

Explanatum explanatum: effect on viability and histology following in vitro treatment with phenyl vinyl sulfone



Zoology

KEYWORDS: Trematode, *E. explanatum*, phenyl vinyl sulfone, antihelminthic, liver, water buffalo

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ABSTRACT

The objective was to study the in vitro effect of the antihelminthic drug phenyl vinyl sulfone (PVS) on the trematode *Explanatum explanatum* (Creplin, 1847) Fukui, 1929; during different periods of incubation. Trematodes infect liver of buffaloes and other cattle and cause extensive damage and have wide geographical distribution in India and other Asian countries. The drug phenyl vinyl sulfone (PVS) is known to have antihelminthic and antiprotozoal properties because it inhibits cysteine protease activities in parasite. Adult *E. explanatum* obtained from the liver of slaughtered buffaloes were treated with PVS in RPMI culture media and incubated at 37°C for periods ranging from 2 to 8 h. The effect of this protease inhibitor on worms was analyzed in terms of viability, motility and death of worms as well as histological changes. The minimum effective concentration of the drug was found to be 50 ppm after 8 h of incubation. 200 ppm concentration of the drug resulted in immediate death of adult flukes in vitro. Histological studies of the treated fluke revealed structural changes in the tegument epidermis and loss of spine. Degenerative changes were also noticed in the testicular follicles, ovaries and vitellaria when compared with untreated controlled group. The results suggest the PVS affects the mobility and histology of the trematode and could also be explored for use as an alternative antihelminthic drug.

Introduction:

Explanatum explanatum (*Gigantocotyle explanatum*) is a parasitic flatworm of the class Trematoda, and is considered as one of the most important trematode causing infection of buffaloes in India and other Asian countries. It (buffaloes) has a wide geographical distribution and plays a crucial role in domestic economy of resource poor rural farmers with respect to production of milk and meat. Among all the domestic animal, Asian buffaloes hold the greatest promise and potential for production of milk and flesh (Cockrill, 1994); however they are very susceptible to many parasitic diseases as they usually graze in the open natural external environment and are subjected to natural calamities such as flood and draught. The authors have recently reported histopathological effect of *E. explanatum* on the host liver tissue and noticed that predominant features were development of multifocal nodules throughout the luminal surface of the bile ducts and there were intense infiltration of inflammatory cells (Haque *et al.*, 2011). The effective strategies for the control of trematode infections are largely based on drugs used on the definitive host (Boray, 1997). Phenyl Vinyl Sulfone (PVS) is a group of drug which is effective protease inhibitors and is reported to be arresting the parasitic infections in mice by inhibiting Cathepsin B, L and S of the parasite (Rasnick, 1996). The concentration at which they are effective for the parasites is not toxic to mammalian tissues (Manson, 1991; Serveau *et al.*, 1996; Lalmanach *et al.*, 1996). The objective of this study is to assess the antihelminthic effects of PVS on *E. explanatum*.

Materials and methods:

The liver of water buffalo *Bubalus bubalis* infected with *E. explanatum* were collected from the local abattoirs and were immediately brought to the laboratory. The bile ducts were cut open and the individual trematodes were recovered, washed and incubated for an hour in Tyrode saline containing 8mM glucose, in order to let them shed gut contents. They were then rinsed with distilled water, blotted dry on filter paper and then incubated in RPMI 1640 culture medium. The trematodes were treated with PVS of specific concentrations ranging from 50, 100, 150 and 200 ppm in four similar experimental sets of the culture media and incubated at 37°C for 2 to 8 h. For this purpose, a measured concentration of PVS was added to 15 ml of culture medium

containing eight *E. explanatum* of approximately similar size in each well. The effects on viability & motility were observed and histological changes were studied under the microscope. Histopathological changes in the trematode parasite and mortality percentage were also recorded. The trematodes were taken out of the culture medium at the specified intervals for histological study, fixed in Bouin's fixative and processed for paraffin sectioning in order to study the effect of PVS. For control, the worms were incubated without PVS under the similar experimental set-up. After 8 hours of incubation, the number of dead and surviving trematodes was counted from each of the treatment sets and the control set followed by statistical analysis.

Results:

Tab. 1 shows the changes in motility and histology of the trematodes after 2, 4, 6 and 8 hours of incubation at 50, 100, 150 and 200 ppm of PVS concentrations, respectively, along with the control. It is apparent that there is no significant change after 2 hours of incubation either in motility or histology except at concentration of 200ppm. The gradual changes in motility as well as histology appear to occur at increasing drug concentrations after four hours of incubation. The changes in motility and histology were more evident after 6 hours of incubation. It was found that 50% of the worms died at 50 and 100 ppm concentration of PVS, 75% died at 150 ppm concentration and approximately 100% death occurred at 200ppm concentration.

Histologically, significant changes occurred in the external and internal morphology of trematode at higher concentration of PVS. The tegument was found to be damaged and in certain region there was a loss of spines. The morphology of testes and ovaries appeared to be greatly disrupted along with a loss of internal tissue mass. The spongy parenchymal tissue was also found to be affected. A cross section of the liver of buffalo heavily infected with *E. explanatum* has been shown in Fig. 1. A transverse section passing through the oral region and the surrounding tegument area of the trematode which were incubated in lower concentration of PVS shows the normal tissue structure (Fig. 2). However there is an apparent disruption of tissue in tegument, testes and ovaries and the surrounding parenchyma at 200 ppm concentration of PVS, which is evident from the loss of tissue mass in these organs

(Fig. 3),with respect to the control (Fig. 4).

Statistical analysis of the data was done. The probability of repetition of the results was quite high as the table 2 shows the mortality at different doses is significant. At 50ppm dose of PVS the average % mortality is 50% (4±0.5) which is significant (P<0.005) with respect to control. At 50 ppm dose of PVS the average % mortality is 50% (4±0.5) with respect to the control. At dose of 100 ppm and 150 ppm the average % mortality further increases to 56.25% (5±0.5) and 75% (6±0.5) respectively. The % mortality increases to 97% (8±0.5) at 200 ppm dose showing that the 200 ppm dose has the maximum lethality and that all the four findings are highly significant [50 ppm(p<0.005), 100 ppm (p<0.004), 150 ppm (p<0.001) and 200 ppm (p<0.001)].

Discussion:

Amphistomiasis caused by various species of amphistome trematodes such as *E. explanatum* infecting liver of buffalo and other cattle in India and other countries of the subcontinent appears to be equally serious disease. The effective strategies for the control of fascioliasis and other trematode infections are largely based on drugs used on the definitive host (Boray J. C., 1997). The trematode parasite *E. explanatum* has tegument as outer body covering which is metabolically active portion of the body; composed of protein, lipid, carbohydrate and RNAs, forming the host- parasite interface. It has absorptive function as well as is the host of a number of enzymes, including cysteine proteases (Cheng, 1986; Mansour and Mansour, 2002). The cells of integument tissue have no distinct boundary, are lined with plasma membrane only which is associated with glycocalyx (fig. 4). Integument has two distinct layers distal and proximal cytoplasm. Distal cytoplasm is the part which is lined with plasma membrane and proximal cytoplasm, is actually cellular region having microtubules. A number of invaginations or surface pits are present in the tegument along with minute bristle like projections or spines, embedded in the basal lamina. Spines are made up of paracrystalline actine filaments (Bogitsh *et al.*, 2005). Triclabendazole is one of the available drugs that affect adult parasites inhabiting the bile duct of the host and immature fluke that migrate through liver parenchyma. Triclabendazole is even absorbed at tegument level of the parasites. It affects the parasites motility. Exposure for longer period produces blabbing of the tegument and finally leads to sloughing of the tegument layer. Scanning electron micrograph and transmission electron micrograph confirm these observations (Robinson and Turdgett, 2002).

More recently the drug PVS, a cysteine protease inhibitor have been studied for antihelminthic properties (Steverding *et al.*, 2006). It has been reported that PVS is a potent cysteine-protease inhibitor (Helmy *et al.*, 2008). In mammalian integumentary system, cystein proteases play a major role in maintaining the stratum corneum (Chapman, *et al.*, 1997; Grzelakowska- Sztabert, 1998; Berdowska and Seiwinski, 2000; Rzychon, *et al.* 2004). Any imbalance in the activity of cysteine proteases leads to pathogenic condition, including skin lesions that provide site for attack by various obligate infections (Berdowska and Seiwinski, 2000). It is responsible for the proper maintenance of the

intact skin by means of replacement of dead part with newly developing underlying basal part. Cysteine protease plays important role in the life cycle of protozoan and helminthic parasites and has been explored as an agent for chemotherapy (Steverding *et al.*, 2006). Cysteine protease inhibitors block parasite replication and differentiation providing an alternative to traditional therapy in drug resistant organism.

Immunological studies carried out in trematodes targeting cysteine proteases by various workers have shown that this group protein is a key role player in maintaining the cellular lining of the gut lumen (Boray, 1997). Alongside, it is also found to be responsible for maintaining vitellaria, the reproductive structure of trematodes and the tegument layer of this parasite (Fairweather and Boray, 1999). Microtubules play important role in maintenance of the tegument layer of the parasites. Inhibition of cysteine protease activity may interface with the proper positioning of the microtubules in the distal cytoplasm and spines (paracrystalline actin filament) in the proximal cytoplasm (fig. 3). Among the various cysteine proteases like Cathepsin L, Cathepsin F, Cathepsin B, found in the trematodes, secretome constitute a group of compounds that help the parasite to penetrate host, dissolve their body proteins so that the break down products i.e. amino acids, could be absorbed by them in order to get nutrients. These secretome also help the parasite to avoid the host immune response generated against them. (Curwen, *et al.* 2006, Robinson, *et al.* 2008)

PVS might have inhibited these categories of cysteine proteases that caused degeneration of the tegument, rendering the parasite reproductively immature and prone to immune attack mounted by the host, when injected into the body of the affected host animals.

In the present study as a result of incubation in 50, 100, 150 and 200 ppm concentrations of PVS at different incubation periods, gradual changes in motility and histology of the flukes began to appear after 4 hours of incubation and were more prominent with increasing PVS concentrations and incubation time. There were disruptions in tegument structure and loss of spine. Loss of tissue mass in parenchyma, testes, ovary and other organs was apparent. These changes are more or less similar to that occurred in *F. hepatica* on exposure to compound alpha-sulphoxide Rivera *et al.* 2004) and by PVS (Helmy *et al.*, 2008). These actions may be responsible for the loss of muscular motility and absorption capacity at the tegument level in PVS treated trematode which ultimately leads to their death. Morphological changes and the histological findings of the *E. explanatum* in the culture media strongly suggest the anti-trematode activity of PVS in vitro condition.

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Tab.1: Analysis of motility and histology as well as percentage of death occurred after 8 hours of incubation at increasing concentrations of PVS.

PVS Conc.	Incubation time							
	2 nd hrs		4 th hrs.		6 th hrs		8 th hrs	
	Motility	Histology	Motility	Histology	Motility	Histology	Motility	Histology
50ppm	No change	No change	Slow	No change	Sluggish, color changed	Changes in tegument, musculature	50% died	significant changes
100ppm	No change	No change	Slower	Not significant	Some shrunk	More evident Changes	50% died	Tegument damage
150ppm	No change	No change	Very slow	Tegument disruption	Greatly shrunk	Spine deformed	75% died	Changes in testes, ovary & vitellaria

200ppm	Less active	Mild change	Very slow	Significant Tissue damage	All died	Spine loss	All died (100%)	Tegument, testes and ovary greatly damaged
Control	No Change	No Change	No Change	No Change	No Change	No Change	One died	Not significant



Fig. 1 A cross section of buffalo liver showing numerous adult flukes *E. explanatum* attached throughout the luminal surface of bile duct which has been cut open.

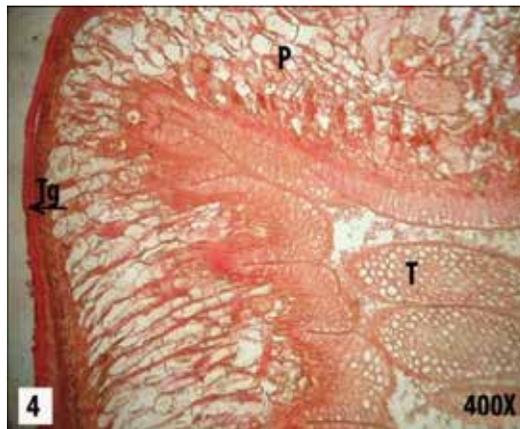


Fig. 4 Micrograph of a haematoxylin and eosin stained longitudinal section of PVS untreated, adult *E. explanatum* showing normal structure Tegument- Tg, Parenchyma- P, Testes- T (H&E x 400).

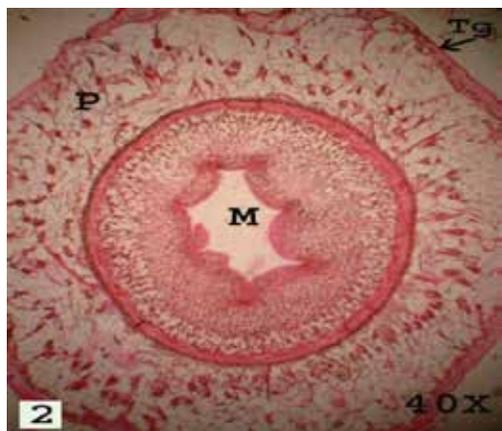


Fig. 2 Micrograph of a haematoxylin and eosin stained transverse section passing through the mouth and surrounding tegument area of the trematode *E. explanatum* showing normal tissue structure. Mouth-M, parenchyma-P, testes-T, (H&E x 100).



Fig. 3 Micrograph of a haematoxylin and eosin stained longitudinal section of PVS treated, adult *E. explanatum* showing overall disruption of Tegument- Tg, Paranchyma- P, Testes- T (H&E x 400).

Tab. 2: % Average mortality and mean with standard deviation of mortality after incubated of *E. explanatum* with different concentration of PVS at 8th h

% Mortality of <i>E. explanatum</i> after 8h of treatment with different concentrations of PVS					
Dose	C	T1	T2	T3	T4
% Mortality	12.5%	50%	56.25%	75%	97%
Mortality in terms of mean \pm SD and p value					
Dose	C	T1	T2	T3	T4
Mean \pm SD & p value	1 \pm 0.5	4 \pm 0.5 P< 0.005	5 \pm 0.5 P< 0.004	6 \pm 0.5 P< 0.001	8 \pm 0.5 P< 0.001

Abbreviation:
C: Control; T1: Treatment of PVS with 50ppm; T2: Treatment of PVS with 100ppm; T3: Treatment of PVS with 150ppm; T4: Treatment of PVS with 200ppm

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