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In vitro Evaluation of Benzimidazole Carbamates on Cystic Larvae of Three Cestode Parasite Models

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Benzimidazole carbamates are broad-spectrum anthelmintics which have limited solubility and hence poor absorption following oral administration. Consequently, their use is limited almost entirely to the treatment of intestinal helminthiasis. This study was designed to compare two different preparations (polyvinylpyrrolidone-drug solutions and dimethylsulfoxide-drug suspensions) of mebendazole, albendazole and ricobendazole (albendazole sulphoxide) by analyzing their *in vitro* efficacy on *Echinococcus granulosus, Mesocestoides corti* and *Taenia crassiceps* parasite models at concentrations of 5, 25, 50 and 100 µg/ml. The effects of the two drug preparations were evaluated on days 2, 4, 7 and 11 post inoculation. The *in vitro* effects of the two preparations on the assayed cystic larvae showed that polyvinylpyrrolidone-drug solutions were more efficacious than dimethylsulfoxide-drug suspensions in the order of mebendazole >albendazole >ricobendazole. Moreover, the three parasite models complement one another.

Key words: *In vitro* efficacy, *Echinococcus granulosus, Mesocestoides corti, Taenia crassiceps*, benzimidazole carbamates, cysts viability

INTRODUCTION

Benzimidazole carbamate derivatives such as mebendazole (MBZ) and albendazole (ABZ) together with its active metabolite marketed as ricobendazole (RBZ) are potent, broad-spectrum anthelminthics which have shown a limited effectiveness against cestode cystic larvae [1-2]. This might be due to their limited water solubility and therefore poor absorption following oral administration [3-4]; hence their use is limited mainly to the treatment of intestinal helminthiasis [5]. However, these drugs are also indicated for neurocysticercosis as well as unilocular and multilocular echinococcosis whereby long-term treatment is required which may cause severe adverse effects [6-10]. High doses of ABZ have been used in the treatment of cysticercosis caused by *Taenia crassiceps* [11] and *T. solium* in HIV/AIDS patients with a significant possibility of recurrence [12]. Thus, in order to improve their therapeutic effectiveness their bioavailability must be increased. A previous pharmacokinetic study [13] reported an improvement on bioavailability after complexation with polyvinylpyrrolidone (PVP). The present study was designed to verify whether the observed increase in efficacy was due to the improved absorption or the intrinsic efficacy of the PVP-drug solutions. In addition, the appropriateness of PVP-dispersed drugs for the treatment of *Echinococcus granulosus* cystic larvae and chemoprophylaxis during cystic hydatidosis surgery to avoid secondary hydatidosis was investigated.

However, there is a difficulty in conducting *in vivo* or *in vitro* assays on some of the larval stages of cestodes such as *E. granulosus*. Consequently, only few *in vitro* studies using daughter cysts [14-18] or cysts derived from laboratory animals [19-22] have so far been reported. For instance, in the mouse model long periods (6-8 months) are required for the development of secondary hydatid cysts suitable for pharmacodynamic screening. Therefore, this study focused on two cystic larval forms of *T. crassiceps* and *Mesocestoides corti* as alternative *in vitro* models to such time consuming chemotherapeutic trials.

MATERIALS AND METHODS

Acquisition of parasitic materials: E. granulosus protoscoleces were removed aseptically from slaughtered sheep in Madrid, Spain. The samples were processed within 24

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hours and stored at 4 ºC. Viability prior to testing was 95-99 % as assayed by the methylene-blue exclusion test and flame cell activity under microscopic observation as described previously [23]. The protoscoleces were counted on the McMaster chamber.

M. corti and *T. crassiceps* cystic larvae were aseptically extracted from *M. corti* and *T. crassiceps* cysts which were routinely maintained in Naval Medical Research Institute (NMRI) mice under laboratory conditions at the Department of Parasitology, Faculty of Pharmacy, Universidad Complutense de Madrid, Spain. The extracted cysts were briefly washed in Hank's balanced salt solution (HBSS) under gentle agitation at a fixed speed. Cyst viability was determined by observing under a light microscope for general contractile movements and morphological integrity [24].

Preparation of Drugs: Preparation of PVP-drug soluble complexes was performed as previously described [25]. The drug concentrations used were 5, 25, 50 and 100 µg/ml, dissolved in CMRL1066 (GIBCO) medium culture, pH 7.2 ± 0.4. The parent drugs were dissolved in distilled water, 1 % dimethylsulfoxide (DMSO) and 0.9 % glycerine before diluting to the required concentrations.

Evaluation of Drug Efficacy and Statistical Data Analysis: The assays on *E. granulosus* were conducted in 24-well plates. The number of protoscoleces was adjusted to 800-1000 protoscoleces/ml in HBSS per well after which the HBSS was replaced by the appropriate culture medium. The blank contained culture medium alone, while DMSO and PVP controls were made up of culture medium with 1 % DMSO and 2 mg/ml PVP, respectively. About 15-20 cystic larvae of either *M. corti* or *T. crassiceps* were inoculated into 50 ml CMRL 1066 flask containing media. All cultures were incubated at 37 ºC and on days 2, 4, 7 and 11 were examined and the results recorded. The media was changed every 2-3 days. The effect of the drugs on *E. granulosus* protoscoleces and *M. corti* and *T. crassiceps* cysts was determined as previously indicated. Each assay was performed in triplicate and repeated three times with the data obtained being expressed as mean percentages of surviving larvae/cysts.The data was analyzed using the SPSS Version 10 (SPSS Inc., Chicago, IL). Differences among the means of various groups were investigated by analysis of uni- and multivariable variances and the significance level was set at $p<0.05$.

RESULTS

Comparison of parasite models with respect to cysts/larvae survival: No significant differences were observed in the mean percentages of surviving cysts among all control groups of the assayed parasite models. In all the models, the effect of the two drug preparations on the viability of the three parasite models was variable. Moreover, reduction of cyst viability was a function of concentration and time-lapse post inoculation (p.i.) for the three parasite models (Figures 1-9). In all the models, no significant differences were observed between the 50 µg/ml and100 µg/ml concentrations as well as between the 5 µg/ml and 25 µg/ml concentrations. However, there was a significant difference between the two sets of concentrations with respect to their effect on cyst viability regardless of type of drug preparation except ricobendazole (RBZ)-PVP and RBZ-DMSO on *E. granulosus* (Figure 2), RBZ-DMSO on *T. crassiceps* (Figure 8b) and mebendazole (MBZ)-DMSO on *T. crassiceps* (Figure 9b)

Pair wise comparison of cyst viability showed no significant differences among the parasite models: *E. granulosus* against *M. corti*, $p = 0.716$; *E. granulosus* versus *T. crassiceps*, $p = 0.152$; and *M. corti* against *T. crassiceps*, $p = 0.272$. Generally, PVP-drug solutions were more efficacious when compared with DMSO-drug suspensions in all the parasite models.

Evaluation of drug effects on parasite models: Effects of the control groups on cyst survival were compared with the blank, and no significant differences were found ($p <0.05$). All the drug treated groups differed significantly from the control groups and there were significant differences between the two drug preparations particularly from day 4 to day 7 post infection (Figures 1-9). The PVP-drug solutions manifested no significant differences among the assayed drugs when their effects on the *E. granulosus* parasite model werecompared. The effects of RBZ were not significantly influenced by the type of preparation (Figures 1-9).

There was a slight difference in the *in vitro* effectiveness of the two ABZ preparations on the *M. corti* parasite model especially on day 7 (Figure 4) with the PVP-drug solutions being

significantly more efficacious ($p < 0.05$). There was a statistically significant difference between the two MBZ preparations from day 4 to day 7 post infection, but not between the RBZ preparations, with regard to their effects on *M. corti* cysts (Figure 5). The ABZ-PVP solutions were more effective in reducing *T. crassiceps* cyst viability compared to ABZ-DMSO suspensions (Figure 7). Nevertheless, no significant differences were observed on days 2 to 4 with concentrations below 100 µg/ml for ABZ-DMSO suspensions. Furthermore, no significant differences were observed in the reduction of cysts viability between the 50 µg/ml and 100 µg/ml concentrations as well as between the 5 µg/ml and 25 µg/ml concentrations for RBZ-DMSO suspensions (Figure 8b). Significant differences in effect were however observed between two equal concentrations of MBZ preparations on *T. crassiceps* cyst viability (Figure 9). All the assayed drug preparations, except the MBZ-DMSO suspensions manifested concentration dependent effects.

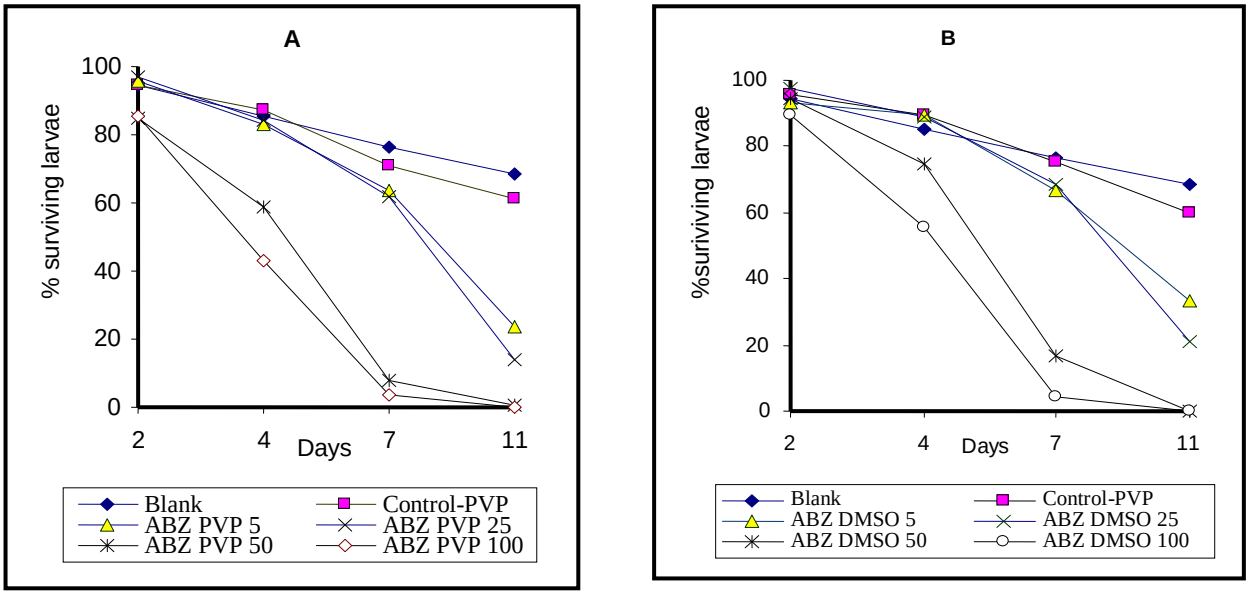

Figure 1: Effects of (A) Albendazole-polyvinylpyrrolidone (ABZ-PVP), (B) Albendazole-dimethylsulfoxide (ABZ-DMSO) on *Echinococcus granulosus* larvae. The number after each preparation denotes the concentration in µg/ml.

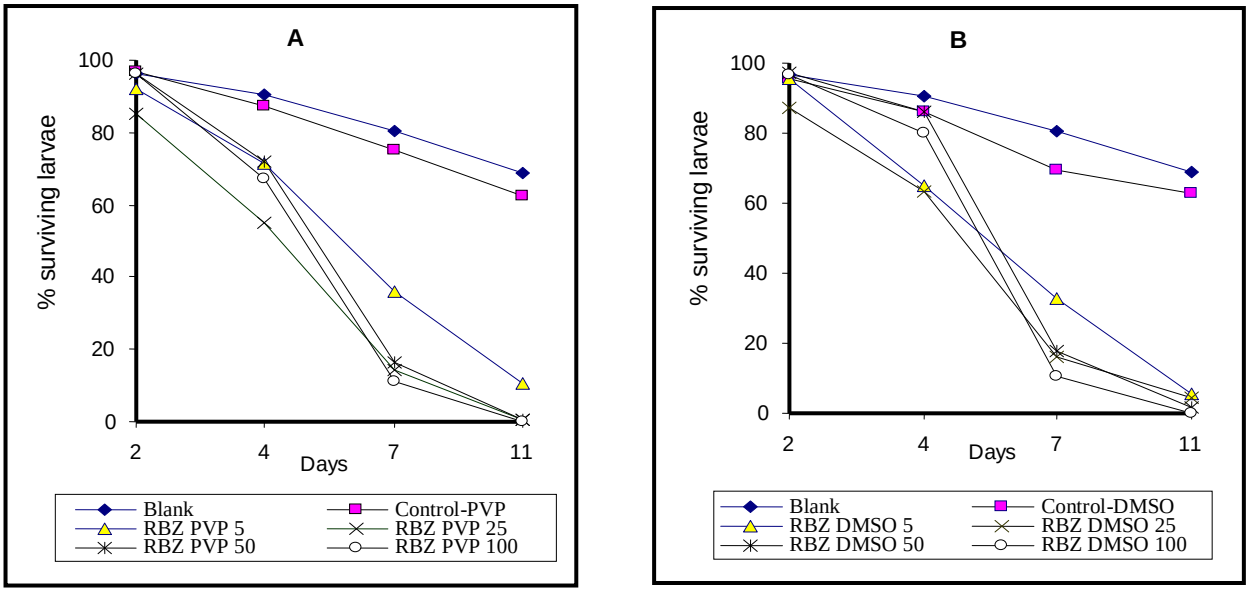

Figure 2: Effects of (A) Ricobendazole-polyvinylpyrrolidone(RBZ-PVP), (B) Ricobendazole–dimethyl-sulfoxide (RBZ-DMSO) on *Echinococcus granulosus* larvae. The number after each preparation denotes the concentration in µg/ml.

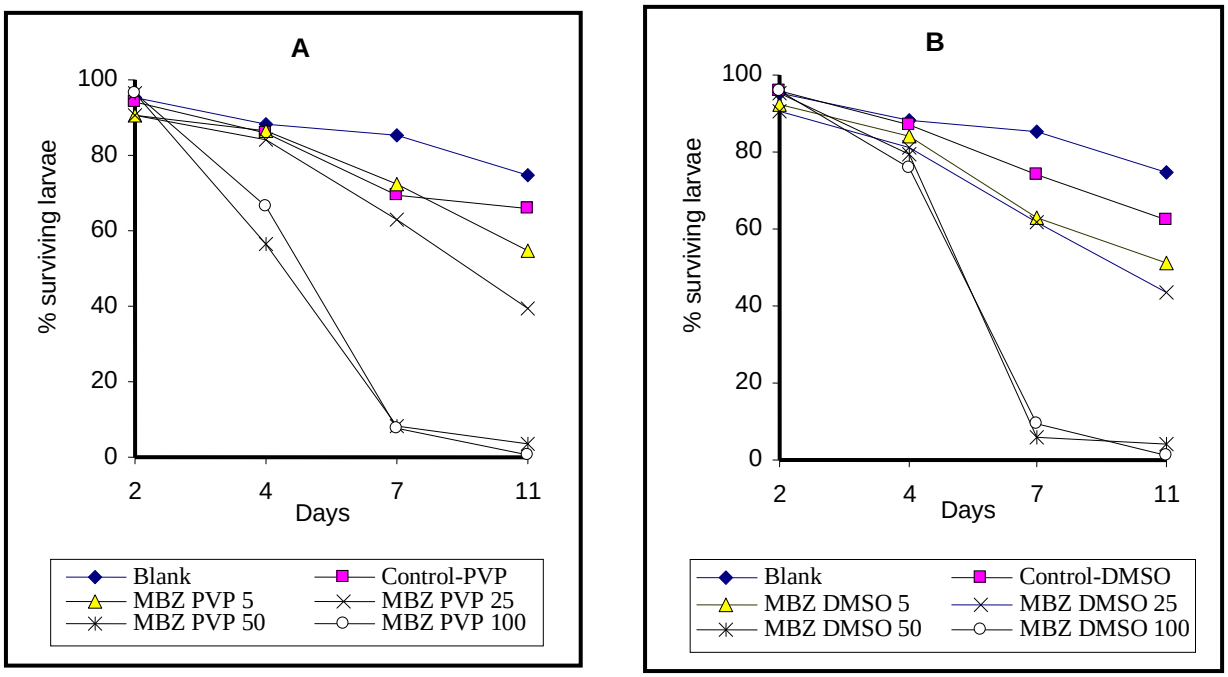

Figure 3: Effects of (A) Mebendazole-polyvinylpyrrolidone (MBZ-PVP), (B) Mebendazole–dimethylsulfoxide (MBZ-DMSO) on *Echinococcus granulosus* larvae. The number after each preparation denotes the concentration in µg/ml.

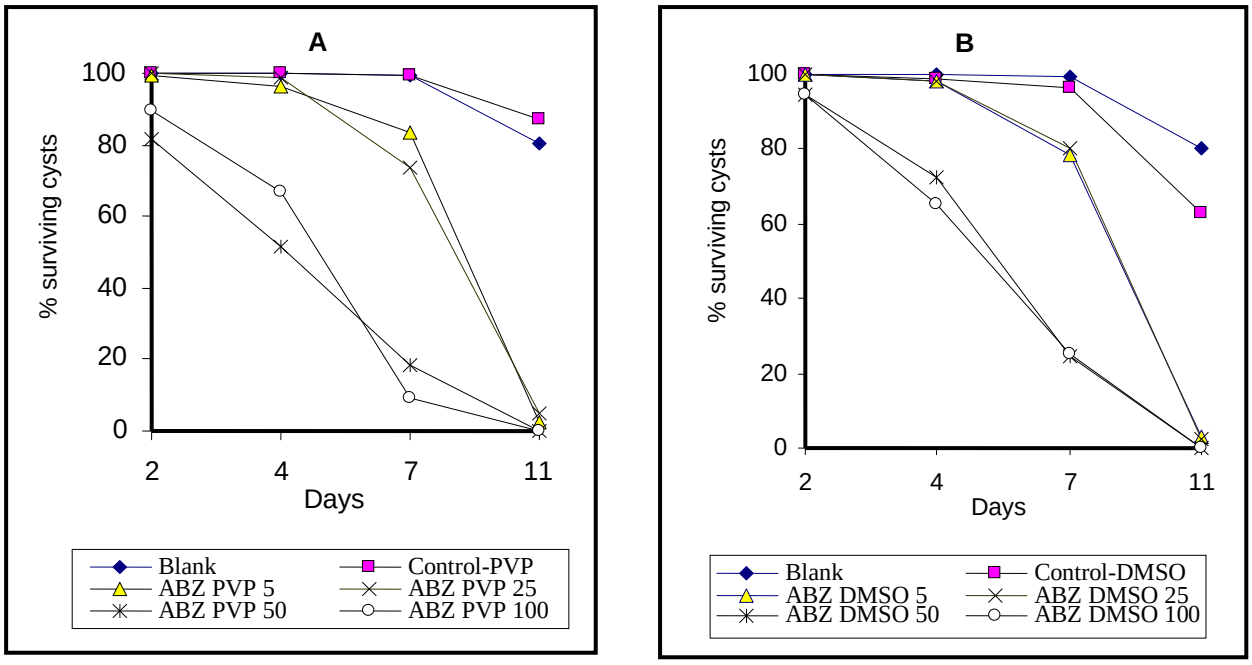

Figure 4: Effects of (A) Albendazole- polyvinylpyrrolidone (ABZ-PVP), (B) Albendazole-dimethylsulfoxide (ABZ-DMSO) on *Mesocestoides corti* cysts. The number after each preparation denotes the concentration in µg/ml.

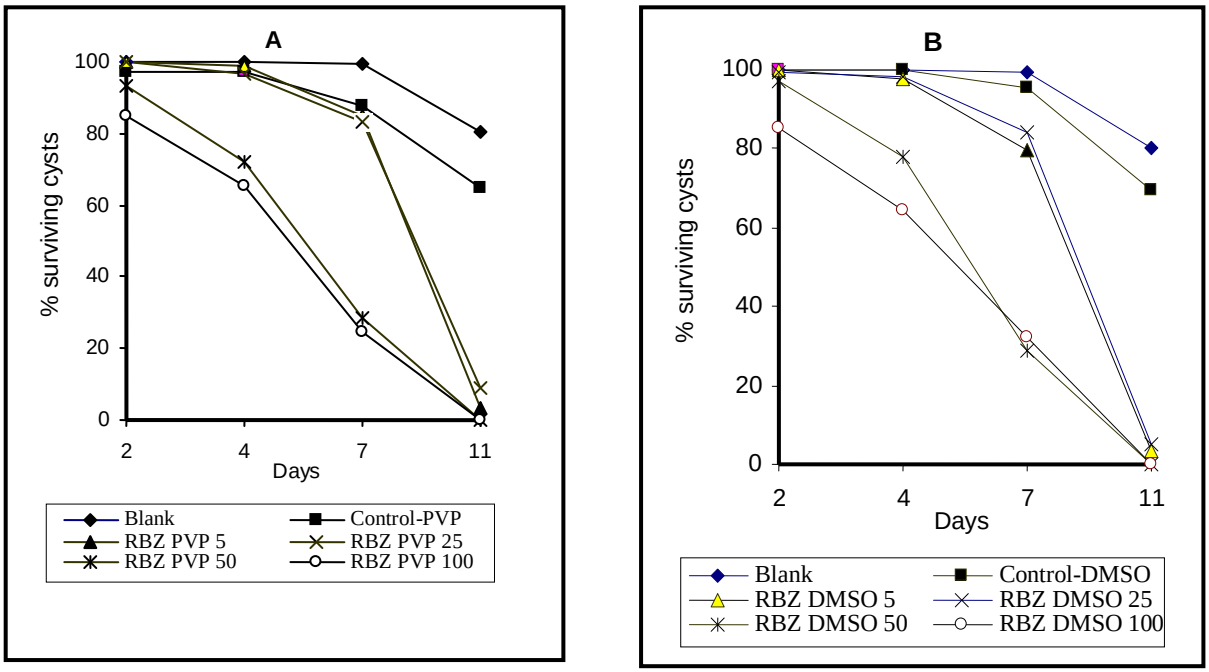

Figure 5: Effects of (A) Ricobendazole- polyvinylpyrrolidone (RBZ-PVP), (B) Ricobendazole - dimethylsulfoxide (RBZ-DMSO) on *Mesocestoides corti* cysts. The number after each preparation denotes the concentration in µg/ml.

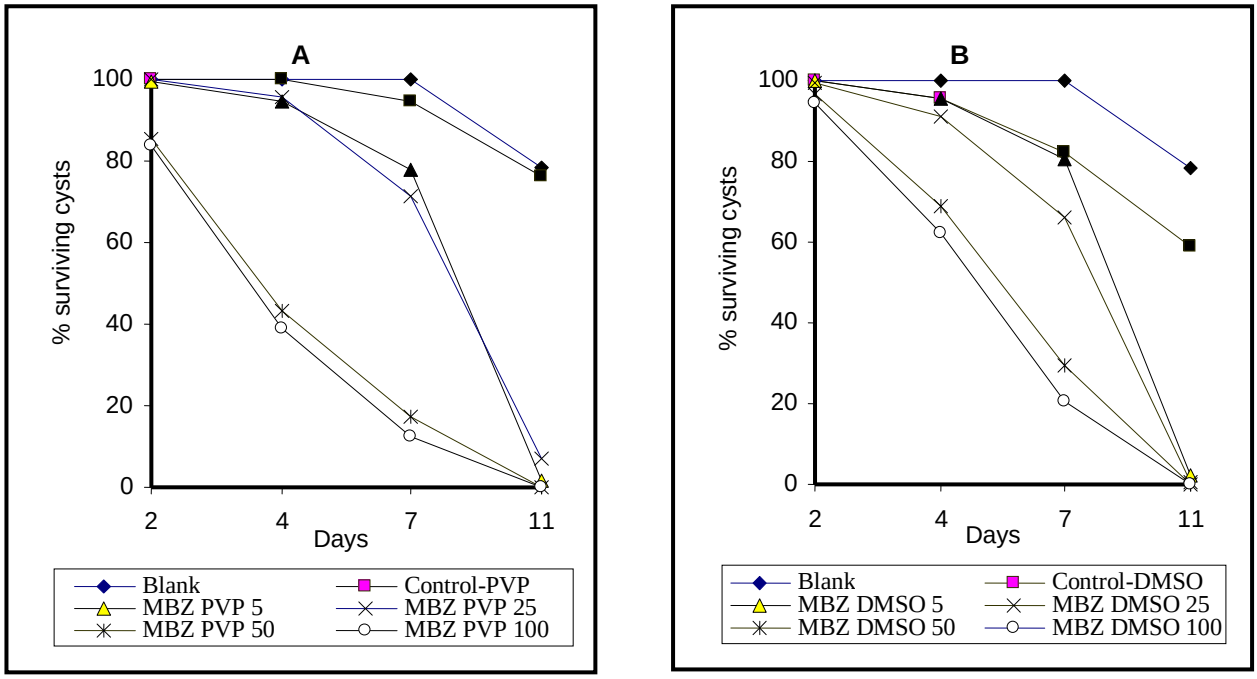

Figure 6: Effects of (A) Mebendazole- polyvinylpyrrolidone (MBZ-PVP), (B) Mebendazole - dimethylsulfoxide (MBZ-DMSO) on *Mesocestoides corti* cysts. The number after each preparation denotes the concentration in µg/ml.

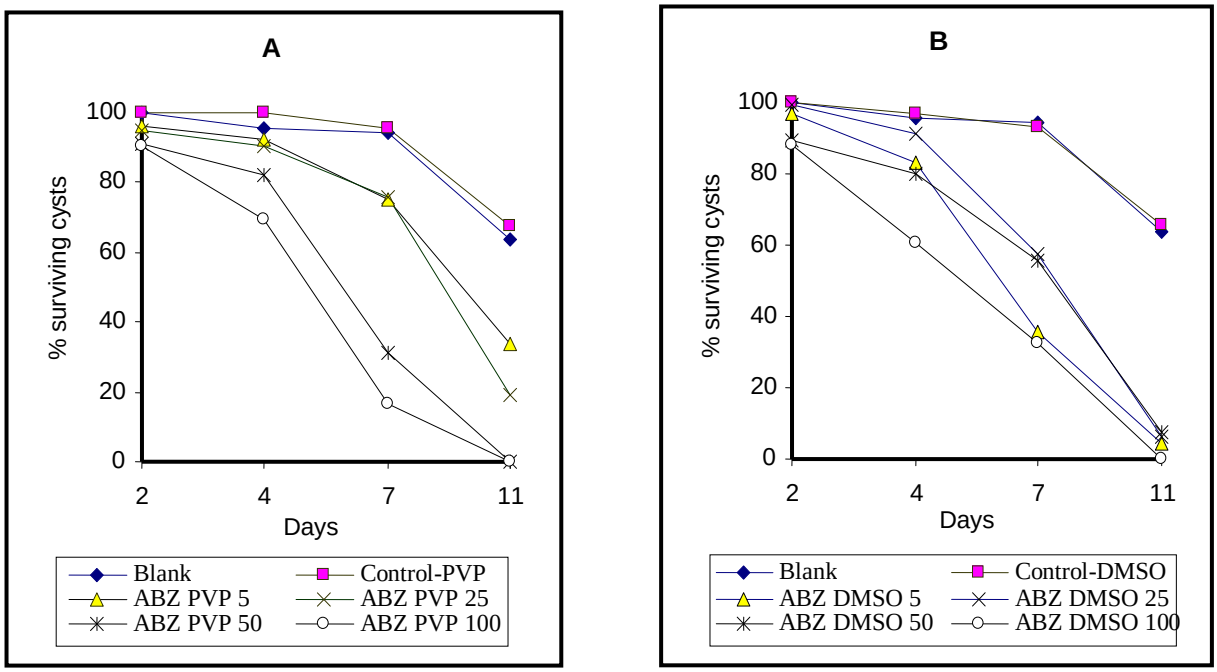

Figure 7: Effects of (A) Albendazole- polyvinylpyrrolidone (ABZ-PVP), (B) Albendazole - dimethylsulfoxide (ABZ-DMSO) on *Taenia crassiceps* cysts. The number after each preparation denotes the concentration in µg/ml.

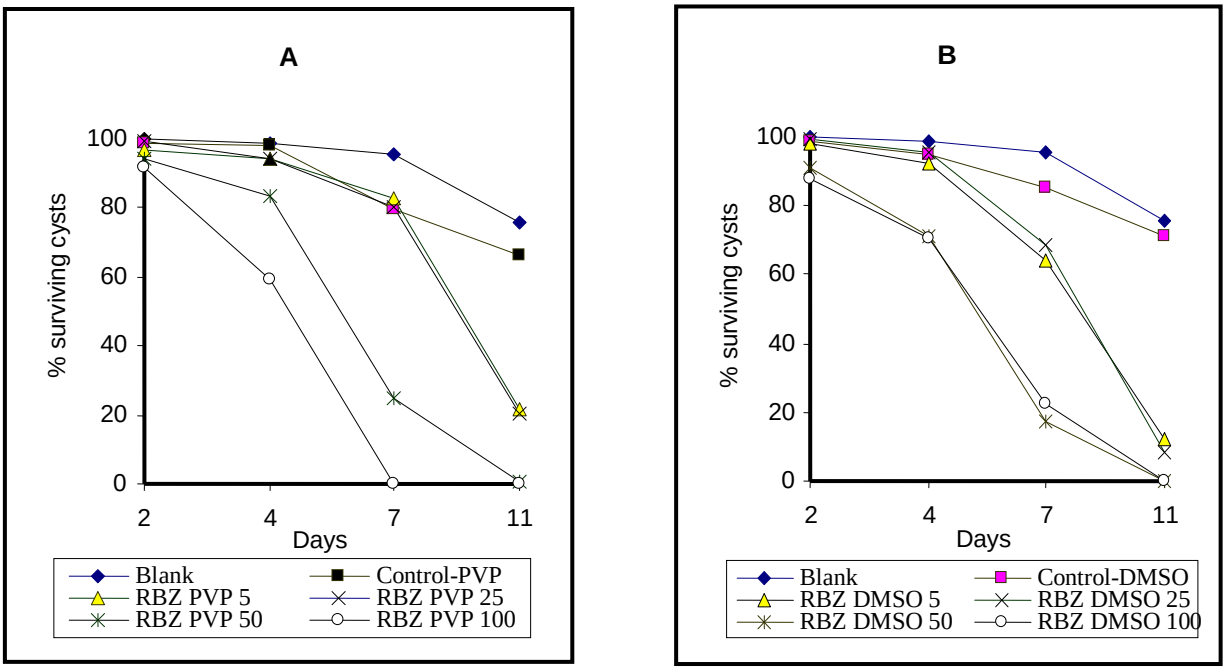

Figure 8: Effects of (A) Ricobendazole- polyvinylpyrrolidone (RBZ-PVP), (B) Ricobendazole - dimethylsulfoxide (RBZ-DMSO) on *Taenia crassiceps* cysts. The number after each preparation denotes the concentration in µg/ml.

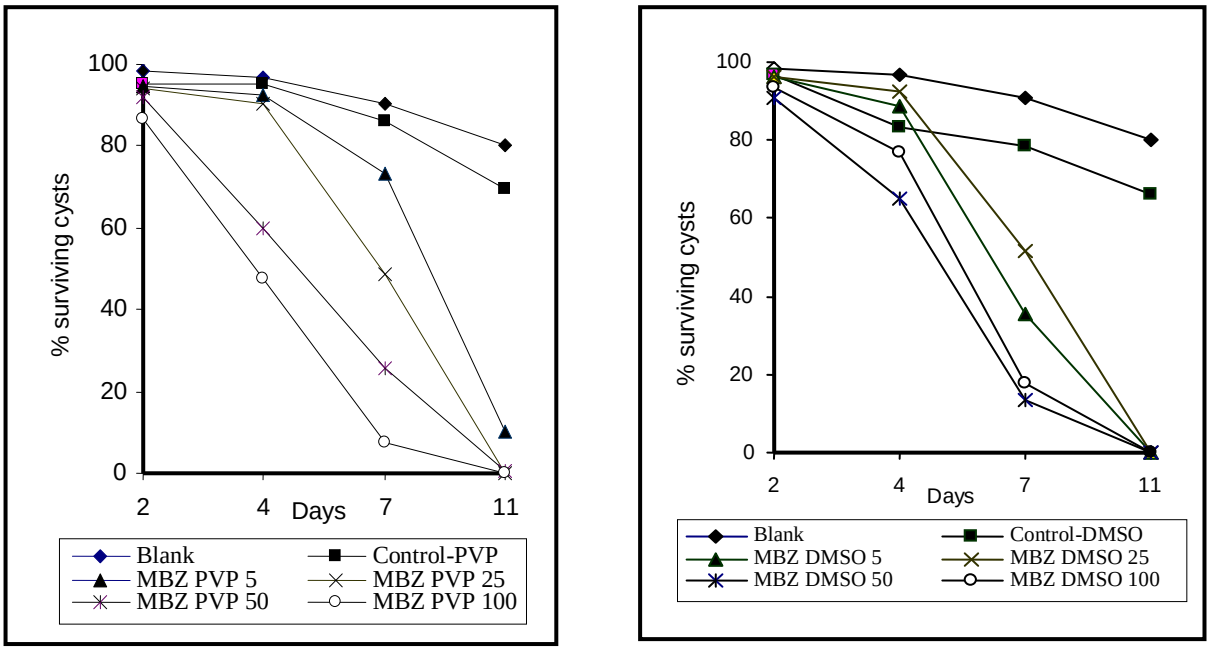

Figure 9: Effects of (A) Mebendazole-polyvinylpyrrolidone (MBZ-PVP), (B) Mebendazole-dimethylsulfoxide (MBZ-DMSO) on *Taenia crassiceps* cysts. The number after each preparation denotes the concentration in µg/ml.

DISCUSSION

The aim of this study was to evaluate the effects of the PVP solutions and DMSO suspensions on the three *in vitro* parasite models, focusing mainly on the *E. granulosus* parasitic characteristics. It was also designed to investigate the *in vitro* efficacy of the resultant solutions and suspensions of the three assayed benzimidazole carbamates taking advantage of the availability and readiness for assay of *M. corti* and *T. crassiceps* larval forms [26]. To date, cystic echinococcosis constitutes a sanitation problem for which there is no simple solution. The therapy for the disease is either by surgery or chemotherapy. Benzimidazole carbamates have been in use for a long time but are not fully effective. Due to their limited therapeutic efficacy, the development of new agents and preparations is inevitable. Moreover, *in vitro* assays of new drugs and preparations on *E. granulosus* larval stage are time consuming thus the need for alternative models.

Taenia crassiceps is wide spread in boreal North America and like a number of other taeniids constitutes a potential risk as a zoonotic parasite. Unfortunately, the route of transmission and effective chemotherapy for *M. corti* (members of *Mesocestoidae*) has not yet been fully elucidated. Furthermore, there are about 25 reported cases world-wide of intestinal *Mesocestoides* tapeworm infection in humans [27] and this figure may increase with the advent of the HIV/AIDS pandemic. Consequently, a greater understanding of the parasite will highlight more effective means for its prevention and chemotherapy.

In the present study, major differences were observed between the two drug-preparations from day 4 post inoculation (p.i) upon comparing two identical concentrations. The PVP drug solutions were more effective compared to DMSO suspended drugs exhibiting significant efficacy from day 4 to 7 p.i. for all the parasite models. The two RBZ preparations demonstrated similar effects on the viability of *E. granulosus* (Figure 2) and *M. corti* (Figure 5) cystic larvae which, to a large extent, could be attributed to the relatively high solubility of RBZ compared to the rest of the assayed drugs. Significant differences ($p < 0.05$) were observed

in efficacy against *T. crassiceps* cyst viability when identical concentrations of different preparations of the same drug were compared. *Taenia crassiceps* cysts were relatively highly susceptible to the drugs which may be due to a larger exposed cystic larval surface area compared to that of the other parasite models. The MBZ-DMSO suspensions were an exception since they did not show the characteristic concentration-dependent effect. This could be attributed to the poor solubility of MBZ. There was a significant difference ($p < 0.05$) when the control groups were compared with the drug treated groups. This could imply that the dispersing or complexation agents had no impact *per sé* on cysts viability.

One previous study on benzimidazole derivatives found that these drugs were rather parastatic *in vivo* than *in vitro* [2]. Another *in vivo* study revealed an improvement in the chemotherapy of cystic infections with benzimidazole carbamates though total eradication of the cystic larvae (metacestodes) could not be achieved in most of the infected patients [6]. For instance, the parasitocidal concentration of MBZ *in vitro* was in the range of 0.1 μM to 1 μM, while the level in plasma that was considered to be effective in the treatment of cystic echinococcosis, 0.25 μM, was within the range [6]. Thus the parastatic effect *in vivo* may reflect differences in the bioavailability of these compounds. Unfortunately, factors that affect efficacy are still unclear and are presumed to be the size and age of cystic larvae, calcification and fibrosis which correlate with the results of the therapy [1, 23]. In addition, the availability of benzimidazole carbamates in insufficient concentrations within the cyst tissue and the possible development of drug resistance may affect the efficiency of chemotherapy [28-30]. Our findings are in agreement with previous pharmacokinetic studies which reported that drug solubility is significantly improved by complexation with PVP for the three assayed drugs. Furthermore, the effect of drug preparation was found to be more pronounced for the less soluble drugs like MBZ as compared to RBZ [13].

In conclusion, this study reports minor improvement in the *in vitro* efficacy of the PVP-drug solutions compared to the DMSO-drug suspensions of benzimidazole carbamates in the order MBZ > ABZ > RBZ. Moreover, the parasite models are comparable to one another such that no significant differences were observed among them with regard to cyst viability. Besides, in all the assayed parasite models, PVP-drug solutions were more efficacious than DMSO-drug suspensions in all the parasite models with the exception of *M. corti*. The observed minor difference, though statistically significant, might be attributed to the absence of physiological barriers and biological interactions which are the real microenvironments that affect drug absorption and consequently bioavailability. Further *in vitro* and *in vivo* studies on benzimidazole carbamates and other anthelminthics, especially the poorly soluble drugs currently used in the treatment of parasitic cysts are recommended.

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