8. *IN VITRO* STUDIES ON HYDATID CYST AND *IN VIVO* ANTICESTODAL EFFICACY

8.1. Objective

The objective of the present chapter was to assess the efficacy of formulated SLN and BSLN on hydatid cyst. The ex-vivo permeation across hydatid cyst membrane and permeation through whole cyst membrane was studied. The *in vitro* protoscolicidal activity was also assessed. Moreover, *in vivo* anticestodal activity was assessed in *H. diminuta* rat model.

8.2. Studies on hydatid cyst and protoscoleces

8.2.1. Collection of cysts

Hydatid cysts of *E. granulosus* were collected aseptically from infected liver and lungs of cattles slaughtered in an abattoir located in Varanasi, India. The intact cysts were immediately placed in an ice-box. Cysts were washed several times in sterile phosphate buffered saline (0.9% PBS, pH 7.2) to yield only viable protoscoleces (Cosado *et al*, 1986). Cyst surfaces were sterilized by 70% ethanol and were cut open and vesicle fluid containing protoscoleces was separated from the metacestode tissue and host adventitia. The fertility of cysts was determined by the presence of free protoscoleces in cystic fluid by microscopic examination of a wet mount drop and characteristic muscular movements. Hydatid fluid along with protoscoleces was collected as described previously (Smyth and Barrett, 1980).

8.2.2. Ex vivo permeation studies through hydatid cyst membranes

Before use, the excised hydatid cysts were incubated three times at room temperature in saline solution (0.9% PBS) to remove impurities. The membranes of fertile cysts were taken off and kept at -40°C in a 10% DMSO (cryoprotectant) aqueous solution until use (Truong *et al*., 2008). Hydatid cyst membrane samples (around $2cm^2$) were cut carefully with a scalpel and mounted on to static Franz diffusion cells of glass containing 9 ml of saline solution (inner face of the cyst membrane in contact with the receptor compartment). The receptor compartment was stirred with a magantic stirrer at 37±0.2°C throughout the study. Mounted membranes were kept on to diffusion cells for 1h before treatment. A sample of PZQ aqueous suspension or PZQ-SLN was then deposited on to haydatid cyst membrane. After 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6 and 24 h, sample (0.5 ml) of receptor fluids were removed and replaced by the same volume of fresh solution. The concentration of PZQ in the receptor fluid was assayed by HPLC.

8.2.3. Permeation studies across whole cyst membrane

The entire hydatid cysts were incubated in either PZQ aqueous suspension or PZQ-SLN. After 3h, the cysts were taken out and punctured by a needle to aspirate the intra-cystic media. These media were the subjected for PZQ assay. The differences between the test and control analyzed with one- way Analysis of variance or Student's t-test. The statistical analysis was performed with Graph Pad InStat software. $P<0.05$ were considered to be significant. As the groups were small (n=3), normal distribution was assumed and not further tested.

8.2.4. Preparation and culture of protoscoleces.

Hydatid fluid containing protoscoleces were allowed to settle completely in to 15 ml Falcon tubes without centrifugation for an hour to obtain hydatid sand at room temperature. The protoscoleces thus obtained were washed in Hanks balanced salt solution (HBSS) and were maintained in a sterile preservative solution RPMI-1640. This preservative medium not amended by any antibiotic or antifungal agents. Viability of the protoscoleces was assessed using the trypan blue exclusion technique, prior to any experiments. A 0.01 ml solution of pooled protoscoleces was transferred over a cavity slide and mixed with 0.01 ml of 0.1% aqueous trypan blue stain and was evaluated by light microscopy after 5 min incubation at 37 °C. Unstained protoscoleces were considered as viable while stained protoscoleces were considered as non-viable (Smyth *et al*.,1980). When 95% or more viable protoscoleces are present in the sediments, it considered to be appropriate for further experiments.

8.2.5. *In vitro* protoscolocidal activity

In this study, PZQ–SLN and PZQ-BSLN at two doses (0.25 and 0.5µg/ml of PZQ) were evaluated for scolocidal activity for 10, 20, 30 and 60 min. Two milliliter of each dose was placed in test tubes, and a drop of protoscoleces-rich sediment was added to the each tube and mixed gently. The tube was then left at room temperature for 10, 20, 30 and 60 min. The supernatant of the solution was then removed with a pipette avoiding settled protoscoleces. Then 2 ml of 0.1% trypan blue stain was added to the remaining settled protoscoleces, mixed gently and incubated at 37ºC for 10 min. After incubation the supernatant was discarded, and

washed with 0.9% PBS to remove the excess stain. The remaining settled protoscoleces were then smeared on a glass cavity slide, covered with a cover glass and examined microscopically (Nikon Eclipse E100 research microscope) for viability. The percentages of dead protoscoleces were determined by counting nonviable protoscoleces. Protoscoleces suspended in 0.9% PBS with no exposure to PZQ were considered as control group, and treated with pure praziquantel suspension (1µg/ml) was considered positive control in each experiment. The experiments were performed in triplicate. For scanning electron microscopy (SEM) studies, a drop containing 100μL of treated protoscoleces was fixed in 2.5% glutaraldehyde for overnight at room temperature. The fixed protoscoleces were then washed again by 0.1M sodium cacodylate buffer pH 7.4 at 4 °C and left for 3h. The settled protoscoleces were then dehydrated by sequential dipping into alcohol from 30 to 90% followed by a dip into absolute alcohol. The dehydrated protoscoleces were sputter-coated with gold under sputtering device and observed by scanning electron microscopy (Quanta 200 MK2, FEI, The Netherlands). The images obtained were processed using imaging software SmartSEM VS10.

8.3. In-vivo anticestodal study

8.3.1. Maintenance of *H. diminuta* infection

The life cycle of *H. diminuta* was maintained in the laboratory in Wistar rats, using flour beetle *Tribolium confusum* as the intermediate host (Dixon and Arai, 1991). In brief, the gravid segments of tapeworm were scratched smoothly on to the filter papers inside the petri dishes, and the

beetles were allowed to feed on flour for 72 h. These beetles were then maintained at room temperature for at least 12–14 days for the cysticercoid larva to develop. Cysticercoids were collected by dissecting the beetles and inoculated to uninfected rats for initiation of infection. After 18–20 days, eggs of *H. diminuta* were detected in the faeces of rats, which were mixed with flour powder and fed to the beetles to continue the life cycle in the laboratory.

8.3.2. Effect of SLN on hymenolepis dimunata

The efficacy of solid lipid nanoparticles were evaluated against the larval, immature and mature *H. diminuta* infections in rats (Yadav *et al*., 2011). For each experiment, animals were divided randomly into three groups comprising of six animals each (n=6). Each animal was infected by oral inoculation with four cysticercoids and maintained in a separate cage harbouring *H.dimunata* infection proved by passage of the eggs in their faeces.

Animals of Group 1 served as untreated control and received only the blank SLN (Control group). While, animals of group 2 (PZQ-control group) were treated with PZQ suspended in 0.5 % methyl cellulose. Animals of group 3 and group 4 were treated with the PZQ -SLN and PZQ –BSLN, respectively.

In order to determine the efficacy against the larval stage of *H.diminuta*, animals were administered with PZQ suspension, PZQ-SLN and PZQ BSLN on day 5 post inoculation (p.i.) of cysticercoids. From day 18 post inoculation, fresh fecal samples of rats were collected from cages and eggs

per gram (EPG) counts were determined for each animal for 3 days (days 18–20).

In case of efficacy against immature stages, animals were treated from day 10 p.i. of cysticercoids, and their EPG counts were determined on days 18–20. Similarly, to evaluate the efficacy against mature stages, animals were treated from day 22 p.i. of cysticercoids. The EPG counts were performed on days 18–20 (pre-treatment period) and days 26–28 (post-treatment period) and faecal egg count reduction (FECR) was calculated as described by Iqbal *et al*., 2004. All animals were sacrificed at day 40 p.i. and reduction in their worm load was calculated as described previously.

$$\text{Faecal egg count reduction (\%FECR)} = \frac{\text{(Pre-treatment EPG - Post-treatment EPG)}}{\text{Pre-treatment EPG}} \times 100$$

8.4. Results and discussion

8.4.1. Permeation studies across cyst membrane

The Figure 8.1 describes the PZQ permeation through hydatid cyst membrane from PZQ-BSLN and free PZQ suspension by using Franz diffusion cell. The drug concentration permeated through the membrane was quite higher in case of PZQ-BSLN than PZQ suspension. However, there are factors like membrane thickness difference plays a vital role in drug permeation. The difference in amount of drug permeated through membrane could be probably explained by the better dispersion of the drug obtained with lipid nanoparticles in comparison with aqueous

suspensions (Truong *et al.*, 2008). The lipid/oil containing compound such as emulsion can penetrate into hydatid cyst wall and produce higher drug concentration in the cyst because of the presence of many lipoidal inclusions in the inner region of the germinal membrane (Xiao *et al.*,2002; Pérez-Serrano *et al.*, 1997; Stettler *et al.*, 2004). The other reason for better permeation across cell membrane might be the lipidic nature of the nanoparticles which in turn reflects upon the greater concentration of drug in the receptor compartment.

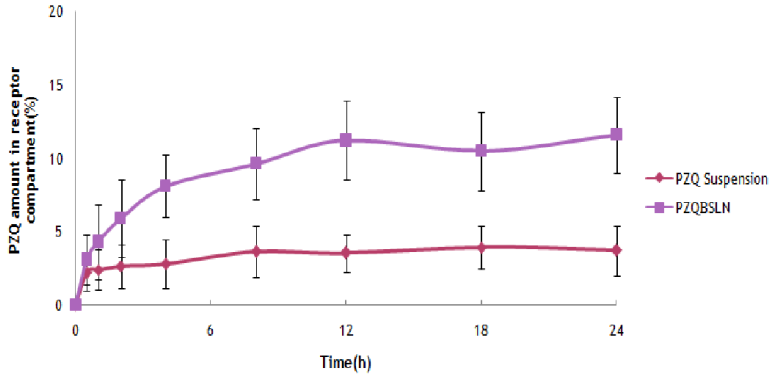

Figure 8.1: *In vitro* delivery of PZQ from PZQ BSLN and PZQ suspension (0.5% w/v methyl cellulose suspension of pure PZQ) through hydatid cyst membrane.

8.4.2. Permeation studies through whole cyst membrane

The success of the chemotherapeutic treatment of hydatid disease requires the ability of the drug to operate on the germinal layer and on the protoscoleces of the hydatid cyst interior at adequate concentrations (Morris *et al.*, 1987). However, PZQ does not penetrate well into the

mature hydatid cyst and therefore does not inhibit cyst growth, but is a highly effective protoscolecidal agent both *in vitro* and *in vivo* and shows activity against early cysts. PZQ is found more effective in combination with albendazole (ABZ). ABZ is rapidly converted to an active metabolite, ABZ sulphoxide, which achieves high concentrations in the cyst and is active against both protoscoleces and the germinal membrane. The action of ABZ is the inhibition of microtubule assembly, with detected alterations in cyst tissue also occurring in the germinal layer (Casado *et al*., 1996). It may be feasible that prior cyst wall damage by ABZ facilitates the penetration and action of PZQ (Bygott and Chiodini, 2009). To study the effect of SLN on hydatid cyst, the whole cysts were incubated in PZQ-SLN and PZQ-BSLN for 3 hours to study the drug amount reaching in the cyst to exert its protoscolocidal action. After 3 hours, the cysts were washed and punctured by a needle to quantify the intra-cystic concentration of PZQ. After three hours of incubation, the intra-cystic PZQ concentration for PZQ SLN was 1.1±0.5 µg/ml while for PZQ suspension was 0.6 ± 0.5 µg/ml.In case of incubation with PZQ-BSLN, , the intra-cystic PZQ concentration for PZQ SLN was 1.24 ±0.5 µg/ml *In vitro*, effective concentration of intra-cystic PZQ is 0.5 µg/ml (Thompson *et al*., 1986). The passive diffusion through the hydatid cyst membrane might be favoured by the partition coefficient and the increased apparent solubility of PZQ in SLN form. The SLN could also be internalized into the syncytial surface cytoplasm either by receptor-mediated endocytosis or by fusion, the mechanisms used by nonphagocytosing cells (Margolis *et al*. 1982, 1984). Since the lipids are preferentially incorporated into metabolically

stable compounds (Meyer *et al*. 1970), their composition in SLN/BSLN as used in the present study could also modify the lipid composition of the tegument, in other words, its fluidity thereby facilitating the incorporation of PZQ (Rumjanek and McLaren 1981; Lima *et al*. 1994).Similar studies with liposomes showed an active transport of liposomes across the membranes could account for a more efficient uptake of drug (Rumjanek and Simpson 1980; Furlong *et al*. 1992). The altered fuidity of membranes or their lipid organization results in perturbation of the worm's homeostasis (Yeagle *et al*.,1989).

8.4.3. Protoscolicidal activity

The appearance of control and treated adult E*. granulosus* as observed by SEM and light microscopy is shown in Figure 8.2 and Figure 8.3, respectively. The control *E. granulosus* appears elongated, have a scolex with a short neck, and consist of three proglottids (Figure. 8.2A). The scolex bears a rostellum armed with a full complement of hooks and typically concave suckers. PZQ suspension, PZQ-SLN and PZQ-BSLN showed significant protoscolocidal activity against *E. granulosus* under *in-vitro* conditions. Treatment with PZQ-SLN and PZQ-BSLN indicated the significant loss in the viability and morphology such as tegumental alterations and disintegration of protoscoleces (Figures 8.2B & 8.2C). Protoscoleces treated with the SLN exhibited morphological changes which were characterized by sloughing of tegument and distortion of distinct body parts (Figures 8.2B, 8.2D &8.3D). Tegument of the

protoscoleces became more porous after treatment with the SLN (Figure 8.3C).

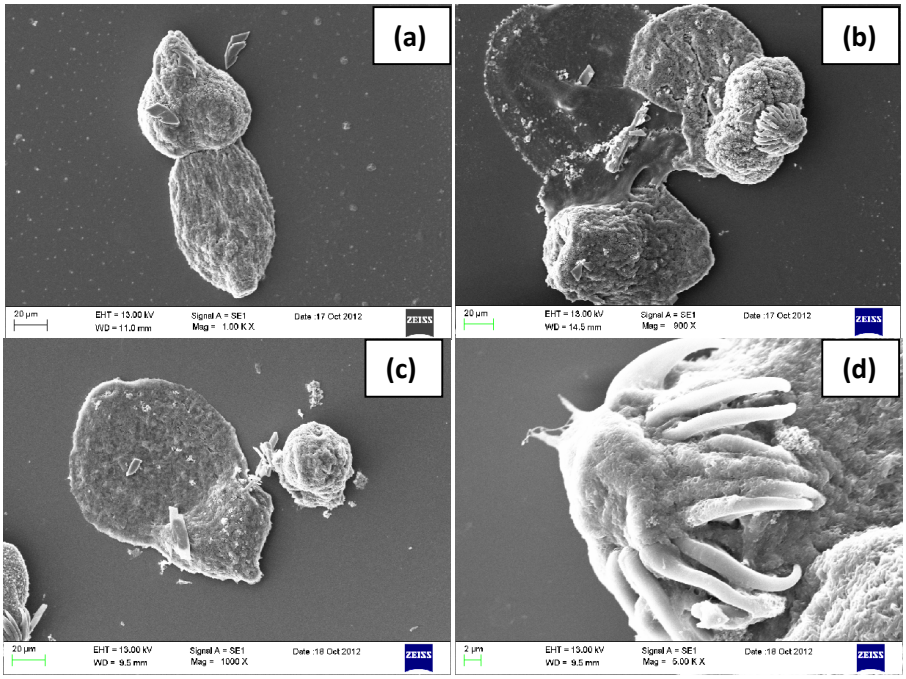

Figure 8.2: Scanning Electron Microscopy of *E. granulosus* protoscoleces (A) Control protoscoleces, (B) Protoscoleces incubated *in vitro* with PZQ SLN (0.5 µg/ml), (C-D) Protoscoleces incubated *in vitro* with PZQ BSLN (0.5 µg/ml).

After 10 min of treatment, PZQ-SLN and PZQ-BSLN at concentrations of 0.5 µg/ml of PZQ showed 48% and 59% mortality, respectively; while 44% mortality in case of PZQ suspension (1µg/ml) (Figure 8.4). SLN at 0.5 µg/ml of PZQ concentration showed 82% mortality while 63% in case of PZQ suspension after 30 min treatment. The SLN at 0.5 µg/ml of PZQ resulted in significant loss of motility and turgidity of cysts. Evagination of the protoscoleces was greater with SLN. However, all protoscoleces, whether evaginated or inevaginated, were stained in trypan blue

exclusion test (Figure 8.3C). Other morphological changes observed were Tegumental vacuolization, loss of motility, loss of hooks and damaged germinal layers (Figures 8.2C and 8.2D). Additionally, the degenerative effect was also observed as loosening of the microtriches and hooks at the scolex region that causes the significant loss in the potential of protoscoleces to attach with host tissues leading to decrease in the infection vigor of the cysts (Figures 8.2D & 8.3D). The scolex region of the cysts was also completely disintegrated.

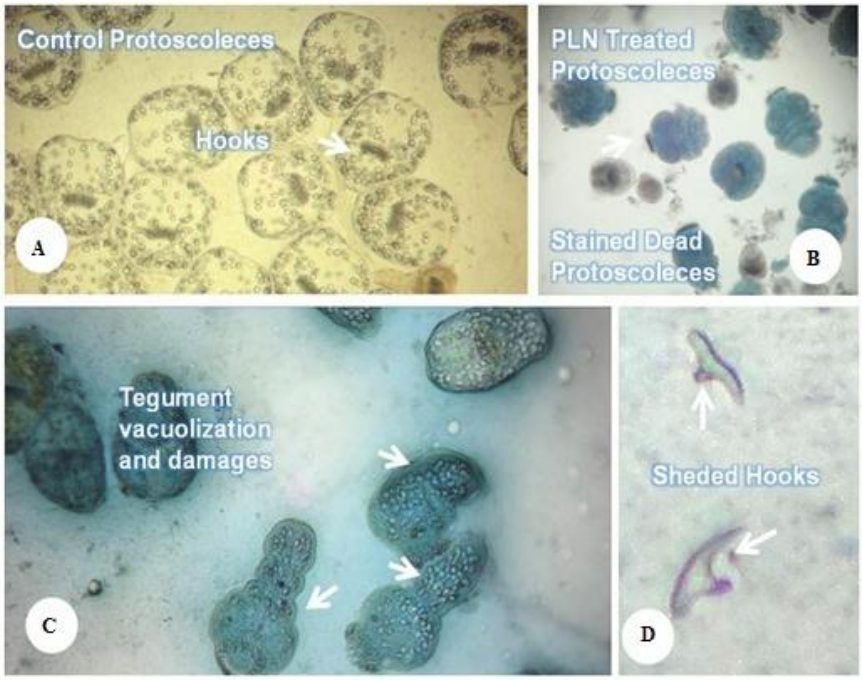

Figure 8.3: Light microscopy of *E.granulosus* protoscoleces. (A) Control protoscoleces, (B-D) Protoscoleces incubated *in vitro* with PZQ SLN (PLN,0.5 µg/ml).

The marked improvement in the efficacy of PZQ using BSLN and SLN at low concentrations may be attributed to increase in surface area and partition coefficient probably due their lipophilic character. It might be

the synergistic effect of the higher concentration of PZQ and the changed membrane fuidity following the exposure to PZQ-SLN and PZQ-BSLN that caused the higher protoscolicidal activity. Distorted ultra-structural changes indicated protoscolocidal activity of PZQ suspension,PZQ-SLN and the PZQ-BSLN (Morris *et al* 1988; Urrea-paris *et al*., 2001)

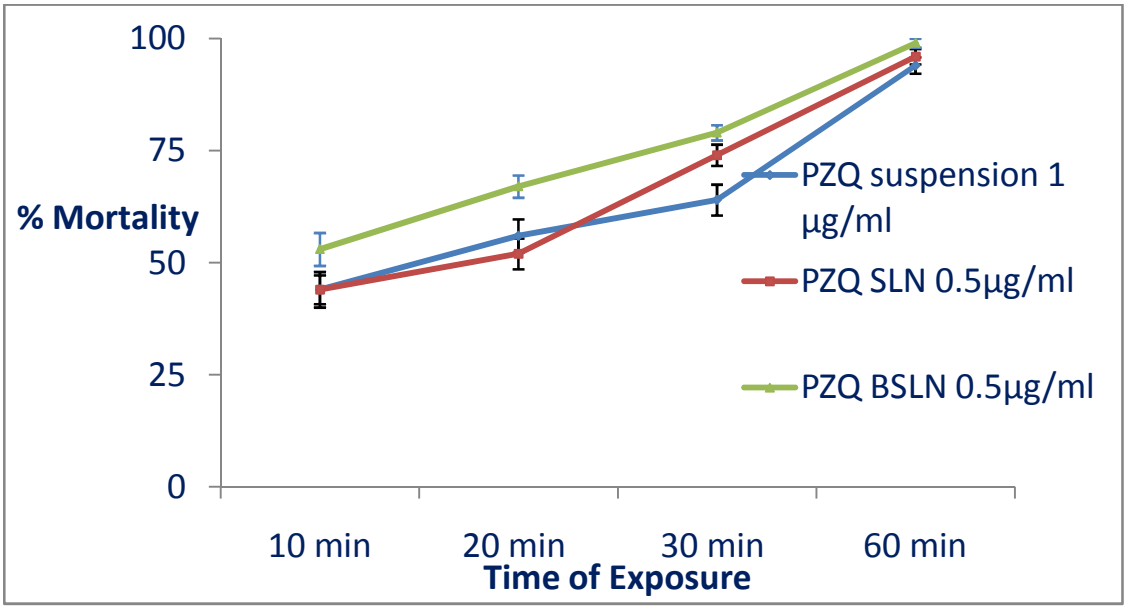

Figure 8.4: Comparative protoscolocidal activity of PZQ-SLN and PZQ-BSLN with respect to PZQ suspension

8.4.4. *In vivo* efficacy against *H.Diminata*

In this study, the efficacy of PZQ-SLN and PZQ- BSLN was investigated against the larval, immature, and mature stages of parasite *H.dimunata*. In the present study both SLN and BSLN showed remarkable efficacy against larval and immature stages in comparison to control PZQ suspension (Table 8.1). However, most previous studies documented that the Praziquantel is effective on mature parasites and found to be less effective on larval stages or immature parasites. The present study shows a

significantly high level of efficacy (P<0.05) against various stages of the parasite.

Moreover, PZQ-SLN and PZQ BSLN exhibited significant activity against mature stages and causing around 97.92 % and 98.35% reduction in EPG count (Post treatment) respectively in comparison to control PZQ suspension (Table8.2).

Table 8.1: Anticestodal activity of PZQ SLN and PZQ-BSLN against larval and immature *H.diminuta* infections in rats

Treatment	**Formulations**	**EPG Count(after 18-20 days)**
Larval *H.diminuta* (5^{th} day)	Control	28300±159
	PZQ-Suspension	7630.00±161
	PZQ-SLN	830±41
	PZQ-BSLN	514±54
Immature *H.diminuta* (10^{th} day)	Control	23962±352
	PZQ-Suspension	7293±83
	PZQ-SLN	787± 69
	PZQ-BSLN	674± 56

Table 8.2: Anti-cestodal activity of PZQ SLN and PZQ-BSLN against mature *H. diminuta* infections in rats

Treatment	**Formulations**	**EPG Count (18-20 days)(Pretreatment)**	**EPG Count (28-30 days) (Post-treatment)**	**Reduction (%) in EPG count(Post treatment)**
Mature *H.diminuta* (22^{nd} day)	Control	28300±275	30433± 238	-7.5371
	PZQ-Suspension	26736±287	6956± 316	73.98
	PZQ-SLN	27673±411	574±42	97.92
	PZQ-BSLN	27942±345	459±46	98.35

The findings of the study are in agreement with other related studies showing huge improvement in antischistosomal activity of PZQ using PZQ loaded phosphatidyl choline liposomes (Murao *et al*, 2005). Anthelmintic efficacy studies typically involve direct counts of worms remaining in the host shortly after drug treatment. The huge improvement in the efficacy of PZQ on *H.Diminuta* using PZQ-SLN and PZQ-BSLN may be due to increased surface area as the Particles are in nano range. Moreover this enhanced effect may be attributed to the increase in bioavailability due to intestinal lymphatic targeting.

The incorporation of PZQ in SLN allowed the drug administration without decreasing the anticestodal effect of the drug which is related with the lipophilic profile of the drug due to the interaction between the drug and the parasite membrane. The incorporation of PZQ in liposomes showed similar effect of the effect on *S. mansoni* cultures.

High concentration of the lipid in SLN also caused cystocidal effect on the *H.diminuta*, which may be due to its ability to capture external lipids through an active process of absorption and metabolism (Tadeusz Moczoñ , 2006). Similar results in culture of *Mesocestoides vogae tetrathyridia and S.mansoni* have been observed using liposomes (Hrckova and Velebny, 1997, Maurao *et al.*, 2005).

The *in vivo* study demonstrated the use of SLN and BSLN improved the PZQ anticestodal activity significantly. Further investigative studies are needed to improve PZQ therapeutic efficacy using alternative administration routes and to examine the mechanism of intestinal absorption of PZQ.