina θ

Batch II: Treated later with Cina 200

Batch III: Treated later with Cina 1000

Figure 1 Percentage of reduction in microfilarial concentration of *D. immitis* in 4 dogs treated with ethanol extract of *Artemisia nilagirica* (Cina θ) at 10 mg/kg/day for 15 days and 20 mg/kg/day for the next 15 days (—○—). The same in 2nd batch of 4 dogs treated with Cina 200 for 30 days (—□—) and in 3rd batch of 4 dogs treated with Cina 1000 for 30 days (—△—).

Percentage changes in microfilarial concentration for the treated dogs were plotted in a graph against days of sampling in Fig. 1. The mean mf density just before treatment of the 3 treatment groups served as the standard with respect to which the percentage change was

Table 2 Spin-lattice relaxation time (T_1) of ^2H of 90% ethanol, Cina θ in 90% ethanol, Cina 200 and Cina 1000. Cina θ is the ethanolic extract of *A. nilagirica* flowering tops. The two potencies, Cina 200 and Cina 1000, were prepared by successive dilution of Cina θ with 90% ethanol 1:100 and manual succession. Measurements were taken by a AMX-400 NMR spectrometer operating at 61.41 MHz at 22°C

	Mean $\pm$ S.E. of T_1 (m sec)			
	Water		Ethanol	
	OH	OH	CH ₂	CH ₃
Ethanol 90%	106.9 ± 0.5 a	110.8 ± 0.8 a	846.5 ± 0.4 a	822.5 ± 0.5 a
Cina θ in 90% EtOH	104.3 ± 0.4 b	—	883.4 ± 0.3 b	776.1 ± 0.4 b
Cina 200	108.2 ± 0.7 c	102.5 ± 0.8 b	867.4 ± 1.7 c	792.7 ± 0.3 c
Cina 1000	106.6 ± 0.8 a	86.8 ± 0.9 c	971.7 ± 2.4 d	857.6 ± 2.3 d

a, b, c, d: Significant difference ($P < 0.01$) by ANOVA (one way) in a column.

Figure 2 Absorption spectra of Cina θ (ethanol extract of flowering meristems of *A. nilagirica*) and its two ultra high dilutions called Cina 200 and Cina 1000, all in 90% ethanol in wavelength range of 190–750 nm. The same of Cina θ in CCl_4 showing a red shift as compared to Cina θ in 90% ethanol. Cina 200 and Cina 1000 show a blue shift. The absorbance intensities in all the 4 are fairly comparable.

calculated in subsequent samples. The mf density showed marked reduction in the treated dogs. The maximum reduction was 78.4% with Cina θ , 63.1% with Cina 200 and 71.40% with Cina 1000. The reduction was 42.4–64.2% on the last day of sampling i.e. day 75 (Fig. 1).

Electronic spectra

Electronic spectra of Cina θ in 90% ethanol, Cina θ in CCl_4 , Cina 200 and Cina 1000 are given in Fig. 2. The absorption spectra of Cina θ in ethanol shows a blue shift as compared to that of Cina θ in CCl_4 (Fig. 2). Absorption spectra of Cina 200 and Cina 1000 show a blue shift as compared to Cina θ in ethanol. The absorbance was fairly comparable in the tincture and its two potencies (Fig. 2).

NMR Spectra

The T_1 values with S.E. of ^2H of water, OH, CH_2 and CH_3 of 90% ethanol, Cina θ in 90% ethanol, Cina 200 and Cina 1000 are presented in Table 2. The T_1 values were compared by ANOVA. T_1 of OH of water was lowest with Cina θ and highest with Cina 200. T_1 of ethanol hydroxyl was absent in Cina θ , highest in 90% ethanol and lowest in Cina 1000. T_1 of CH_2 was highest in Cina 1000 followed by Cina θ , Cina 200 and 90% ethanol. T_1 of CH_3 was highest in Cina 1000. This was followed by 90% ethanol, Cina 200 and Cina θ in decreasing order (Table 2).

DISCUSSION

It is evident from the results that both the crude extract as well as the two potencies of *A. nilagirica* proved highly effective against the microfilariae of *Dirofilaria immitis* in dogs. Species of *Artemisia* are reported to contain various types of essential oils and sesquiterpene lactones including santonin (Carnat *et al.*, 1992; Rucker *et al.*, 1992; Nin *et al.*, 1995; Todorrova and Krasteva, 1996). These compounds in the crude extract may have a direct effect on the microfilariae. The effect of the two potencies, which have no drug molecules, can be explained in a different way. Electronic spectra of Cina θ in 90% ethanol and that in CCl_4 are different with the former showing a blue shift as compared to the latter. This suggests molecular complexation and charge transfer (CT) interaction between the compounds of *A. nilagirica* and ethanol molecules. Here ethanol molecules served as electron donors and *Artemisia* compounds as electron acceptors (Singh and Dikshit, 1995). Alcohols donate electrons from the highest occupied nonbonding, n (b_1), orbital of the Oxygen atom to the π orbital of the acceptors in *Artemisia* (Frey *et al.*, 1994). Absorption occurs well into the near UV region. CT is further evidenced by the absence of T_1 value at the ethanolic hydroxyl site of Cina θ (Table 2). Relaxation at this site was too efficient and the n.m.r. signal was too broad to be observed (Banwell and McCash, 1994). We have already observed CT interaction in other potentized homeopathic drugs like Iodine and Nux vomica (Sukul, 1999). Other plant extracts such as tea contain a mixture of potential complexing agents which form molecular complexes with other compounds (Hernaiz *et al.*, 1997). For intermolecular electron-transfer systems, a number of competing acceptors may exist, as in *Artemisia* extract, in a complicated spatial array about the donor. The back transfer process is coupled to the

forward transfer in a complex fashion (Weidemaier and Fayer, 1996). Thus the electronic configuration of the donor molecules, i.e. aqueous ethanol, would undergo a change according to the nature of the electronic acceptors of *Artemisia* extract. With successive dilution, the acceptor molecules are progressively depleted and fresh molecules of the donor occupy their place. Finally, it is the molecules of aqueous ethanol which exist in the form of ethanol molecules surrounded by the hydration shell of water molecules.

UV spectroscopy is highly sensitive to the distortion of chromophores and auxochromes (Banwell and McCash, 1994). The UV spectra of Cina 200 and Cina 1000 show a blue shift as compared to that of Cina θ thereby suggesting a possible change in the electronic configuration of the medium, i.e. aqueous ethanol (Fig. 2). The altered T_1 values of the deuterium nuclei of Cina 200 and Cina 1000 as compared to those of aqueous ethanol and Cina θ (Table 2) suggest that the rate of tumbling in the relevant parts of the molecule in potentized Cina has undergone a change obviously due to CT interaction and mechanical agitation (Sukul, 1999). Haseba *et al.* (1993) reported that the thermal motion of water molecules in sonicated aqueous ethanol was greater than that in unsonicated one, and this change in the solution structure produced significant biological effects.

Living microfilariae of *D. immitis*, injected intravenously, disappeared rapidly from the peripheral circulation of uninfected dogs, which had received and cleared previous infections of microfilariae. Eosinophilia and antibodies to microfilaria were demonstrable in the blood of the dogs (Wong, 1964, 1966 cited by Wong and Guest, 1969). Filarial worms are known to cause immunosuppression (Ottesen, 1980). It is possible that potentized Cina might have removed immunosuppression resulting in vigorous responsiveness of the host to parasite antigens thereby clearing microfilariae from the blood.

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