Experiment 1

Effects of tetracycline, azithromycin and acaciasides on D. immitis microfilaria in vitro

Introduction:

Lymphatic filariasis has become a global concern; it is responsible for major health problems throughout tropical and subtropical countries. Most of the filarial nematodes affecting human and animals are infected with endosymbiotic bacteria, Wolbachia. It is evident that these bacteria play an important role in the pathogenesis and immune response to the filarial infection (Bandi et al; 2001). In adult D. immitis, Wolbachia is present in the lateral chords of hypodermis, in the ovaries of females and in the developing embryos, but is not reported from male reproductive apparatus (Sacchi et al., 2002).

Many studies in animal model have shown that antibiotic eradication of the bacteria from filarial worms resulted in embryotoxicity, inhibition of moulting and development and eventually, the death of adult parasites (Taylor, 2000; Hoerauf et al., 2000a; Langworthy et al., 2000).

Treatment with doxycycline in human onchocerciasis leads to a profound embryotoxicity and sustained clearance of bacteria for several months (Hoerauf et al., 2003a). There are several in vitro studies on motility of filarial worms with DEC or tetracyclines. Court et al. (1986) reported that DEC did not
immobilize *B. pahangi* females at more than 100 µM. Doxycycline reduced *B. malayi* adult motility more clearly than tetracycline (Rao and Weil, 2002). A single use of oxytetracycline (50 µM) reduced motility levels of *O. gutturosa* males (Townson *et al.*, 2000).

The aim of this study was to evaluate the effects of tetracycline, azithromycin and acaciasides on *D. immitis* in vitro. The second aim was whether adding acaciasides to the effective dose of either antibiotic tested could enhance the microfilaricidal activity.

**Materials and methods:**

**Animals and preparation of microfilaria stock:**

Adult dogs naturally infected with *D. immitis* were selected for the study. Blood was collected from each dog for the determination of microfilaria (mf) load per 1ml of blood.

From all the experimental dogs, blood was taken in heparinised tubes and immediately diluted (1:1) with prechilled PBS (0.01 M phosphate buffer, 0.15 M sodium chloride, pH 7.4) and was filtered through a 5 µM filter membrane (Millipore, USA). Mf was separated by spinning at 5000 x g for 5 min. The mf pellet was then resuspended with RPMI 1640 containing 100 U/ml streptomycin, 100 µg/ml penicillin and 40 µg/ml gentamycin.

Medium was replaced with fresh medium containing antibiotics in every 6 h.
Preparation of drugs:

The drugs used for the experiments were tetracycline and azithromycin and acaciasides. Tetracycline was obtained from Aventis Pharma Ltd., India and azithromycin from Alembic Ltd. Tetracycline and the mixture of two acaciasides were dissolved in small volume of sterile double distilled water and azithromycin in a small quantity of ethanol and then diluted in RPMI 1640.

Effects of drugs on mf viability:

Mf were acclimatized in complete medium (CM) containing RPMI 1640 supplemented with 25 mM HEPES buffer, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 40 µg/ml gentamycin and 10% fetal calf serum in BOD for 6 h at 37°C.

The effects of antibiotics on mf were studied by incubating 100 mf in 200 µl of CM containing drugs at 5, 10, 20, 40, 60, 80 and 100 µg/ml in 96-well flat-bottomed microtiter plates. Mf were viewed with an inverted microscope and their motility was scored at 0, 1, 3, 4, 6, 12, 24 and 48 h of incubation. For each set of drugs one drug free control was kept. The mf cultures with or without antibiotics were carried out in triplicate and the result was expressed as means of the replicate experiments.

Assessment of mf motility by microscopic observation:

Mf motility and death were assessed visually by microscopy. Survivality was based on the motility of the mf according to a Movability Index (MI)
scoring method by Zaridah et al. (2001). Movability Index (MI) scores were then assessed with movements, as whole body movement (score of 3), partial movement (score of 2), immobile but responding to stimuli (score of 1) and no response (score of 0). The experiment was repeated in triplicates.

Microfilaricidal activity was described as relative movability (RM) with both MI and RM values were calculated using the equations given below.

\[
\text{Movability Index, } \text{MI} = \frac{\sum nNn}{\sum N} \quad \text{where, } n = \text{score 0, 1, 2 or 3}
\]

\[
\text{Relative Movability, } \text{RM} = \frac{\sum N}{\sum N} \times 100 \quad \text{X 100}
\]

where, RM value of 100 = no microfilaricidal activity

RM value of 0 = strongest microfilaricidal activity

**MTT reduction assay:**

Mf viability was assessed quantitatively by the 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT, Sigma) reduction assay (Comley et al., 1989). After microscopic assessment of mf viability, 200 µl PBS containing 0.5 mg MTT/ml was added to each tube and incubated for 2 h at 37°C.
C in the dark. Mf was then pellet down and the supernatant was discarded. The dark blue formazan crystals formed were then dissolved in 200 µl dimethyl sulphoxide (DMSO) at room temperature for 1 h. Formazan was measured with a microtiter plate reader by measuring optical density at 595 nm, using DMSO as a blank. The minimum concentration of drugs that caused a 50% reduction in formazan crystal production was considered the minimum effective concentration (MIC) in the MTT assay.

**In vitro cytotoxicity assay:**

In vitro cytotoxicity assay for acaciasides was performed on Vero cells (monkey kidney cells) in the laboratory of Dr. Mrs. Shailja Mishra-Bhattacharya, Senior Deputy Director, Central Drug Research Institute, Lucknow.

**Statistical analysis:**

The data were analysed by one way ANOVA method using MS excel software to identify differences among treatment groups compared with control. Differences were considered significant with $P$ values <0.05.
**Results:**

**Effects of antibiotics and acaciasides on mf movability:**

All the drugs/compounds tested did not produce any significant change in RM (%) at 5, 10, 20, 40 and 50 µg/ml after 48 h of incubation (one way ANOVA; P < .05). These are the least effective concentrations.

After 6 h of incubation with tetracycline microfilaricidal activity with a non-linear curve over time was observed with 50, 60, 80 and 100 µg/ml (Fig. 5). Dose-dependant microfilaricidal activity was observed with the highest concentration (100 µg/ml): the strongest microfilaricidal activity (RM 3%). After 48 h of incubation with 50 µg/ml, the RM value reduced to 35%, the minimum effective concentration (MEC) in this series (one way ANOVA, P < .05). However, RM value reduced to 50% much earlier (12 h) with 60 µg/ml (one way ANOVA, P < .05; Fig. 5). Strongest microfilaricidal activity with reduced RM of 3% was obtained at higher dose (100 µg/ml) after 48 h of incubation (one way ANOVA, P < .001; Fig. 5).

Maximum microfilaricidal activity (RM 65%) was observed with 100 µg/ml azithromycin after 48 h incubation (Fig. 8). The minimum effective concentration in this series was the combination (1:1) of azithromycin and acaciasides at 50 µg/ml, demonstrating reduced RM to 52% and 37% after 24 and 48 h of incubation, respectively (Fig. 9).
Acaciasides at 50 µg/ml alone reduced the RM to 52% after 48 h of incubation (Fig. 7). This is the MEC in this series. Whereas at the same dose (50 µg/ml) azithromycin and tetracycline yielded RM value 85% and 35%, respectively by the same hour of incubation. The combination of tetracycline and acaciasides (50 µg/ml) effectively reduced RM value to 30 % (48 h, Fig. 6). The combination of azithromycin and acaciasides resulted RM value of 52 % much earlier (24 h) than acaciasides (48 h) alone (Fig. 6). This may be due to the synergistic effects of azithromycin and saponins. So the combination of tetracycline and acaciasides is more potent as a microfilaricide than the individual/combination drugs tested.

Effects of antibiotics and acaciasides on mf viability (MTT assay):

Mf viability was reduced to 55% after 12 h of incubation with tetracycline at 80 µg/ml. However, maximum reduction (68%) was observed after 48 h of incubation with 100 µg/ml (Fig. 10). MEC for tetracycline was 80 µg/ml (55% reduction after 12 h). Mf viability was reduced to 50% after 48 h of incubation with acaciasides at 60 µg/ml. MEC for acaciasides was 60 µg/ml (50% reduction after 48 h, Fig. 12). Though 50% reduction in viability was achieved earlier (24 h) at a higher concentration (100 µg/ml). But the combination (1:1) of tetracycline and acaciasides reduced viability to 51% at a lower dose (50 µg/ml) after 48 h of incubation (Fig. 11).
Mf viability was significantly decreased to 35% after 48 h of incubation with 100 µg/ml azithromycin (Fig. 13). After 48 h of incubation with azithromycin and acaciasides at 50 µg/ml reduced the viability of mf to 21% and 40%, respectively. The drugs used in combination (1:1) produced almost the same result (Fig. 14).

**In vitro cytotoxicity assay:**

*In vitro* cytotoxicity assay revealed that acaciasides at 300 µg/ml and above did not produce any toxic effect on Vero cell line (monkey kidney cells).

**Discussion:**

The microfilarial activity was assessed by microscopy (movability) and by the MTT assay. The present study was an attempt to investigate whether antibiotics could enhance the activity of saponins on mf of *D. immitis* *in vitro*. The results reveal that all the active compounds tested showed increased microfilaricidal effects on mf with higher drug concentrations, and the same activity increased eventually over time. Different drug concentrations were required to achieve MECs for different parameters.

For azithromycin no significant change in microfilaricidal activity was observed even with the highest dose (i.e. 100 µg/ml) indicating that higher dose and longer exposure time may be required for the antibiotic to produce the
desired result. The combination (1:1) of azithromycin and acaciasides (50 µg/ml) resulted in maximum 37% RM value by 48 h of incubation whereas acaciasides at the highest dose (100 µg/ml) produced the same result by 24 h of incubation. Data from MTT assay revealed that azithromycin even at the highest dose (i.e.100 µg/ml) was not able to kill the mf significantly and addition of acaciasides to azithromycin (1:1) could not exert better effect. This seems that azithromycin may enhance the activity of saponins so that microfilaricidal activity was achieved at a lower dose in the combination (1:1) of azithromycin and acaciasides compared to either drug alone. From the data it is very much likely that higher dose of azithromycin alone and in combination with acaciasides may be needed to kill the parasites rather than to immobilise them.

The lower concentration of acaciasides and tetracycline (5-40 µg/ml) did not significantly differ from each other with respect to microfilaricidal activity. One possible explanation is that at these concentrations (5-40 µg/ml) the activity of the drug reached to a plateau, therefore it could not exert further effect on the target organisms. It is very much likely that worms may require longer period of exposure (beyond 48 h) to the drugs used for obtaining the desired results. Data from MTT assay demonstrates that tetracycline below 80 µg/ml and acaciasides below 60 µg/ml did not significantly affect the viability of mf. Though these concentrations of tetracycline and acaciasides were effective for immobilising the worms, as evident from microscopy but could not induce cell death. The ‘straight pose’ may not be confused with the death of an
organism. It is also possible that motility reflects a total outcome of coordinated muscular cell activities rather than a simple sum of cell viability. Comley et al. (1989) discussed that motility was a sensitive measure of ‘worm health’, while the MTT assay was more indicative of irreversible degenerative changes. It may be due to drug-induced paralysis. The apparently immobilised mf may regain its activity after 48 h of incubation.

The combination (1:1) of tetracycline and acaciasides resulted in 30% RM value at a lower dose (50 µg/ml by 48 h of incubation) compared to either drug alone. Tetracycline at 80 µg/ml by 12 h of incubation and acaciasides at 100 µg/ml by 48 h of incubation produced the same result. From MTT assay it is clear that the combination (1:1) of tetracycline and acaciasides reduced mf viability to 51% at a lower dose (50 µg/ml) compared to tetracycline (80 µg/ml) and acaciasides (60 µg/ml) given alone.

The activity of tetracycline has been confirmed by earlier observations. Tetracycline (50 µg/ml) effectively reduced microfilaria release from adult females of B. pahangi from day 1 of culture and the motility of adult females reduced gradually and reached a significantly different level (Gunawardena et al., 2005). Smith and Rajan (2000) showed that tetracycline at 25 µg/ml is capable of blocking the L3 to L4 molt of B. malayi, B. pahagi and D. immitis in a serum-free in vitro system.

The result on in vitro tests do corroborate our in vivo experiments where we have shown that the drugs in combination (tetracycline and acaciasides)
provided the best antifilarial efficacy by totally (99.8%) clearing the circulating mf compared to individual drug. This indicates that tetracycline enhances the microfilaricidal activity of saponins. This effect may be additive or synergistic as the worms are weakened by the depletion of *Wolbachia* and then these weakened mf are killed by saponins.

The antibiotics tested were often above the safety levels used for humans (5µg/ml tetracycline, 0.4 µg/ml azithromycin) (Kucers *et al*., 1997). Rao and Weil (2002) have demonstrated that mf release from adult *B. malayi* tended to be inhibited at lower drug concentrations than those required for killing of mf and adult worms and the antibiotics affected filarial worms within a few days. This suggests that the worms have a constant need for the bacteria and that there is no reservoir of bacterial products in treated worms that can substitute for metabolically active, live bacteria.

Antibiotics concentrations needed to kill *Wolbachia* in insect cell cultures (Hermans *et al*., 2001) were lower than those required to kill the bacteria in intact nematodes in the present study and this also correlates the previous reports (Rao and Weil, 2002). This implies that antibiotics may diffuse more easily into cells than into intact nematodes. The same antibiotics may require different times to become active on different nematode species due to differences in drug penetration and methodological alterations (Townson *et al*., 2000; Rao and Weil, 2002).
The combination of the three drugs tested offers the best treatment regimen than individual one. The use of acaciasides in the combination may reduce the need to use high doses of conventional drugs for treatments and prophylaxis and thus indirectly reducing the potential risks of adverse reactions expressed by these drugs. All the tested drugs showed antifilarial activity against *D. immitis* *in vitro*, the most effective is the combination (1:1) of tetracycline and acaciasides.

*In vitro* cytotoxicity assay revealed that acaciasides (at 300 µg/ml and above) did not produce any toxic effect on Vero cell line (monkey kidney cells).

![Graph showing the relative movability (RM%) value of *D. immitis* mf against time for different concentrations (µg/ml) of tetracycline. Data were analysed by one way ANOVA and expressed as mean±S.E. (n=3). Significance was compared with the negative control group. Asterisks denote level of significance: *P<0.05.](image-url)
Fig. 6: Relative movability (RM%) value of *D. immitis* mf against time for 50 µg/ml each of tetracycline, tetracycline+acaciasides and acaciasides. Data were analysed by one way ANOVA and expressed as mean±S.E. (n=3). Significance was compared with the negative control group. Asterisks denote level of significance: *P<0.05.
Fig. 7: Relative movability (RM%) value of *D. immitis* mf against time for different concentrations (µg/ml) of acaciasides. Data were analysed by one way ANOVA and expressed as mean±S.E. (n=3). Significance was compared with the negative control group. Asterisks denote level of significance: *P<0.05.
Fig. 8: Relative movability (RM%) value of *D. immitis* mf against time for different concentrations (µg/ml) of azithromycin. Data were analysed by one way ANOVA and expressed as mean±S.E. (n=3). Significance was compared with the negative control group. Asterisks denote level of significance: *P<0.05.
Fig. 9: Relative movability (RM%) value of *D. immitis* mf against time for 50 µg/ml each of azithromycin, azithromycin +acaciasides and acaciasides. Data were analysed by one way ANOVA and expressed as mean±S.E. (n=3). Significance was compared with the negative control group. Asterisks denote level of significance: *P<0.05.
Fig. 10: Percent reduction in viability of *D. immitis* mf relative to control against time for different concentrations (µg / ml) of tetracycline. Data were analysed by one way ANOVA and expressed as mean ± S.E. (n=3). There was a significant difference between the control and treated groups. Asterisks denote level of significance: *P<0.05.
Fig. 11: Percent reduction in viability of *D. immitis* mf relative to control against time for 50µg / ml each of tetracycline, tetracycline+acaciasides and acaiasides. Data were analysed by one way ANOVA and expressed as mean ± S.E. (n=3). There was a significant difference between the control and treated groups. Asterisks denote level of significance: *P<0.05.
Fig. 12: Percent reduction in viability of *D. immitis* mf relative to control against time for different concentrations (µg / ml) of acaiasides. Data were analysed by one way ANOVA and expressed as mean ± S.E. (n=3). There was a significant difference between the control and treated groups. Asterisks denote level of significance: *P<0.05.*
Fig. 13: Percent reduction in viability of *D. immitis* mf relative to control against time for different concentrations (µg / ml) of azithromycin. Data were analysed by one way ANOVA and expressed as mean ± S.E. (n=3). There was a significant difference between the control and treated groups. Asterisks denote level of significance: *P<0.05.*
Fig. 14: Percent reduction in viability of *D. immitis* mf relative to control against time for 50µg / ml each of azithromycin, azithromycin +acaciasides and acaciasides. Data were analysed by one way ANOVA and expressed as mean ± S.E. (n=3). There was a significant difference between the control and treated groups. Asterisks denote level of significance: *P<0.05.
Fig. 15: *In vitro* cytotoxicity assay of acaciasides was performed on cultivated Vero cells (monkey kidney cells). Initial concentration of drug was 300 µg/ml and diluted three fold. Data was transferred to Excel to calculate the CC$_{50}$ values. The CC$_{50}$ value was > 300 µg/ml.
Experiment 2

Improved antifilarial efficacy of antibiotics by acaciasides on microfilaria of *D. immitis* in vivo

Introduction

Lymphatic filariasis is a major tropical disease and one of the major common causes of global disability. More than 120 million people are infected with filarial worm and about 1 billion people are at the risk of infection (Rao, 2005). According to 1995 estimates, in India 533 million people were exposed to filarial infection of which 21 million people with symptomatic filariasis like lymphangitis, hydrocoel, lymphoedema or elephantiasis and 27 million microfilaria carriers (Rao, 2005). Present day antimicrofilarial or macrofilaricidal treatment regimens do have certain well documented limitations. Diethylcarbamazine and ivermectin are effective at killing microfilariae but are associated with systemic and inflammatory adverse reactions. Albendazole increases the efficacy of DEC and ivermectin and is used in combination with either of the drugs as the basis of long-term intervention programme. Thus, the present day requirement for filarial chemotherapy is a cheap, non-toxic and novel antifilarial drug with long term
antimicrofilarial or macrofilaricidal activity. Ivermectin has been used as standard antifilarial drug for comparing the results.

One potential target is to use anti-rickettsial antibiotics to deplete *Wolbachia* endosymbionts that exist in the lateral cords of adult female and microfilaria of most filarial nematodes including *D. immitis*, *Litomosoides sigmodontis*, *O. volvulus*, *W. bancrofti* and *B. malayi* (Bandi *et al.*, 1999; Taylor *et al.*, 1999). In recent years, studies have linked tetracycline treatment of filaria infected animals with reduced worm burdens and decreased level of microfilaremia. This has been demonstrated in animal models with *L. sigmodontis*, *D. immitis* and recently confirmed in patients with *O. volvulus* (Hoerauf *et al.*, 1999; 2000b; Taylor *et al.*, 1999; 2000b). We have earlier reported that tetracycline at 35 mg/kg/day for 31 days causes a steady depletion in dirofilarial count in the blood of treated dogs (Das *et al.*, 2006).

The antifilarial activity of two triterpenoid saponins acaciaside A and acaciaside B, originally isolated from the funicles of *A. auriculiformis* were observed earlier (Mahato *et al.*, 1992). The saponins were found effective against both microfilaria and the adult worm of *S. cervi* in rats (Ghosh *et al.*, 1993). An ethanol extract obtained from the funicles of the plant proved effective against both microfilaria and the adult worm of *D. immitis* in dogs (Chakraborty *et al.*, 1995). The crude ethanol extract at the effective dose did not show any apparent toxicity in the treated dogs in terms of lethargy, food
intake and change in body temperature; however serological tests revealed some mild transient effects in liver function (Sarkar, 1997). Recently we have reported absence of Wolbachia in S. cervi collected from local abattoirs (Datta et al., 2007). Since this cattle parasite does not harbour Wolbachia, it is likely that the filaricidal activity of saponins may be mediated through a different target altogether. These two saponins are known to interact with the membrane, thus inflicting membrane damage (Sinha Babu et al., 1997). Our findings on the mechanism of action of saponins further revealed that super oxide anion is probably involved in the expression of membrane damaging effect of saponins (Nandi et al., 2004). Since tetracycline acts on the clearance of Wolbachia, it is of great interest to know whether saponin-induced membrane damage could increase the efficacy of tetracycline on Wolbachia. In the present study, the mixture of acaciaside A and acaciaside B was used for testing its effects on the antifilarial efficacy of tetracycline. We have already observed antifilarial activity of tetracycline, azithromycin and acaciasides and their combination on D. immitis in vitro in the previous experiment. These results promoted us to study the effect of tetracycline, azithromycin and acaciasides and their combination on D. immitis in vivo. Our primary aim is to assess the impact of tetracycline and azithromycin on Wolbachia loads within microfilaria and of the combination of tetracycline + acaciasides and azithromycin + acaciasides on microfilaria level over the observation period.
Materials and methods:

Acaciaside A and Acaciaside B

The mixture of acaciaside A and acaciaside B, which is water soluble, was used for testing its effects on *D. immitis* in dogs.

Drugs used for the experiment:

Tetracycline (hostacycline) used in the experiment was obtained from Aventis Pharma Limited, India. Azithromycin was obtained from Alembic Limited. The antibiotics were given orally. Ivermectin was obtained from Ochoa Laboratoy Limited and injected subcutaneously in a single shot.

Experimental animal and study design

Twenty one stray dogs (15 males and 6 females) naturally infected with *D. immitis* were used in the present experiment. Blood samples from all the dogs were collected every week for a period of ten weeks and mf density per 0.25 ml of blood was determined in each sample. Six mf dogs (males) were administered orally with tetracycline at 10 mg/kg/day for 40 days. Three male tetracycline treated (for 40 days) dogs were given saponins of *A. auriculiformis* at 10mg/kg/day orally for 7 consecutive days and the other three received the placebo.
Six mf dogs (males) were administered orally with azithromycin at 10 mg/kg/day for 40 days. Three male azithromycin treated (for 40 days) dogs were given saponins of *A. auriculiformis* at 10mg/kg/day orally for 7 consecutive days and the other three received the placebo. Among the remaining nine mf dogs, three females received no treatment, three males were treated orally with acaciasides at 10mg/kg/day for 7 days (day 41- day 47) only and three females were given ivermectin at 2 mg/ kg body weight (single subcutaneous injection).

**Collection of microfilaria from blood**

Animal trials were duly approved by the institutional animal ethics committee of the University. Blood samples were obtained separately from each dog with the help of 5ml heparinised disposable syringe. Blood was drawn on day 15, 30, 40 and 47 from the commencement of treatment. Additional samples were taken at quarterly intervals up to day 75 and last sampling was done at 120 day post-treatment. From all the experimental dogs, 5 ml of blood was taken in heparinised tubes and immediately diluted (1:1) with pre-chilled PBS (0.01 M phosphate buffer, 0.15 M sodium chloride, pH 7.4) and was filtered through a 5µm filter membrane (Millipore, USA). Microfilariae were separated by spinning at 5000 ×g for 5 min and the pellet was stored immediately in 100 µl Tris-EDTA-sodium chloride (0.1 M Tris; 0.2 M NaCl, pH 7.5, 0.005 M EDTA; pH 8.0) and kept at -20ºC.

**Extraction of DNA and PCR**
Total genomic DNA was extracted from *D. immitis* microfilariae, collected from blood drawn from naturally infected stray dogs following the method by Smith and Rajan (2000), with slight modifications. The mf pellet was resuspended in 500 µl of lysis buffer, pH 8.0 containing 20 mM Tris-HCl, 50 mM EDTA, 0.5% SDS, 100 mM NaCl, 1%(v/v) β-mercaptoethanol and proteinase-K 0.1 mg/ml. Then the mixture was incubated at 55°C for 3 h. To inactivate proteinase-K, samples were heated at 95°C for 10 min. After phenol-chloroform-isoamyl alcohol (25:24:1) extraction and ethanol precipitation, the pellet was washed with cold ethanol (70%) and then resuspended in sterile 25 µl 10 mM TE buffer (pH 8.0). Total genomic DNA was also extracted from adult worms of *S. cervi* collected from the peritoneal cavity of freshly slaughtered cows at local abattoirs (Kashipur, Birbhum), washed briefly with modified Ringers medium at 37°C and stored immediately in 1 ml of TEN buffer (100 mM Tris, 5 mM EDTA, 200 mM sodium chloride, pH 7.5) at −20°C. Finally DNA was isolated as described above.

In both treated and untreated dogs PCR was performed to check DNA integrity and to assay for the presence of *Wolbachia* in *D. immitis*. PCR was performed in 50µl of reaction mixture having 1× PCR buffer containing (NH₄)₂SO₄, 2 mM dNTP mix, 2 mM MgCl₂, 20 ng/ml each of forward and reverse primers and 1.5 units *Taq* polymerase (Fermentas). PCR products were visualized by running 5 µl of reaction mixture in 1% agarose (SRL, India) gel followed by staining with ethidium bromide (SRL, India). To confirm filarial
DNA, 28S rRNA primers (BD1A-F and BD1A-R) were used (Smith and Rajan, 2000). Presence and integrity of *Wolbachia* DNA was confirmed by using *Wolbachia* *wsp* primers (WSPintF and WSPintR) derived from conserved regions between *D. immitis* and *B. malayi* *wsp* genes (Bazzocchi et al., 2000a) and *Wolbachia* 16S rRNA primers FIL-5 and FIL-6 (Smith et al., 2000). Annealing was done at 51°C for all three sets of primers. As negative control, sterile distilled water or DNA extracted from *Setaria cervi*, which does not have *Wolbachia* endosymbionts (Datta et al., 2007) was used.

**Primer sequences:**

<table>
<thead>
<tr>
<th>Filaria 28S rRNA primers</th>
<th>BD1A-F</th>
<th>5′-ATGAAAGGCGTTGATATATAG-3′</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BD1A-R</td>
<td>5′-GCAAGCCATGCAAGCGTTGAG-3′</td>
</tr>
<tr>
<td><em>wsp</em> primers</td>
<td>WSPintF</td>
<td>5′-TAG(CT)TACTACATTCCGCTTGCA-3′</td>
</tr>
<tr>
<td></td>
<td>WSPintR</td>
<td>5′-CCAA(CT)AGTGC(CT)ATAAAGAAC-3′</td>
</tr>
<tr>
<td><em>Wolbachia</em> 16S rRNA primers</td>
<td>FIL-5</td>
<td>5′-TGAGGAAGATAATGACGG-3′</td>
</tr>
<tr>
<td></td>
<td>FIL-6</td>
<td>5′-CCTCTATCCTCTTTCAACC-3′</td>
</tr>
</tbody>
</table>

**Side effects of drugs:**

Following treatment with the test drugs the animals were kept under observation and their body weight, food intake and movement was recorded at
regular intervals. The treated animals did not show any toxic effects in terms of change in body weight, food intake and movement.

Serological tests were applied to pariah dogs naturally infected with *D. immitis* before and after tetracycline, azithromycin, acaciasides and their combination treatment. The same serological tests were performed for control dogs. Blood was analysed before and after treatment with respect to the following parameters: SGOT, SGPT and % haemoglobin.

**Results:**

PCR amplification of *S. cervi* and *D. immitis* genomic DNA yielded distinctive bands at 150 bp corresponding to filarial 28S rRNA gene product (lane 2, Fig. 16 and lane 2, Fig. 17) respectively, establishing the quality of the template DNA and authenticity of the experimental protocol. However, use of 16S rRNA primer specific for *Wolbachia* did not produce any amplified product (band) when *S. cervi* genomic DNA preparation was used as template even after 35 cycles of amplification (lane 3; Fig. 16). These results suggest that *Wolbachia* sp. are either absent from *S. cervi* or present in extremely low numbers. In a similar condition, *D. immitis* genomic DNA used as template gave a distinct 16S rRNA or *Wolbachia*-specific amplified product in agarose gel (lane 3; Fig. 17) confirming the presence and integrity of *Wolbachia* DNA prepared from *D. immitis*. 
Fig. 16: Polymerase chain reaction of *Setaria cervi* total genomic DNA to assay for the presence of *Wolbachia* endosymbiont. PCR using primers specific for filarial 28S rRNA (lane 2) yielded a product of 150 bp. No band corresponding to *Wolbachia*-specific 207 bp found through PCR amplification using *Wolbachia* 16S rRNA primer in *S. cervi* (lane 3). Migration pattern of DNA ladder was shown in lane 1.
Fig. 17: Polymerase chain reaction of microfilaria of *Dirofilaria immitis* total genomic DNA using primers specific for filarial 28S rRNA (lane 2) and *Wolbachia* 16S rRNA (lane 4). Presence of a distinct band of size approximately 207 bp confirms the presence of *Wolbachia* in *D. immitis*. Migration pattern of DNA ladder (lane 1) was shown at extreme left.
Experiment 2.1: Improved efficacy of azithromycin by acaciasides on *D. immitis*

**Results:**

**Parasitological findings:**

The mf count per 0.25ml of blood did not vary appreciably during the 10 week period of observation before the commencement of treatment. Treatment with azithromycin or azithromycin + acaciasides or acaciasides alone at the effective dose levels did not produce any apparent side effects in the treated dogs in terms of lethargy, food intake and serological tests including SGPT SGOT and % Hb (Table 2). The percent occurrence of mf/ 0.25ml of blood following treatment with azithromycin or azithromycin + acaciasides or acaciasides alone (Table 1) in comparison to placebo at various time intervals are shown in Figures 18,19 and 20 respectively.

Treatment of microfilaremic adult dogs (body weight range 8-12 kg) with azithromycin at 10 mg/kg/day for 40 days resulted in 54% (P< 0.05, one way ANOVA) and 57% (P< 0.05, one way ANOVA) reduction in mf count on day 15 and 30, respectively, and the maximum reduction in mf count (70%, P< 0.01) was achieved on 40 day treatment (Fig. 18).
However, treatment with azithromycin (10 mg/kg/day for 40 days) followed by acaciasides (10 mg/kg/day for 7 days) resulted in 90% clearance of mf at a faster rate on 45 day post-treatment (Fig. 19).

In dogs treated with acaciasides only for seven days, the mf count was reduced by more than 64% (P< 0.05) on day 7 (the last day of treatment), thereafter, the mf density increased gradually to 34% reduction level on day 120 post-treatment (Fig. 20).

In dogs treated with single dose ivermectin at 2 mg/kg body weight the mf population in blood disappeared totally as observed on day 15 post-treatment.

**PCR of microfilaria samples:**

Using filaria specific primers, PCR amplification of *D. immitis* mf DNA from both pre-treated (0 day, lane 2; Fig. 21) and azithromycin treated (sampling on 30 and 45 days) dogs yielded distinctive bands at 150 bp (lanes 3 and 4; Fig. 21). A comparison between pre-treated and treated dogs reveals that there was a trace of filarial specific amplified product in both treated dogs on 45 day post-treatment but the band intensity was higher in azithromycin treated dogs (lane 4; Fig. 21) than azithromycin + acaciasides treated dogs (lane 5; Fig. 21) as evident from the image J analysis of the gel photographs (Gel doc system, Bio-Rad). The template DNA prepared from a calculated number of
300-1200 mf was used to determine the presence of *Wolbachia* by PCR. The data obtained were normalized against filarial 28s rRNA gene. The *Wolbachia* 16S rRNA primers produced amplified product at 207 bp after 35 cycles of amplification (Fig. 22). No depletion was found in *Wolbachia* population on day 30 (lane 3, Fig. 22) and 45 day post-treatment compared to 0 day samples (lane 2; Fig. 22) from both azithromycin (lane 4; Fig. 22) and azithromycin + acaciasides (lane 5; Fig. 22) treated groups.

**Discussion:**

The principal of anti-wolbachial chemotherapy of filariasis relies on either depletion or a significant reduction of the *Wolbachia* endobacteria in the adult worms leading to sterility and eventually death of female worms (Debrah *et al.*, 2006; 2007; Hoerauf *et al.*, 2007). The ability to provide riboflavin, flavin adenine dinucleotide, heme and nucleotides is likely to be *Wolbachia*’s principal contribution to the mutualistic relationship, whereas the host nematode likely supplies amino acids required for *Wolbachia* growth (Foster *et al.*, 2005). The lack of nucleotide synthesis would particularly affect cell division during oogenesis and embryogenesis, and this is the first parasitological feature that can be observed after *Wolbachia* depletion (Hoerauf *et al.*, 2003a).

It is safe to administer azithromycin to the children indicating the rationale for including azithromycin in the anti-wolbachial chemotherapy.
Treatment of onchocerciasis patients with a 5-day course of rifampicin or azithromycin or both did not cause depletion of Wolbachia, reduction of microfilariae in the skin or degeneration of adult worms even after 9 months of treatment indicating that a short term course with these antibiotics will not clear Wolbachia (Richards et al., 2007). A 6-week regimen of azithromycin at 250 mg/day significantly reduced the worm’s burden in onchocerciasis patients but there was no change in the Wolbachia population in the treated worms (Hoerauf et al., 2008). In the present study a 40-day treatment of azithromycin at 10 mg/kg body weight/day resulted in a maximum 70% reduction in mf count compared to untreated control on 40 day post-treatment. However, the Wolbachia population was not reduced significantly as evident from the PCR using Wolbachia 16S rRNA primers. But a 40-day regimen of azithromycin at 10 mg/kg body weight/day followed by a 7-day regimen of acaciasides at the same dose reduced the mf count (90% clearance) at a faster rate on 45 day post-treatment with no effect on Wolbachia population. This implies that azithromycin has effect on a minority of worms and the reduction of Wolbachia is not enough to play any role on the parasites.
Table 1: Mean mf count / 0.25 ml blood in control and treated groups

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*: Azithromycin was given for 40 days at 10 mg/kg/day followed by either 7-day placebo (azithromycin group) or acaciasides (azithromycin+acaciasides group) treatment at 10 mg/kg/day.

**: Acaciasides was given for 7 days (from 41-47 days) at 10 mg/kg/day.

Data were analysed by one way ANOVA. Significant difference was found between control and treated groups (P<0.05).
Table 2: Side effects of drugs

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*: Tetracycline was given for 40 days at 10 mg/kg/day followed by either 7-day placebo (tetracycline group) or acaciasides (tetracycline +acaciasides group) treatment at 10 mg/kg/day.

**: Azithromycin was given for 40 days at 10 mg/kg/day followed by either 7-day placebo (azithromycin group) or acaciasides (azithromycin+acaciasides group) treatment at 10 mg/kg/day.

***: Acaciasides was given for 7 days (from 41-47 days) at 10 mg/kg/day.

Data were analysed by one way ANOVA. No significant difference was found between control and treated groups (P<0.05).
Fig. 18: Percentage of microfilaria (*D. immitis*) per 0.25 ml of blood in control and treated dogs. Three dogs were kept as control and three were treated orally with azithromycin (40 days, at 10 mg/kg/day) followed by 7-day placebo treatment. Each bar represents the mean±S.D. Data were analysed by one way ANOVA. There was a significant difference between control and treated groups and among the treated groups. Asterisks denote level of significance: *P<0.05.
Fig. 19: Percentage of microfilaria (D. immitis) per 0.25 ml of blood in control and treated dogs. Three dogs were kept as control and three were treated orally with azithromycin (40 days, at 10 mg/kg/day) followed by 7-day acaciasides (10 mg/kg/day) treatment. Each bar represents the mean±S.D. Data were analysed by one way ANOVA. There was a significant difference between control and treated groups and among the treated groups. Asterisks denote level of significance: *P<0.05, **P<0.01.
Fig. 20: Percentage of microfilaria (*D. immitis*) per 0.25 ml of blood in control and treated dogs. Three dogs were kept as control and three were treated orally with acaciasides (7 days, at 10 mg/kg/day). Each bar represents the mean±S.D. Data were analysed by one way ANOVA. There was a significant difference between control and treated groups. Asterisks denote level of significance: *P<0.05.
Fig. 21: PCR of mf of *D. immitis* total genomic DNA using primers (BD1A-F and BD1A-R) specific for filarial 28S rRNA before (lane 2) and after azithromycin treatment (sampling as on 30 day treatment and 45 day post-treatment; lanes 3, 4 respectively). Lane 5 is 45 day post-treatment with the azithromycin+acaciasides and yielded products of 150 bp. Electrophoretic migration pattern of DNA ladder (lane 1) is shown.
Densitometric analysis of band intensity (through Image J software)  
(PT= post-treatment, Az=azithromycin, A=acaciasides)

Fig. 22: PCR of mf of *D. immitis* total genomic DNA using primers (FIL-5 and FIL-6) specific for *Wolbachia* 16S rRNA before (lane 2) and after azithromycin treatment (sampling as on 30 day treatment and 45 day post-treatment; lanes 3, 4 respectively). Lane 5 is 45 day post-treatment with the azithromycin+acaciasides. Presence of a distinct band of approximately 207 bp size confirms the presence of *Wolbachia* in all the lanes. Migration pattern of DNA ladder (lane 1) is shown at extreme left.
Experiment 2.2: Improved efficacy of tetracycline by acaciasides on *D. immitis*

**Results:**

**Parasitological findings:**

The mf count per 0.25ml of blood did not vary appreciably during the 10 week period of observation before the commencement of treatment. Treatment with tetracycline or tetracycline + acaciasides or acaciasides alone at the effective dose levels did not produce any apparent side effects in the treated dogs in terms of lethargy, food intake and serological tests including SGPT SGOT and % Hb (Table 2). The percent occurrence of mf/ 0.25 ml of blood following treatment with tetracycline or tetracycline + acaciasides or acaciasides alone (Table 3) in comparison to placebo at various time intervals are shown in Figure 23, 24 and 20 respectively.
Tetracycline treatment resulted in 72% (P< 0.01, one way ANOVA) and 83% (P< 0.01, one way ANOVA) reduction relative to control in mf count on day 15 and 30, respectively, however, the maximum reduction (91%, P< 0.01) in mf count was observed on 75 day post-treatment. Thereafter, the mf density started to increase and on day 120 post-treatment 40% reduction was observed compared to day1 (Fig. 23). Tetracycline treatment for 40 days followed by treatment with acaciasides for 7 consecutive days resulted in gradual decline in mf count and finally a total clearance of mf was recorded on day 75 post-treatment. This condition was maintained even 120 days after the last dose. Although a reappearance of mf was recorded but a maximum 99% (P< 0.01) reduction relative to control was obtained on day 120 post-treatment (Fig. 24).

In dogs treated with acaciasides only for seven days, the mf count was reduced by more than 64% (P< 0.01) on day 47 (the last day of treatment), thereafter, the mf density increased gradually to 34% reduction level on day 120 post-treatment (Fig. 20).

In dogs treated with single dose ivermectin at 2 mg/ kg body weight the mf population in blood disappeared totally as observed on day 15 post-treatment.

**PCR of microfilaria samples:**
Using filaria specific primers, PCR amplification of *D. immitis* mf DNA from both pre-treated (0 day, lane 2; Fig. 25) and tetracycline treated (sampling on 30 and 45 day) dogs yielded distinctive bands at 150 bp (lanes 3, 4; Fig. 25). A comparison between pre-treated and treated dogs reveals that there was a trace of filarial specific amplified product (lane 4; Fig. 25) in tetracycline treated group but no such band was observed in tetracycline + acaciasides treated dogs (lane 5; Fig. 25) on 45 day post-treatment. The template DNA prepared from a calculated number of 300-1200 mf was used to determine the presence of *Wolbachia* by PCR. The data obtained were normalized against filarial 28s rRNA gene. The *Wolbachia* *wsp* primers produced amplified product at 590 bp after 35 cycles of amplification (Fig. 26). A clear depletion in *Wolbachia* population due to tetracycline treatment on day 30 compared to 0 day samples was evident (lanes 2 and 3; Fig. 26). Using *Wolbachia* primers we could not detect any amplified product from blood samples collected on 45 day post-treatment from both tetracycline (lane 4; Fig. 26) and tetracycline + acaciasides (lanes 5; Fig. 26) treated groups.

**Discussion:**

The marked reduction of mf count following treatment for 47 days may be attributed to the antifilarial activity of both tetracycline and saponins from *A. auriculiformis*. Treatment with ethanol extract from *A. auriculiformis* alone, at
15 mg/kg/day for 45 days resulted in a 98% fall in mf count on day 75 after treatment, the mf count started rising thereafter, and on day 120 post-treatment, a 59% reduction was observed (Chakraborty et al., 1995). We have further shown that tetracycline at 35 mg/kg/day for 31 days caused a dramatic reduction in dirofilarial count in the blood of treated dogs (Das et al., 2006). Here we report that the drugs in combination (i.e. tetracycline + acaciasides) provided the best antifilarial efficacy by totally eliminating the circulating mf from day 75 onwards with 99.8% suppression even on day 120 post-treatment. Thus, the combination is more effective than the individual drug. Our results have established that a 40-day course of tetracycline in combination with saponins from A. auriculiformis is sufficient to ensure long-term reduction in mf level.

Further it has been shown by many workers that 3-5 weeks of tetracycline/doxycycline will cause filarial worms to become sterile after their Wolbachia are depleted (Bandi et al., 1999; Debrah et al., 2007; Turner et al., 2006). In human trials this sterility is apparently permanent. However, in several animal trials the sterility has been transient. There could be a hypothesis that the sterility becomes transient due to incomplete tetracycline therapy, which leaves enough Wolbachia to repopulate the worms.

We have selected the dose at 10 mg/kg because it is not sufficient to induce a permanent block in embryogenesis in D. immitis, rendering the parasite susceptible to saponin mediated killing effect. Our results also support this view as tetracycline alone could not eliminate mf from blood during treatment and
post-treatment period. In our previous investigations it was suggested that the conjugated unsaturated system of the saponins is involved in producing the damaging effect of saponins, probably by resulting free radicals that induce membrane damage through peroxidation (Sinha Babu et al., 1997). Our findings on the mechanism of action of saponins further revealed that the superoxide anion is probably involved in the expression of membrane-damaging effect of the saponins (Nandi et al., 2004). The present data indicate that prior tetracycline treatment enhances microfilaricidal activity of saponins. This effect may be additive or synergistic as the worms are weakened by Wolbachia depletion and then these weakened microfilariae are possibly killed by the saponins. Although we observed long-term suppression of microfilaremia after administration of tetracycline in combination with saponin treatment, there was no evidence to support a macrofilaricidal effect. More than 40 days of treatment to rodents and 9 months treatment in cattle is recommended for achieving macrofilaricidal activity of tetracycline (Taylor et al., 2000). A recent study with regard to human filariasis has reported that a treatment of doxycycline for 3 weeks is more effective in mediating a long-term microfilaremia but is insufficient of curing the disease (Turner et al., 2006). Inflammatory reactions to antifilarial treatment are associated with levels of mf and Wolbachia released in plasma. Thus antibiotic treatment, irrespective of direct effect on the nematodes, may prove beneficial in alleviating the sign and symptom of lymphatic filariasis. Antifilarial therapy preceded by antibiotic treatment may
also prevent adverse reactions (Jayakody et al., 1993). The present findings support further the saponins as a new class of microfilaricide for antifilarial therapy.

Table 3: Mean mf count / 0.25 ml blood in control and treated groups

* Tetracycline was given for 40 days at 10 mg/kg/day followed by either 7-day placebo (tetracycline group) or acaciasides (tetracycline+acaciasides group) treatment at 10 mg/kg/day.

** Acaciasides was given for 7 days (from 41-47 days) at 10 mg/kg/day.

Data were analysed by one way ANOVA. Significant difference was found between control and treated groups (P<0.05).
Fig. 23: Percentage of microfilaria (*D. immitis*) per 0.25 ml of blood in control and treated dogs. Three dogs were kept as control and three were treated orally with tetracycline (40 days, at 10 mg/kg/day) followed by 7-day placebo treatment. Each bar represents the mean±S.D. Data were analysed by one way ANOVA. There was a significance difference between control and treated groups. Asterisks denote level of significance: **P<0.01, ***P<0.001.
Fig. 24: Percentage of microfilaria (D. immitis) per 0.25 ml of blood in control and treated dogs. Three dogs were kept as control and three were treated orally with tetracycline (40 days, at 10 mg/kg/day) followed by 7-day acaciasides (10 mg/kg/day) treatment. Each bar represents the mean±S.D. Data were analysed by one way ANOVA. There was a significance difference between control and treated groups. Asterisks denote level of significance: **P<0.01, ***P<0.001.
Fig. 25: Polymerase chain reaction using total genomic DNA of *D. immitis* mf as template. PCR amplification using filarial 28S rRNA specific primers before (lane 2) and after tetracycline treatment (sampling as on 30 and 45 days; lanes 3, 4, respectively) followed by agarose gel electrophoresis yielded products at 150 bp. No visible band was found in tetracycline+acaciasides-treated dogs on day 45 post-treatment (lane 5). Lane 1 shows electrophoretic migration pattern of 100 bp DNA ladder.
Densitometric analysis of band intensity (through Image J software)  
(PT= post-treatment, T=tetracycline, A=acalasisdes)

Fig. 26: Polymerase chain reaction of total genomic DNA of *D. immitis* mf using *Wolbachia* wsp specific primers (WSPintF and WSPintR) before (lane 2) and after 30-day tetracycline treatment (lane 3) followed by agarose gel electrophoresis yielded a product of approximately 590 bp size and confirms the presence of *Wolbachia* in microfilariae of *D. immitis*. No detectable bands were found from both tetracycline (lane
4) and tetracycline+acaciasides (lane 5) treated groups (sampling as on 45-day post-treatment). Lane 1 shows migration pattern of 100 bp DNA ladder.

<table>
<thead>
<tr>
<th>arbitrary densitometric unit</th>
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<tbody>
<tr>
<td>0 DAY</td>
</tr>
<tr>
<td>30 DAY</td>
</tr>
<tr>
<td>45 DAY PT (T)</td>
</tr>
<tr>
<td>45 DAY PT (T+A)</td>
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</table>

Densitometric analysis of band intensity (through Image J software) (PT= post-treatment, T=tetracycline, A=acaisides)

**Conclusion:**

In the present study a 40-day treatment of azithromycin at 10 mg/kg /day resulted in a maximum 70% reduction in mf count compared to untreated control on 40 day post-treatment (Fig. 27). However, the *Wolbachia* population was not reduced significantly as evident from the PCR using *Wolbachia* 16S rRNA primers. But a 40-day regimen of azithromycin at 10 mg/kg body weight/day followed by a 7-day regimen of acaciasides at the same dose reduced the mf count (90% clearance) at a faster rate on 45 day post-treatment with no effect on *Wolbachia* population (Fig. 27). This implies that azithromycin has effect on a minority of worms and the reduction of *Wolbachia* is not enough to play any role on the parasites.

Here we report that the drugs in combination (i.e. tetracycline + acaciasides) provided the best antifilarial efficacy by totally eliminating the
circulating mf from day 75 onwards with 99.8 % suppression even on day 120 post-treatment. Thus, the combination is more effective than the individual drug. Our results have established that a 40- day course of tetracycline in combination with saponins from *A. auriculiformis* is sufficient to ensure long-term reduction in mf level.

The present findings support further the saponins as a new class of microfilaricide for antifilarial therapy.
Fig. 27: Percent occurrence of mf per 0.25 ml blood in control and treated groups during treatment and post-treatment. Each data represents the mean±S.D. Data were analysed by one way ANOVA. There was significant difference between control and treated groups (P<0.05).

Experiment 3

Mechanism of action of acaciasides on microsomes of Setaria cervi

Introduction:

Cell membranes are made up of unsaturated lipids which are susceptible to oxidative damage. Oxidative damage can lead to a breakdown or even hardening (peroxidation) of lipids. Lipid peroxide formation can lead to membrane damage as has been shown for erythrocytes (Tsen and Collier, 1960) and lysosomes (Wills and Wilkinson, 1966). Formation of lipid peroxides in vivo would therefore cause severe cellular damage. Under certain conditions lipid peroxides may be formed in vivo but its measurement in vivo has been
difficult and results are controversial (Zalkin and Tappel, 1960; Philpot, 1963). It is firmly established that homogenates of most animal tissues form thiobarbituric acid reactants (peroxides) when incubated in vitro in aerobic conditions (Zalkin and Tappel, 1960). These substances are formed by the oxidation of unsaturated fatty acids of lipid constituents of the tissue probably via hydroperoxides.

Saponins are known to interact with membrane inflicting membrane damage. Acaciasides A and B are unique molecules because they contain a conjugated unsaturated system which is highly susceptible to peroxidation (Mahato et al., 1992). Acaciasides A and B, two acylated triterpenoid bisglycosides isolated from the funicles of A. auriculoformis, are known to have antihelmintic properties (Mahato et al., 1992). The saponins were found effective against both microfilariae and adult worms of S. cervi in rats (Ghosh et al., 1993). An ethanol extract of the funicles, when administered orally to dogs naturally infected with D. immitis found effective against both microfilaria and adult worm (Chakraborty et al., 1995). The cestocidal activity of the saponins has also been reported (Ghosh et al., 1996).

In the present study it was observed that incorporation of acaciasides along with antibiotics gave better results on mf of D. immitis both in vitro and in vivo. In the present chapter we have selected the bovine filarial worm S. cervi which does not harbour the Wolbachia endosymbiont and observed the effects of acaciasides on membrane damage in vitro.
The acaciasides either cause damage to the membrane of the filarial worms or may facilitate the entry of the antibiotics that may prove to be an effective mass chemotherapeutic regimen in future.

This prompted us to investigate the interaction of saponins and membrane, with *S. cervi* microsomes as our model to understand the mode of action of the saponins.

**Materials and methods:**

**Preparation of microsomes:**

Adult *S. cervi* were collected from the peritoneal cavity of freshly slaughtered cows at local abattoirs. The worms were then repeatedly washed with PBS (0.01 M phosphate buffer, 0.15 M sodium chloride, pH 7.4) to remove blood and remnants of any host tissue. The weighed worms were homogenized in 250 mM sucrose containing 10 mM Tris – HCl, pH 7.4 with a glass homogenizer to prepare a 20% homogenate at 4°C. The homogenate was filtered through a filter paper to eliminate cell debris. The filtrate was centrifuged at 12,000 X g for 10 min at 4°C. Solid CaCl$_2$ (8 mM final concentration) was added to the resultant post-mitochondrial supernatant (PMS) and thoroughly mixed until homogeneity for complete aggregation of
microsomes. The microsomes were pellet down by centrifugation of the CaCl$_2$-PMS mixture at 25,000 X g for 15 min. The pellet was then resuspended in 0.5 ml wash buffer (10mM Tris-HCl, pH 7.4 containing 150 mM KCl) and resedimented at 25,000 X g for 15 min. Final pellet was reconstituted in 0.5 ml wash buffer.

**Preparation of Acaciasides A and B:**

Discussed earlier.

**Treatments:**

To 50µl microsome preparation (1mg/ml protein), acaciasides at 1mg/ml and Kreb’s Ringer bicarbonate solution were added to a final volume of 1.5 ml. The mixture was incubated for 2h at 30°C. The mixture was then centrifuged at 25,000 X g for 15 min and the pellet was redissolved in 1.5 ml wash buffer from which 0.5 ml was taken for MDA assay and 1ml for CD assay (Sato and Nagai, 1986).

To study the effects of SOD (500 U/ml), catalase (2000 U/ml) and thiourea (1 mM), these agents were added to the membranes prior to the addition of saponins. Three dilutions of SOD (200, 400 and 500 U/ml) were
used to study whether it could block the effect of saponin-induced enhanced lipid peroxidation in a concentration dependent manner. These particular doses were selected from our previous work (Nandi et al., 2004; Sinhababu et al., 1997). Cadmium chloride was used as a standard toxicant because it is known to increase peroxidation in isolated hepatocytes (Jamall and Smith, 1985; Sato and Nagai, 1986).

Assay of malondialdehyde (MDA):

The thiobarbituric acid (TBA) test is performed to measure the amount of thiobarbituric acid reactive substances (TBARSs) or malondialdehyde (MDA) present in the sample. MDA is generated as a degradation product from peroxidised lipids (Janero., 1990) and as a side product of enzymatic metabolism of thromboxanes and prostaglandins (McMillan et al., 1978; Shimizu et al., 1981). The basis of the TBA method is the reaction of MDA with TBA at low pH and high temperature to form a coloured complex, the MDA-TBA complex, with an absorption maximum at 532–535 nm that can be measured by visible absorption spectrophotometry (Sinnhuber et al., 1957; Ottolenghi., 1959). The test works well in defined membrane systems (e.g. microsomes and liposomes), but its application to body fluids has produced a host of problems.
One ml of microsome fraction (1mg of membrane protein) was combined with 2 ml of trichloroacetic acid (TCA)-thiobarbituric acid (TBA)-HCl and mixed well. The solution was heated for 15 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000 X g for 10 min. Supernatant was collected and optical density was measured at 535 nm against a blank that contains all the reagents minus lipid. The MDA concentration of the sample was calculated using an extinction coefficient of 1.56 X 10^5 M^{-1} cm^{-1} (Buege and Aust, 1978) and expressed in terms of µM MDA/ mg membrane protein/ min.

**Assay of conjugated diene (CD):**

Conjugated diene (CD) structures with a double-single-double bond (-C=C-C=C-) arrangement absorb UV light in the wavelength range 230–235 nm and can thus be detected by UV absorption spectrophotometry (Gutteridge and Halliwell., 1990; Halliwell and Chirico., 1993; Corongiu *et al*., 1994). Conjugated diene measurement has successfully been used to study peroxidation in isolated lipoprotein fractions.

To one ml of microsome fraction (1mg of membrane protein), 5 ml chloroform- methanol (2:1) mixture was mixed to extract the membrane lipids. The mixture was then centrifuged at 1000 X g for 5 min to separate the phases. Most of the upper layer was removed by suction, and 3 ml of the lower chloroform layer was taken in a test tube and evaporated to dryness in a 45^0C
water bath. The lipid residue was dissolved in 1.5 ml cyclohexane, and absorbance was measured at 233 nm against a cyclohexane blank. The CD concentration of the sample was calculated using an extinction coefficient of $2.52 \times 10^4 \text{M}^{-1} \text{cm}^{-1}$ (Buege and Aust, 1978) and expressed in terms of $\mu$M CD/mg membrane protein/min.

**Results:**

Incubation of microsomal membrane with saponins at 20, 50 and 100 $\mu$g/ml increased MDA formation by 12, 25 and 38%, respectively (Fig. 28) compared to control indicating that saponins enhanced lipid peroxidation (paired t-test, $P < 0.05$). Cadmium chloride a known toxicant that causes membrane damage by peroxidation increased MDA formation by 43% (paired t-test, $P < 0.05$, Fig. 28).

Incubation of microsomal membrane with saponins at 20, 50 and 100 $\mu$g/ml increased CD formation by 31, 51 and 52%, respectively (Fig. 30) compared to control indicating that lipid peroxidation is enhanced by saponins (paired t-test, $P < 0.05$). Cadmium chloride increased CD formation by 58% (paired t-test, $P < 0.05$, Fig. 30).
SOD significantly blocked the effects of saponins-induced membrane damage in a concentration dependent manner. Catalase had a minor effect (6%) on saponins-induced membrane damage and thiourea had no effect (paired t-test, P < 0.05, Fig. 29 and 31).

**Discussion:**

Acaciasides A and B have a conjugated diene system which is susceptible to peroxidation. The double bonds present can form allylic radicals at two centres that are resonance stabilized. These allylic radical reacts with the molecular oxygen present in microsomal membrane forming peroxide i.e., hydroperoxide. Due to the formation of hydroperoxide, peroxidation is enhanced. The conjugated unsaturated system is involved in the the damaging effects of saponins probably by resulting free radicals that labilize parasite membrane through peroxidation. The results clearly reveal that saponins have significantly enhanced membrane peroxidation in terms of both MDA and CD formation. The increased rate of peroxidation may lead to the formation of peroxyl radicals that may react with the lipid, probably by hydrogen abstraction. To further investigate the mechanism of saponins-induced membrane damage, SOD, thiourea and catalase were added to membranes prior to the addition of saponins. Since SOD completely blocked the saponin-induced membrane damage, it may be suggested that superoxide anions are probably involved in
the expression of membrane damaging effect of saponins. The experimental results suggest that such an interaction may also occur between the parasite membrane and saponins in vivo. Superoxide dismutase converts superoxide radicals to $\text{H}_2\text{O}_2$, which in turn is broken down to water and oxygen by catalase. SOD and catalase constitute the first coordinated unit of defense against reactive oxygen species (Chang et al., 1999). Hydrogen peroxide is not probably involved in these reactions since thiourea had no effect on saponin-induced damage.
Fig. 28: Effects of acaciasides on malondialdehyde (MDA) formation in *S. cervi* microsomal membrane preparation. Acaciasides were given at 20, 50 and 100 µg/ml (A20, A50 and A100 respectively) of microsomal membrane preparation. Cadmium chloride was added at a final concentration of 10 µM as positive control. Each bar represents the mean±S.E. (n=3). Data were analysed by paired t-test between the control and treated sets and among the different treated sets. Mean values with significant differences (P<0.05) were shown with different superscripts.
Fig. 29: Effects of acaciasides, superoxide dismutase (SOD), catalase and thiourea on malondialdehyde (MDA) formation in S. cervi microsomal membrane preparation. Acaciasides were given at 100 µg/ml (A100) of microsomal membrane preparation. Each bar represents the mean±S.E. (n=3). Data were analysed by paired t-test between the control and treated sets and among the different treated sets. Mean values with significant differences (P<0.05) were shown with different superscripts.
Fig. 30: Effects of acaciasides on conjugated diene (CD) formation in *S. cervi* microsomal membrane preparation. Acaciasides were given at 20, 50 and 100 µg/ml (A20, A50 and A100 respectively) of microsomal membrane preparation. Cadmium chloride was added at a final concentration of 10 µM as positive control. Each bar represents the mean±S.E. (n=3). Data were analysed by paired t-test between the control and treated sets and among the different treated sets. Mean values with significant differences (P<0.05) were shown with different superscripts.
Fig. 31: Effects of acaciasides, superoxide dismutase (SOD), catalase and thiourea on conjugated diene (CD) formation in *S. cervi* microsomal membrane preparation. Acaciasides were given at 100 µg/ml (A100) of microsomal membrane preparation. Each bar represents the mean±S.E. (n=3). Data were analysed by paired t-test between the control and treated sets and among the different treated sets. Mean values with significant differences (P<0.05) were shown with different superscripts.